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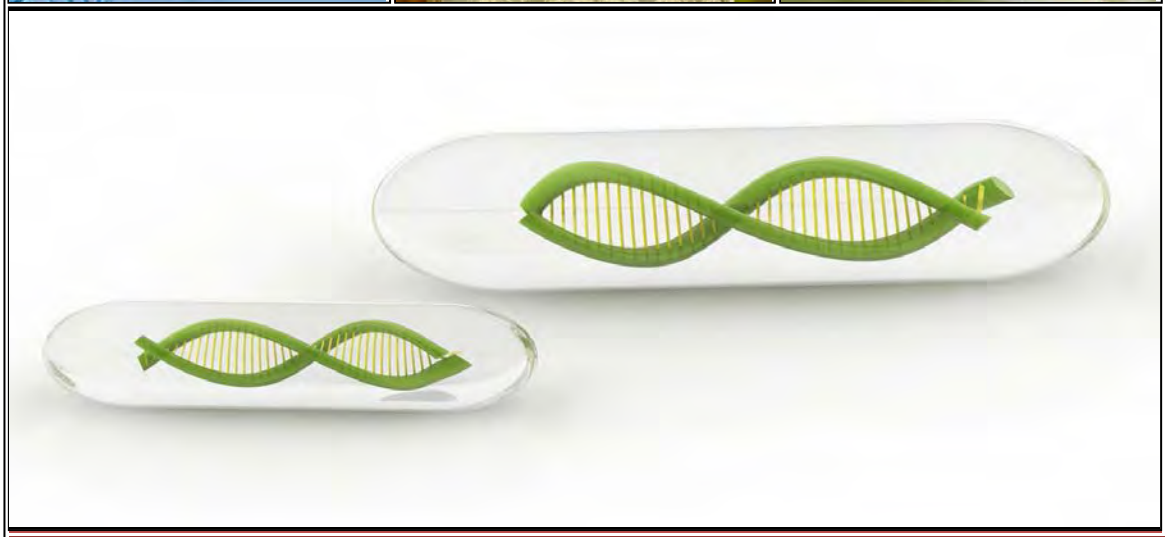


First International Conference on Biotechnology Applications in Agriculture

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Invited Papers

Transgenic Crops
Global Picture: Opportunities and Risks

Ali Shams El Din

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Abstract

This article was written to overview the historical implementation of transgenic crops as well as to give a short talk about the world food supplies. Pesticides applications and their negative and positive side effects could be carefully clarified. Pest resistance and environmental pollution is considering the very important issue in the world wide which should be reduced. New trends in pest control such as bio-control will be the promising techniques. These new techniques offer new opportunities for impacting the harmful insects and pathogens and could help to present safe food for human beings. The potential of genetic engineering as plant protection and crop improvement tool could be concerned to estimate the actual efficacy in field applications. In addition, the updated genetically modified plants major risks and concerns which published annually could be concluded as well. Also, commercialization of the first transgenic crop world wide as a first record was noticed. Finally, this article concluded the recommendation focused on wider cooperation and development of trade negotiation. Multilateral, regional and bilateral coordination, monitoring of innovation, technological platform and network should be clarified. Furthermore, information and knowledge diffusion, local markets' administration and financial mechanisms are indeed.

Key word: Transgenic Crops, Risks, Pesticides, Bio-control.

Biotechnology in Egypt: Current Status and Future Scenarios

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Abstract

According to United Nations world population prospects (2008), the population of Egypt rose from 44.4 million in 1980 to about 77.1 millions in 2008 and is expected to increase to 129.5 millions in 2050. These absolute numbers mean that we must increase our food production by, at least, 33-40% in order to keep the current existing food gap not to achieve food security for Egyptians. This increment must be achieved using the limited available water resources and land suitable for agriculture. Moreover, strategic studies and foresights point to shortage in water resources and desertification due to global warming and climatic changes. Therefore, somebody has to expect dramatic decrease in natural resources needed for food production in arid and semi arid regions of the world, including Egypt. This background necessitates decisive actions, governmental and society commitments to rely on recent advances in genetic engineering and biotechnology to cope with this dilemma. Analysis of published data and ongoing activities in the biotechnology domain in Egypt indicates that biotechnology research in Egypt can be divided into three main eras. The first era was focused on plant tissue culture, production of some industrial enzymes, fertilizers and biopesticides and is dating back to late seventy's. The second era started in the early 1990s and dealt with the deployment of molecular markers and DNA fingerprinting in genetic analysis of tissue culture-derived plants, cultivars identification, development of molecular markers, diagnosis and characterization of diseases and pathogens and recombinant proteins. The current (third era) of biotechnology is focusing on transgenic crops, genome mapping, bioactive substances and molecules, bioenergy and bioremediation. In my opinion, future trend of biotechnology will be focused on improvement of nutritional quality, development of nontraditional crops and new breeds more tolerant to adverse conditions and with low water demands, fisheries, domestic farm animals, molecular farming and converging sciences (nanobiotechnology). In this lecture, an overview on ongoing biotechnology research in Egypt and future prospects will be presented with special emphasis on plant biotechnology.

Key words: Egypt, biotechnology, industrial enzymes, fertilizers, bio-pesticides.

Biosafety and Conservation of Plant Genetic Resource Regulatory Policies: Issues and Considerations for Developing Countries

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Abstract

Conservation and safe use of plant genetic resources (PGR) are the foundation of food and drug production, and the biological basis for food security, livelihoods and economic development. In the last three decades, the increasing appearance and commercialization of products from recombining DNA of plants constructed in the North, has created a need for access to PGR of the South and raised a flux of biosafety concerns of unintended consequences on human and the environment (5). On one hand, the conservation of PGR in world's Centers is a complex initiative and often contentious. The declared objective of the North's initiatives in the conservation of PGR is perceived with suspicion by the South due to the emerging disputes over the sovereignty of new food; resulting from the current modus of access to this resource and inability to trace its use under the patent law (5). On the surface, it appears to be a simple issue of protecting plants from forces beyond the control of the owning nations in the South. Closer inspection reveals a complex tangle of conflicting issues concerning the South states' control over its PGR that is profoundly the base for biotechnology development by the North. On the other hand, the current world economic and legislative climate, competitive entry of the South into the biotechnology industries is fraught with many difficulties; such as lacking of technological and legal capacities for developing innovative biotechnology R&D, and relevant biosafety regulatory policy, inability to access new technology tools attached to intellectual property rights and missing the share of benefits regenerated from existing patents and infringements of owned PGR. In view of the continuing coalescence of the industry under the control of limited multinational companies, these difficulties seem to intensify. This paper discusses the conservation and regulations for a fare access to PGR and safe use of biotechnology in a sustainable and efficient manner, devoid of potential non-tariff/technical trade barrier as central to the principles of free trade under General Agreement on Tariffs and Trade, GATT.

Keywords: agriculture trade policy, biosafety, conservation, food security, GATT, GMO, trade negotiation,

Introduction

Plant Genetic Resource (PGR) is profoundly the base for biotechnology development by the North and for securing Global Food Security. By 2050, the world needs to produce twice as much food for the world as was produced in 2000 from the same land area and using less water. The Second Report on the State of the World's PGR for Food and Agriculture (SoWPGR-2) documents the current status of PGR diversity, conservation and use, as well as the extent and role of national, regional and international efforts that underpin food security. It provides the basis for the updating of the Global Plan of Action for the conservation and sustainable utilization of PGR for Food and Agriculture (GPA). The flanking issue here is linking aid to national trade policies under the General Agreement on Tariffs and Trade (GATT).

In this regard, the South is faced by a number of regulatory challenges including achieving national Food Security under the free trade climate, holding grips on the diversity of PGR and controlling its conservation activities, handling the regulatory issues of biosafety of crops driven from biotechnology. In this paper, these challenges are discussed.

Food Security and the GATT:

Ever since the national debates in the South on whether or not their economies should ratify the General Agreement on Tariffs and Trade (GATT) Uruguay Round in 1994, food security has been a controversial issue. In this globalised agricultural markets, international trade is increasingly becoming the central determinant of food security in the South including the ability to produce most of food necessities, securing the welfare of producers, respecting cultural preferences of consumers, and achieving political stability of rural society (8). The shift from the cultivation of food crops to the cultivation of export crops has the farmers' preference for achieving higher return on their crops

and crop-derivatives; integrating food production into the international market. As a result, the agricultural sectors in the South became less and less capable of supplying the national's food needs. In the 1980's and 1990's, United States (US) and the European Union (EU) demonstrated food security by means of dumping their surpluses of food crops and agricultural products on the markets of the South, resulting in crippling agricultural production and threatening national food security initiatives (2). Thus, free trade reflects only the interests of the agricultural exporting countries, while neglecting the special conditions in other countries. The clash between the two paradigms of free trade and national food security became especially bitter during the last stages of the negotiations of the Uruguay Round of the GATT (10).

Attention should be paid to what the US Secretary of Agriculture John Block said at the start of the Uruguay Round in 1986, the "idea that developing countries should feed themselves is an anachronism from a bygone era. They could better ensure their food security by relying on US agricultural products, which are available, in most cases at lower cost." Under this climate, the North adopted domestic and trade policies to protect domestic food production in pursuit of food self-sufficiency thereby reducing the contribution that trade could have made to economic efficiency and development. Such policies are costly and thus turned to be more damaging to the economies and to food security. South economies receiving aid from the North were met with protestation by the North for wasting aid money to secure food self-sufficiency. The US position paper for the World Food Conference in Rome in November 1996 went even further to say that pursuing food self-sufficiency could in fact be detrimental to food security; i.e. access to food available from the North which should urban consumers, for whom the provision of food is seen as the key component of food security (10).

The World Trade Organization (WTO) meeting in Chiang Mai in March 2001 evoked widespread protest against probably the world's most unpopular organization. Initially, the formal agenda of the meeting was about trade and the environment, but the informal agenda was to pressure government officials to commit themselves to launching a new trade round. Most developing countries are opposed to a new round because they have not yet absorbed the demands on them by the Uruguay Round, and their believe that the United States may use it to push for a revision of the Agreement on Sanitary and Phytosanitary Standards (SPS) along lines that would support its concept of "sound science" as a methodology in order to force other economies to accept genetically modified organisms (GMOs).

Free trade monopoly partners of the North

The negotiation of the GATT Agricultural Accord in 1992 in Washington resulted in an agreement negotiated principally between the EU and the US that would regulate their monopolistic competition for the South markets. The monopoly partners managed to commit the South to reducing their domestic support subsidies by 20 percent and cutting their export subsidies by 36 percent over 10 years, which might finally become almost irrational for farmers to cultivate their land. However, in 1995, the first year of the implementation of the GATT Uruguay Round, the overall subsidy transfers in the US and Europe rose by five percent instead of going down. For that year, 20 percent of the cost of US farm production was financed by government subsidies totaling $25 billion.

Direct income subsidies to US farmers have been exempted under the so-called "Green Box" provisions of the agreement on the specious grounds that they are "decoupled" from production and thus "non-trade distorting." In the European Union, these direct income payments are mainly based on output, the bulk of it via a "land set-aside program" which entitles each farmer to a subsidy when he/she withdraws 15 percent of his/her land from cultivation. The idea behind the set-aside program is to restrict output, thus raising prices. However, in the US, direct income subsidies have taken the form of "deficiency payments," which bridge the gap between the market price for the year and a politically determined target price set by the United States Department of Agriculture (USDA) to support farm incomes. Regardless of the given name to the direct payments to EU and US farmers, the truth is that the payments are anything other than "decoupled" from production. It is just the intention of the EU and US to define the concept of subsidy to bring world trade rules into line with their perceived self-interest and surplus dumping practices. The institutionalization of the system of subsidization of the Northern agriculture by the GATT Agricultural Accord has had two major implications for the South economies which are now required to eliminate trade restrictions and lower tariffs to food exports to the North.

First of all, since the US and the EU are such massive grain producers and exporters, the world market price of grains has been greatly determined by the subsidization of agricultural production in these two areas. International prices are thus depressed relative to prices of domestically produced grain in most South economies. Interestingly, the pressure on world prices of wheat is exercised by the subsidized US

price, where a 10 percent decline in the US wheat crop would reduce world export supplies by six percent, with the consequent upward movement of international prices. Second, these massive subsidies have led to a more intense struggle between the EU and the US, to stake out South economies markets on which to dump their products. The conclusion of the GATT or World Trade Organization (WTO) Agricultural Accord, which was supposed to stabilize this monopolistic competition, did not make any difference in this regard. Indeed, export markets have become even more central to European agriculture following the institutionalization of direct income subsidies in the GATT-WTO. In the US, the massive subsidization centered on direct income payments permissible under GATT had created an export-dependent economy. Today, one out of every three farm acres in America is dedicated to exports, including wheat, rice, soybean, corn, fruits and vegetables.

Food security policy

Despite a rhetoric of concern about the fate of small farmers, the government's policy on agriculture and food security is driven by doctrinal liberalization, by a strong belief in the idea that greater exposure to the impersonal forces of the international market will guarantee greater efficiency in agriculture, bring about food security, promote consumer interests, and match farmers to activities in which they have a "comparative advantage." (17). There is only one major problem with this strategy. Given the fact that the free market in international agricultural trade is scarcely a reality, doctrinal liberalization would only result in expanding the position of subsidized US producers in the local market while consolidating the positions of EU and US producers internationally. The likely result is not local competitiveness but the worst of all possible worlds: domination by subsidized foreign agricultural systems, loss of competitiveness in most commodities, and the silent destruction of vulnerable rural communities.

Conservation of PGR

Conservation of PGR necessarily entails the imposition of regulations over access to the resources with specific people or institutions attempting to define who has access to those resources and on what terms. There are critical questions revolving around the need to employ accurate science in the conservation of PGR and its management, and the links between power and knowledge influence. Therefore, the conservation of PGR in world's Centers is a complex initiative and often contentious. On the surface, it appears to be a simple issue of protecting plants from forces beyond the control of the owning nations in the South. The declared objective of the North's initiatives in the conservation of PGR is perceived with suspicion by the South due to the emerging disputes over the sovereignty of new food; resulting from the current modus of access to this resource and inability to trace its use under the patent law. While the declared objective of holding gene banks among nations of the North and the South is to preserve biodiversity, the hidden purpose adopted by the North is to monopolistically use as a source of desirable genes for improving plants for commercial enterprises. The outcome of negotiated access to resources is largely a reflection of power relations at the local, regional or national level; with little or no consent from the South. Conservation practice is, therefore, a profoundly political process.

Biosafety issue and constraints in the South

The Cartagena Protocol on Biosafety (CPB) that followed the Convention on Biological Diversity (CBD) is an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another. It was adopted on 29 January 2000 as a supplementary agreement to the Convention on Biological Diversity and entered into force on 11 September 2003. The Protocol was ratified by the 171 member countries of the CBD, and became a legally binding instrument and its implementation became mandatory (4). This has become pivotal to trade and a potential trade barrier and therefore, required countries to develop the necessary legal and technical capacities in biotechnology for safe transfer, handling, use and identification of GMOs and their derivatives; particularly by protecting against unwanted human health and environmental outcomes. Environmental and food safety risk assessments were recommended to be contracted out, where appropriate, on a cost recovery basis from applicants, as not to over burden the national regulatory agencies. While considering the need for developing relevant National Biosafety Guidelines to safeguard the environment and public health and to protect national interests and concerns, the North faces a number of major constraints, among them; (1) understanding the potential impact of GMOs/ transgenic organisms and their products, including pharmaceuticals and genetically modified food (GMF), upon the environment, biodiversity and human health; (2) dealing with public perception issues related to GMF, and the environmental impact of GMOs; (3) assessing possible social and economic implications of the import of GMOs and their products in light of availability of safer substitutes; (4) coping with the workload regulators are likely to face in preparing new regulatory policy and the cost

of implementing relevant legislation; and (5) relying on domestic public sector with limited support resources as opposed to strong private-sector industry that are in support of legislation in developed countries.

Considerations in the South's National Biosafety Guidelines

The development of National Biosafety Guidelines in biotechnology is based on certain global, regional and bilateral considerations that may intervene with or pressurize national interests in the course of optimizing a national framework for regulatory policy. Some of these considerations are:

The implications of World Trade Agreements (GATT), specially provisions concerning non-tariff trade barriers

The principles of free trade set at the final round of the GATT (21) that was completed in Marrakech in 1993 is administered by the World Trade Organization (WTO), which became responsible for undertaking GATT obligations in the discrimination between justified non-tariff trade barriers, i.e. for reasons of environmental prudence and/or adverse effect on human health, and restrictions that are unjustified under the principles of GATT. In this respect, the World Trade Agreements (WTAs) serve as a *de facto* means for harmonizing biosafety legislation and decisions through curtailing national sovereignty to rule unilaterally on new GMOs and their products under the principles of free trade. Evidently, the implication of GATT compromises the sovereignty of individual nations, where its biosafety legislation might be construed as a spurious non-tariff trade barrier. In other words, the requirement is that national biosafety measures adopted for reasons of environmental protection are 'legitimate' and 'scientifically justifiable'. In case where national provisions deviate from international guidelines, a country must, if challenged, produce scientific evidence justifying such deviation. Under GATT, the potential Technical Barriers to Trade (TBT) were addressed in two agreements reflecting environmental biosafety issues, relying upon a range of international standards and guidelines. These agreements are: Agreement on Technical Barriers to International Trade (TBT); and the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS).

The TBT Agreement requires that "*Members shall ensure that technical regulations are not prepared, adopted or applied with a view to, or with the effect of creating unnecessary obstacles to international trade*" (18). However, the Agreement does itemize particular 'legitimate objectives', according to which trade restrictions may be permitted. These objectives include exceptions, which were also made in Article XX of the GATT. Obstacles to trade based on the National Biosafety Guidelines may not be considered necessary or legitimate under TBT. This is obviously a potential of conflict of interest between principal exporters of biotechnology products (mainly developed countries), and developing countries. Therefore, many developing countries consistently supported the need for a protocol to cover not just the transboundary transfer of GMOs, but also their post entry safe handling and use. In the course of biosafety risk assessment and risk management, developing countries anticipate commitment from developed countries for more modest capacity building in biosafety.

The SPS Agreement requires members to base their sanitary or phytosanitary measures on international standards, guidelines or recommendations, where they exist. But, an option is available under Article 5.7, which allows adoption of provisional measures "*Members may introduce measures, which result in a higher level of protection than would be achieved by SPS measures, if there is a scientific justification.*" (1). Substantial evidence is not a condition for applying restriction on imported GMOs or their products. Least-developing member countries were given till this year, 2000, to implement SPS obligation. Otherwise, a member country might apply the restriction with some evidence, and within a reasonable time, the member must provide additional evidence to justify further restriction. However, providing adequate scientific justification in a reasonable duration would be a problem case when risk is uncertain, impossible, or cannot be identified. This could mean that an embargo on an import of GMO or its products, based on provisions of the Biosafety Protocol, by one country is likely to bring trade disputes to the WTO. For this reason, the appropriateness of the 'Biosafety Protocol' has been challenged on the basis of its possible conflict with the GATT principle of free trade (17).

Socio and economic issues have little scope in the GATT. In the Article 5.3 of the SPS, there is a little consideration for economic factors, but a restriction must be least-trade restrictive. The fact that there is no explicit provision under GATT for excluding an import on the basis of the possible social or economic ramifications, socioeconomic factors may be considered at a national level to serve as legitimate restrictive reason, at least for now. However, the acceptance of GMOs imports remains obligatory. In

Italy, for example, Bt-maize was banned from cultivation; however, seeds were imported into the country. It was argued that the arrangement infringes national sovereignty and disregards the wishes of the public majority. Nevertheless, in cases when the public votes in favor of a proposed ban, the country itself will finally fall in a direct conflict with WTO obligations. Despite the possible conflict with WTO, socio-economic, ethical and public demands have come out clearly in the biosafety guidelines in many countries around the world including the Scandinavian countries, Switzerland and Italy. The fact that the environmental impact assessment and the risks associated with the release of a GMO vary between ecosystems, the socioeconomic impact of the introduction of LMOs varies widely between countries. Also, the socio-economic benefits assume a different weighting to developing countries and the implications will evidently depend upon a plethora of local factors. Therefore, experiences of environmental releases in industrialized countries are not transferable, *in toto*, to non-industrialized countries, where different environmental conditions prevail, in particular where local biodiversity differs. This emphasizes that the interest of developing countries in a mechanism for the regulation of GMOs and their products, may not be best served by a straightforward assimilation of regulatory models taken from industrialized nations.

The commitment to an international 'Biosafety Protocol', which was ratified by Parties that have previously ratified the CBD.

The provision of Article 19.3 of the CBD for member countries to consider a legally binding international biosafety protocol is about to be materialized. The proposed Biosafety Protocol specifies obligations for international transfer of LMOs/GMOs and sets out means for risk assessment and risk management, technology transfer and capacity building.

Whilst developed countries realizing the importance of regulatory convergence amongst trading partners, they remained concerned that none of the international "Biosafety Protocol" would adversely affect their biotechnology exports. The US and the World Bank, for example rejected the need for such protocol. Although the US has not ratified the CBD, and, therefore, not a party to the 'Biosafety Protocol', it continues to participate in the negotiations, both directly (14, 8), and by advising countries receiving US aid on the deliberations of the Conference of the Parties.

Advance Informed Agreement (AIA) is a provision, which was made central to the Biosafety Protocol. It offers mechanisms by which exporters of GMOs or their products inform the competent authority in the importing country prior to export, as to allow the importing countries informed decision making prior to importation of such commodities, which may raise concerns regarding the effect on conservation and sustainability use of biodiversity'. Because of the reduced capacity and resources of developing countries for biosafety risk assessment and risk management, the AIA offers the only affordable negotiating power in the trade of GMOs and their products. In the absence of risk assessment, member countries are prohibited from applying permanent restrictions on GMOs and their products.

Liability and compensation: If GMOs have the potential to cause serious environmental damage or pose an unanticipated public health risk, the issue of liability becomes important. In the negotiations for the international Biosafety Protocol, it has been suggested that there should be a requirement for liability and compensation as part of the AIA process. For a more far-reaching protocol, which would include provisions for consideration of the liability and compensation following environmental damage, an African proposal was tabled at a meeting of the Open-Ended Ad Hoc Working Group on Biosafety as late as 1997 (21). This was met with the opposition of both the EU and the US (18). However, in AIA procedures, a commitment to fault-based, civil liability would conceivably be commensurate with risk posed by GMOs. In the absence of evidence for a negative outcome from a GMO release, requirements for public liability, care or mandatory compensation may not be satisfied under the SPS requirement.

Labeling of Foods driven from GMOs (GMF): An International standard for GMO labeling has been prepared under the Codex (2). In the absence of a Codex standard, the TBT Agreement permits mandatory labeling. This is referred to as incorporation of Process and Production Methods (PPMs). Labeling for unincorporated PPMs may only be introduced on a voluntary basis, and then only under guidelines specified in the TBT Agreement. Mandatory labeling for unincorporated PPMs (known as negative labeling) is prohibited. The criteria used to determine that foods are not familiar or substantially different as a result of genetic modification is important to justify any exclusion decision, however, will depend on what normative standards were used. Opponents of mandatory GMO/GMF labeling maintain that the consumers should not be bordered with unnecessary labeling and the regulators and manufacturers should not be unnecessarily over burdened (2). May be against the Codex standards, the European Parliament has

adopted GMO-labeling requirements under council Directive 90/220/EEC, and under legislation for products of GMF soybeans and maize. The EU requires mandatory labeling for GMFs and foods that may contain GMOs (6). In comparison, the United States only requires labeling of GMF that are unfamiliar and /or substantially different from the unmodified counterpart (7).

Trade bans against non-Parties to the Biosafety Protocol: Trade bans against non-Parties was proposed to the CBD, however, this would penalize countries not taking part in the protocol. Under the principles of GATT, the most favored nation and national treatment principles (Article I and III of the GATT) would be breached by such a ban, unless the mandated measures under a multilateral agreement fulfilled the terms of the GATT Exception clause (Article XX). However, a more WTO-consistent provision would be one that permitted trade with exporting interests prepared to enter into AIA procedures, or trade with non-Parties who had legislation consistent with the spirit of the Biosafety Protocol.

Pressure under regional trade agreements towards harmonization of biosafety regulations, such as that under APEC, EU, NAFTA etc.

All 132 members of the WTO are also members of some form of regional trade agreement (21), such as Asia Pacific Economic Co-operation (APEC), European Union, North American Free Trade Agreement (NAFTA) etc. Membership of 'free trade areas' (or aspiration to membership) may place certain constraints upon the national biosafety legislation, due to the need for harmonization in the presence of large differences among the biotechnology capacities of member countries. Regardless of being a member of a trade agreement, the geographical proximity of some economies leads to ecological similarities, which may be reflected in their biosafety provisions. It is a fact that, regulations cannot avoid judgment about strategic advantages or disadvantages of a product; presumed benefits may influence how regulators define harm. Thus, an implicit technology assessment enters their safety judgment.

Pressure from multinational biotechnology companies to introduce biosafety regulations prior to local investment.

It is an imperative for multinational companies that a country has biosafety regulatory policy or guidelines in place prior to investing or experimental introduction of transgenic organisms in such country. This arises, partly, from an anxiety to minimize the risk of liability in the event of environmental or human health problems arising from the release, and partly from a concern to avert potential criticism from public interest groups that the company is exploiting a lack of regulation in choosing to develop their products in these countries. There is recognition that where existing legislation cannot be easily adopted to cover biotechnology, multinational companies are anxious to see a new legislation *per se* been introduced, irrespective of the system from which it is drawn.

Pressure from foreign aid agencies to introduce biosafety regulations under 'Aid for Regulation' deals.

The development of national biosafety legislation, in some cases, may become a prerequisite for bilateral aid. The USAID requires the introduction of regulatory measures by developing countries prior to condition for aid for biotechnology capacity building. The tying of bilateral aid packages to the development of biosafety regulations necessarily amounts to the transfer of legislative approaches from donor to recipient countries. An USAID sponsored project in Egypt provides a good example. A binding code of conduct for biosafety in Egypt, approved in 1995, was developed by the Egyptian Agricultural Genetic Research Institute (AGERI) specifically to facilitate bilateral research projects (9). However, although this code of conduct was produced with the collaboration of representatives from the USAID funded Agricultural Biotechnology for Sustainable Productivity (ABSP) Project, it does not closely follow a US model.

Confusion caused by deregulation/relaxation of regulation in some leading countries, such as the US

Recently, there has been a relaxation of legislation (deregulation) in the US, which may be followed by other countries. This relaxation is reflected in the strengthening of a 'notification system' rather than a 'permit system' for the interstate movement or field-testing of particular GMO, with a provision for extension of the list of exempts from full regulatory control through petition. Presently, researchers need simply to notify the USDA Animal and Plant Health Inspection Services (APHIS) of their intention to move the GMOs, or to conduct a field test. The deregulation will generate potential problems, as hybrids from deregulated GMOs and their products will not be officially recognized as genetically modified. Developing countries and the EU do not favor deregulation, because it neglects the perception

of risk and the possible social and economic harm of the new products.

Concluding remarks

Agricultural policy for Egypt is in crisis. The current posture of doctrinal deregulation and liberalization of agriculture policy, that are yielding ground to uncontrolled market forces, is permissive and needs to be changed. There is a need for reforming the agriculture sector as a key to a healthy and prosperous smallholder agriculture that is also efficient and productive. A new approach to food security is urgently necessary and it could not have come from the former administration. Today, however, this might be an appropriate moment for NGO's and other groups and individuals concerned with the future of the Egyptian agriculture to intervene in the political process to formulate and push for the adoption of a new strategy. The new approach must be part of an integrated policy for sustainable agricultural development that serves the interests of smallholding producers, consumers, and future generations of the Egyptian people. This approach is not going to be possible without a strong government will to pursue an offensive strategic trade policy covering the interrelated areas of agriculture, agricultural trade, and food security. The government must also be aggressive in invoking provisions of the GATT Uruguay Round to defend its farmers by resorting to anti-dumping provisions. Sanitary and phytosanitary must be considered to protect the health of the people and the environment. Egypt must approach the WTO rules to exploit the ambiguities of the system for the country's small farmers. A strategic policy for food security, agricultural trade, and agricultural development must not be put into motion by government alone. A structure of participatory decision-making on food security and agricultural issues must be created that includes the multiple actors with a stake in a sound strategic agricultural policy to draw up the national food security, trade, and agricultural development plan; create clear cut criteria to determine situations of grain shortage that might justify the higher importation of grain and other foodstuffs; monitor import price levels and make decisions on invoking "anti-dumping measures"; monitor and accelerate agrarian reform; determine and monitor implementation of government expenditures for agriculture; and determine and promote research in agricultural biotechnology that ensures sustainable agricultural development.

After the GATT, the Egyptian governments are under pressure to manage trade in agricultural commodities for maximum comparative advantage. The interests and concerns of developed and developing countries over the development and introduction of GMOs and their products vary greatly. In the South, countries are currently lacking biosafety regulatory policy, need to understand the implications of ratification of an international 'Biosafety Protocol' and need to adopt national biosafety guidelines/framework in biotechnology. In the process of developing such guidelines in a manner that protects national interest and concerns, policy makers are reminded to avoid direct assimilation of biosafety frameworks developed in industrialized countries, and to observe the various global, regional and bilateral considerations that may intervene with or pressurize national interests and compromise national sovereignty. Trade impediments based on socio-economic consideration contained in national biosafety regulation are likely to be challenged. In order to formulate realistic national biosafety guidelines, and for the effective safe transfer, handling use and identifying biotechnology products, especially GMOs and their derivatives, developing countries need to, seriously consider: 1. Develop relevant legal and administrative frameworks for biotechnology, 2. Acquire scientific and technical training, and institutional capacity in biotechnology and 3. Develop strategies for the training of biotechnologists to assist in the harmonization of guidelines at sub-regional, regional, and international levels, and in monitoring the implementation of the International Biosafety Protocol.

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Applications of bio-techniques in genetic improvement programs to synthesize new lines of small animals in the Arabian countries

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Abstract

A crossbreeding program between Ardi Saudi goats (A) with the Syrian goats (Damascus, D) was started in 2006 in two experiments (Jouf and Qassim) applying bio-techniques of estrous synchronization and artificial insemination to improve milk and meat production. In rabbits, few numbers of new lines were synthesized in our Arabian countries using different criteria and methods of selection and crossbreeding. In this concept, Saudi 2 and APRI rabbits (as maternal lines) and Alexandria and Saudi 3 (as paternal lines) and Moshtohor (as multipurpose line) were being formed to be convenient in hot climate. The most common selection criteria used in selection programs to develop new maternal lines were related with litter size at birth or at weaning and milk production, while in paternal lines, post-weaning daily gain or marketing weight are commonly selected individually. Spanish V-line rabbits genetically selected for more than 35 generations were introduced in various developing countries (as alive animals or as frozen embryos) and by using recent bio-techniques and applying selection and /or crossbreeding programs with local lines, this line was widely distributed in some hot countries of the world like Egypt and Saudi Arabia.

In rabbits, direct selection had little or moderate effects on litter and lactation traits, while it had considerable effects on post weaning growth and feed conversion. Direct heterotic effects were evidenced for litter size, litter weight, and milk yield. Crossbred does and dams gave favourable heterotic effects on litter traits, milk yields and components and milk conversion ratio. Direct heterosis for body weights raised in hot countries were mainly positive and ranging from 1.3 to 14.5 %, but the estimates for maternal heterosis were mainly low and ranging from 0.2 to 5.3 %. Crossbred dams gave moderate maternal heterotic effects ranging from 4.8 to 18.7%. Neither individual heterosis, nor maternal heterosis were evidenced for meat quality traits. Crossbred bucks were associated with an existence of heterotic effects in ejaculate volume (11.6%), sperm concentration (10.5 %), percentages of motile (9.8%) and living sperms, along with a reduction in percentages of abnormal (-10.8%) and dead sperms (-23.5%). Crossbred dams gave maternal heterotic effects on some semen parameters in their progeny of crossbred bucks. The recent molecular technologies were used only in developing countries to detect the associations between phenotypic traits and genetic markers and three markers were detected for litter and milk traits and body weights.

In goats, The favourable estimates of direct and maternal heterosis for some litter and lactation traits and most growth traits would be an encouraging factor for the goat producers in hot climate countries to use crossbred does and dams on commercial scale. Crossbred dams had considerable maternal heterotic improvements in their crossbred daughters in terms of larger litter size, heavier litter weight at birth and weaning, pre-weaning mortality and milk production and components (2.4 to 28.1 %). Crossbred dams could produce crossbred bucks characterized by higher volume of ejaculate, higher semen quality with more concentration and motile sperms, along with lesser percentages of abnormal sperms and dead sperms than their crossbred daughters. Direct recombination losses were of little importance for the majority of the traits and they were generally lower than the estimates of direct heterosis.

Keywords: Small Animals, Bio-techniques, Genetic improvement programs, Selection, Crossbreeding, Synthetic lines.

Introduction

Nowadays, synthetic lines are being developed in hot climate countries by crossbreeding and selection for defined objectives (El-Raffa, 2007; Khalil and Al-Saef 2008; Youssef *et al.*, 2008; Iraqi *et al.*, 2009). These lines, depending on their specialization, perform better than the standard of the original breeds and the current production tends to rely on them. In our Arabian countries, little numbers of synthetic maternal and paternal lines of rabbits were synthesized using different criteria and methods of selection and crossbreeding (Al-Seaf *et al.*, 2008).

Long-term selection experiments carried out in rabbits for more than 10 generations were few (El-Raffa, 2000; Khalil *et al.*, 2005; Youssef *et al.*, 2008; Iraqi *et al.*, 2009). Nowadays, synthetic lines are being formed in Egypt and Saudi Arabia by crossbreeding and

selection for defined objectives (El-Raffa, 2007; Khalil and Al-Saef 2008; Youssef *et al.*, 2008; Iraqi *et al.*, 2009). Some attempts to develop new lines already done and **V**-line has proved to be advantageous to standard Californian and New Zealand White for litter size traits and daily gain (Iraqi *et al.*, 2006). Recently in Egypt, new synthetic lines of rabbits called Moshtohor and APRI as maternal lines and Alexandria as a paternal line were being formed to be convenient in hot climate. Till now, multi-purpose synthetic lines were not formed to be used on small and large scales. Three-way crossing will be beneficial in this concept. This scheme has the advantage of exploiting at each generation the entirety of the effect of heterosis (direct and maternal), but it requires a complex scheme, based on the maintenance and selection of the pure stocks and the multiplication and diffusion of the crossbred bucks and does. This program makes possible to provide farmers with improved animals, while ensuring their independence.

In order to improve the productivity of goats, there is a great deal to increase the productivity of native goat breeds in our Arabian area through crossbreeding programs. In the last two decades, new assisted reproductive technologies in goats had been applied successfully (Lazaris *et al.*, 2002; Wheeler *et al.*, 2003; Baldassarre and Karatzas, 2004). In this concept, artificial insemination and estrus synchronization could be used as new powerful and successively tools in planned breeding programmes to increase the rates of genetic improvement in goats (Thibier, 1996; Leboeuf *et al.*, 2000, 2003).

The main objectives of this article are concentrated in evaluating sustainable genetic improvement programs in the Arabian countries in terms of: (1) programs of selection and crossbreeding used to develop new synthetic lines; (2) estimates of selection responses obtained; (3) direct and maternal heterotic improvements obtained from crossbreeding; (4) Molecular analyses and attempts to identify the specific genetic markers.

Genetic improvement programs achieved in the Arabian countries

In practice, the following programs were used to improve the farm animals genetically in our Arabian countries (Khalil and Al-Saef, 2008; Khalil *et al.*, 2011):

Producing specialized strains that are selected to produce F1 females, which are crossed with a sire of another strain to produce terminal product. This method has the advantage of exploiting at each generation the entirety of the effect of heterosis (direct and maternal), but it requires a complex scheme, based on the maintenance and selection of the pure stocks and the multiplication and diffusion of the crossbred females. It is the reason why these turnkey programs are rarely used in developing countries, except in some small or large scale projects in Egypt and Saudi Arabia. They need an important investment and create a technical and economical dependence which does not usually fit the social and economical environment.

Creating a synthetic line by crossing females of a local population or breed (well adapted to the environment) with imported males or semen from a selected strain to produce F1 population; it will be bred without selection during few generations, avoiding consanguinity, and constitute a nucleus submitted to selection. This program makes possible to provide farmers with improved animals, while ensuring their independence. It lets to the farmers to adapt their strategy of renewal of their herd and they can practise self-replacement without loss of the genetic level, buying males or the two sexes to the nucleus.

Selection within pure local breeds. This program is less used, for different reasons: local populations performances are usually very low, even if they are well adapted and imported breeds often do not adapt to poor environments and, in both cases, heterosis are not completely exploited.

In rabbits:

Summaries for lines developed in Egypt and Saudi Arabia through crossbreeding and selection are presented in Tables 1, 2 and 3.

In Saudi Arabia, a national project of rabbit production was established to detect the possibilities of producing meat rabbits under industrialized and hot conditions (Khalil *et al.*, 2002, 2005). For this reason, special emphases were paid to construct a genetic improvement programme to develop new lines of meat rabbits convenient for this hot country. Accordingly, V-line rabbits were imported in 2000 from Spain and were crossed with desert Saudi rabbits (Gabali, G). This program was based on an evidences stating that V-line rabbits and their crosses could produce efficiently under hot climatic conditions (Khalil *et al.* 2002; Al-Sobayil and Khalil 2002). From this program, two synthetic lines (Saudi-2 as a maternal line with the structure of $((¾V¼S)^2)^2$ and Saudi-3 as a paternal line with the structure of $((¾S¼V)^2)^2$) were developed from crossing Saudi Gabali with V-line rabbits, both selected for litter weight at weaning and individual weight at 84 d (Table 1). Details concerning the development of these new lines were presented by Khalil *et al.* (2002, 2005) and Al-Saef *et al.* (2008).

In Egypt, great efforts have been made since 1998 to select for one exotic maternal line under local conditions and to develop and select local lines based

partially on local breeds. An Egyptian-Spanish programme was established involving Alexandria University, Animal Production Research Institute (APRI, Cairo) and Benha University. V-line rabbits were imported in 1998 from Spain and various selection experiments were practiced. The first line was developed from crossing Baladi Red with V-line and this maternal line named APRI and it was selected for litter weight at weaning (Youssef *et al.*, 2008; Table 1). A synthetic paternal line named Alexandria was originated in Alexandria University from crossing V line with Baladi Black and selection was practiced for daily weight gain during 28-63 days of age (El-Raffa, 2007; Table 2). In March 2003, a selection program was started to produce a synthetic multi-purpose line named Moshtohor resulting from crossing Sinai Gabali with V-line and selection was practiced for litter weight at weaning and live weight at 56 days (Iraqi *et al.*, 2007, 2008; Table 3).

In goats:

In Saudi Arabia, a crossbreeding program between Ardi Saudi goats (A) as a native breed with Syrian goats (Damascus, D) was started in 2004 in two experiments (Jouf and Qassim) applying bio-techniques of estrous synchronization and artificial insemination (Khalil *et al*. 2012). Breeding does of Ardi goats were randomly divided into two groups, 120 doe in each experiment. Each group of Ardi does was subdivided into two subgroups to be inseminated artificially from semen of elite bucks of the same breed and of Damascus breed. Crossbred does of ½D½A were backcrossed with Damascus bucks to get the genetic group of ¾D¼A and such breeding plan permitted to produce four genetic groups of AA, DD, ½D½A and ¾D¼A in each experiment separately. The main objectives of this study were: (1) To characterize genetically the native Ardi breed of goats, (2) Improving the productivity of such breed for meat production through crossbreeding, and (3) Applying updated technologies of estrous synchronization and artificial insemination to shorten the generation interval in such genetic improvement program.

Table 1: Programs used to create synthetic maternal lines

Synthetic line and origin	Founder breeds	Selection criteria	Selection methodology	Number of generations	Selection response per generation
Saudi-2, Saudi Arabia (Khalil *et al*., 2005)	V line and Saudi Gabali	LWW + W12	BLUP animal-repeatability model	11	LSB= 0.18 kit/litter; LSW= 0.16 kit/litter; LWW= 62g/litter; WW= 8.6g/kit
APRI, Egypt. (Youssef *et al.*, 2008; El-Raffa, 2007)	V line and Baladi Red	LWW	BLUP animal-repeatability model	7	

LSB = litter size at birth; LSW = litter size at weaning; LWW= litter weight at weaning; WW = weaning weight; W12: weight at 12 weeks**.**

Table 2: Programs used to create synthetic paternal lines

Synthetic line and origin	Founder breeds	Selection criteria	Selection methodology	Number of generations	Selection response per generation
Alexandria, Egypt (El-Raffa, 2007)	Line V and Baladi Black	ADG (28-63d)	Individual selection using BLUP	7	
Saudi-3, Saudi Arabia (Khalil *et al*. 2002, 2005)	Line V and Saudi Gabali	LWW + W12	Individual selection using BLUP	10	W12= 38g; ADG= 0.6g; LSB= 0.14 kit/litter; LSW= 0.12 kit/litter; LWW= 35g/litter

LSB = litter size at birth; LSW = litter size at weaning; LWW= litter weight at weaning; ADG= average daily gain.

Table 3: Programs used to create synthetic multi-purpose paternal line

Synthetic line and origin	**Founder breeds**	**Selection criteria**	**Selection methodology**
Moshtohor, Egypt (Iraqi *et al*., 2008)	Sinai Gabali and line V	LWW+ 56-d weight	Two-stage selection using BLUP

LSB = litter size at birth; LWW= litter weight at weaning; ADG= average daily gain**.**

Selection responses

In rabbits:

In selection experiments carried out in developing countries, definite methodologies have been proposed to estimate selection responses. One of them is based on regressing the estimates of the breeding values on generations and this approach depends on the genetic parameters and the model used (Moura *et al.,* 2001). The other methodologies are not depend on the genetic parameters and the model itself but they are dependent on another approach through using the control population which could be an unselected population (Khalil *et al.,* 2002, 2005), or using the population selected divergently (Moura *et al.,* 1997).

As presented in Table 1, genetic responses obtained from long-term selection experiments for litter size and other litter traits were found to be moderately considerable. Selection experiments for growth rate notifying successful responses in most experiments carried out in Brazil (Moura *et al.,* 1997). Khalil and Al-Saef (2008) stated that does selected for litter size at weaning presented significant responses in feed intake (3%) and milk yield (6%). A response of 62 g per litter was recorded when selecting for litter weight at weaning. Estimates of direct selection responses per generation were moderate and ranged from 8.7 to 12.6 g for weaning weight, and 18 to 68 g for marketing weight (Moura *et al.,* 1997; Khalil *et al.,* 2005). Selection for growth rate has little or somewhat moderate effects on carcass characteristics and meat quality when the rabbits were selected at the same stage of maturity. Selection for litter weight at weaning achieved considerable responses in growth rate with maintaining high litter components and feed conversion.

Sustainable direct and maternal heterotic improvements

In rabbits:

Different crossbreeding experiments carried out in Egypt (e.g. Khalil *et al.,* 1995; Khalil and Afifi, 2000; Abd El-Aziz *et al.,* 2002; Iraqi *et al.,* 2007; Youssef *et al.,* 2009) indicated that direct heterotic effects were evidenced for litter size, litter weight, and milk yield in most of the possible crossbred does obtained. Consequently, both producers and processors in this area could potentially benefit economically through using crossbred does. Also, estimates of maternal heterosis were favourable and indicating that crossbred dams had considerable maternal heterotic effects in terms of larger litter size, heavier litter weight at birth and weaning, favourable feed conversion ratio, and efficient milk to litter gain conversion ratio than their crossbred daughters (Khalil *et al.,* 2005), *i.e.* crossbred does and dams gave favourable heterotic effects on litter traits, milk yields and components and milk conversion ratio.

For postweaning growth, estimates of direct heterosis for body weights raised in hot countries were mainly positive and ranging from 1.3 to 14.5 %, but the estimates for maternal heterosis were mainly low and ranging from 0.2 to 5.3 %, i.e. crossbred dams had little heterotic maternity over their purebred dams in these growth traits. Abdel-Ghany *et al.* (2000a,b) and Afifi *et al.* (1994) for crossing New Zealand White with Baladi Black or Baladi Red in Egypt found that heterosis percentages ranged from 2.7 to 9.5% for post-weaning body weights and gains.

For carcass traits, Afifi *et al.* (1994) found that direct heterosis percentages from crossing New Zealand White X Baladi Red in Egypt ranged from 1.0 to 4.7 % and this indicating that crossbreeding in rabbits was associated with a little improvement in the carcass performance. In Saudi Arabia, Al-Saef *et al.* (2008) showed non-favorable negative estimates of maternal heterosis of -65.5 g, -6.7 g, -5.3 g and –12.2 g for hot carcass, offal, fat and bone weights, respectively; i.e. crossbred dams gave significant negative maternal heterotic effects on carcass traits ranging from 4.8 to 18.7%. For meat quality traits, neither individual heterosis, nor maternal heterosis were significant.

For semen parameters, direct heterosis given by Khalil *et al.* (2007) indicated that crossbred bucks were associated with an existence of heterotic effects in some semen parameters. Such crossing was associated with an increase in ejaculate volume (11.6%; $P<0.05$), sperm concentration (10.5 %; $P<0.05$), percentages of motile (9.8%) and living sperms, and libido of bucks ($P<0.05$) along with a reduction in percentages of abnormal (-10.8%) and dead sperms (-23.5%; $P<0.05$). Reviewed estimates of maternal heterosis for semen characteristics were favourable and moderate (Khalil *et al.,* 2007); indicating that crossbred dams gave maternal heterotic effects on some semen parameters in their progeny of crossbred bucks. Consequently, crossbred dams could produce crossbred bucks characterized by higher volume of ejaculate with more concentration and motile sperms, along with lesser percentages of abnormal sperms and dead sperms than their crossbred daughters.

In goats:

Khalil *et al.* (2012) found that the heterotic increments were mostly significant and ranging from 3.0 to 11.4 % for body weights and daily body gains and 2.4 to 28.1 % for carcass traits relative to the parental purebreds, while the estimates for meat composition traits were mostly non-significant although crossbred have shown reductions in moisture, ether extract and ash in meat of the carcass. The maternal heterotic

increments in body weights and daily body gains were mostly significant and the estimates ranged from 1.0 to 27.0 %.

The estimates of direct heterosis expressed as percentages relative to the parental purebreds have shown a range of 4.9 to 26.5 %, 3.4 to 39.5 %, 3.1 to 6.9 %, and 3.0 to 11.4 % for semen parameters, litter traits, milk traits and body weights and gains, respectively. Direct heterosis in crossbred kids relative to the founder breeds were mostly significant and ranging from 2.4 to 48.2 % for edible carcass traits and 3.5 to 14.6% for non-edible carcass traits, while the estimates for meat composition traits were mostly non-significant although crossbred have shown reductions in moisture, ether extract and ash in meat of the carcass. Significant direct heterotic improvements were recorded in the dairy and meat experiments for ejaculate volume (0.075 ml vs 0.085 ml), sperms concentration (0.25 x 10^9 per ml vs 0.11 x 10^9 per ml), total motile sperms (0.275 x 10^9 per ml vs 0.125 x 10^9 per ml), and total sperms output (0.33 x 10^9 per ml vs 0.155 x 10^9 per ml), associated with significant reduction in percentage of dead sperms (5.5% vs 1.55 %). For litter and milk traits, crossbred litters were associated with significant heterotic improvements in litter size at birth, litter size at weaning, litter weight at birth, litter weight at weaning, pre-weaning mortality, daily milk yield, fat in milk, solids not fat, protein, and ash; being 0.08 kid, 0.09 kid, 0.245 kg, 1.560 kg, -7.65 %, 71 g, 0.12%, 0.30%, 0.14%, and 0.025 % respectively in the dairy experiment, while the corresponding estimates in the meat experiment were 0.07 kid, 0.045 kid, 0.195 kg, 1.03 kg, -2.5 %, 52 g, 0.15%, 0.21%, 0.14% and 0.015 %. The actual heterotic increments in body weights of the dairy experiment were 0.205, 0.362, 0.729, 0.481, 0.497, 0.435 and 0.712 kg at 0, 4, 8, 12, 16, 20 and 24 weeks of age, while the respective significant heterotic increments in meat experiment were 0.212, 0.551, 0.711, 1.125, 1.497 0.797 and 0.875 kg. For daily body gains, heterotic increments were 18, 55, 45, 60, 45 and 60 g in the dairy experiment and 15, 85, 40, 40, 40 and 50 g in the meat experiment during age intervals of 0-4, 4-8, 8-12, 12-16, 16-20 and 20-24 weeks, respectively.

Crossbred dams in the dairy and meat experiments showed significant heterotic improvements in their crossbred bucks for ejaculate volume (0.058 ml vs 0.055 ml; $P<0.05$), sperms concentration (0.15 x 10^9 per ml vs 0.09 x 10^9 per ml; $P<0.05$), total motile sperms (0.225 x 10^9 per ml vs 0.085 x 10^9 per ml; $P<0.05$), and total sperms output (0.58 x 10^9 per ml vs 0.115 x 10^9 per ml; $P<0.01$), associated with favourable significant increases in motile sperms (3.3 % vs 4.05 %; $P<0.05$) and live sperms (3.7 % vs 2.25 %; $P<0.05$) along with a reduction in percentage of dead sperms (4.3% vs 2.25%; $P<0.05$) in the semen of the dairy experiment; the estimates of maternal heterosis for semen parameters ranged from 3.3 to 34.1 %. Crossbred dams showed significant heterotic improvements in litter and milk traits of their crossbred does, being 0.15 kid, 0.11 kid, 295 g, 2.340 kg, -4.9 %, 82 g, 0.35 %, 0.23 % and 0.03 % for litter size at birth, litter size at weaning, litter weight at birth, litter weight at weaning, mortality, daily milk yield, SNF, lactose, and ash, respectively; the estimates of maternal heterosis for litter and milk traits ranged from 2.8 to 25.5%. The actual maternal heterotic increments in body weights of the dairy experiment were 0.130, 0.202, 0.219, 0.288, 0.305, 0.300 and 0.554 kg at 0, 4, 8, 12, 16, 20 and 24 weeks of age, while the respective significant heterotic increments in the meat experiment were 0.165, 0.859, 2.135, 1.421, 1.375, 1.286 and 1.298 kg. For body gains, maternal heterotic daily increments were 7.5, 4.5, 6.5, 2.0, 3.5 and 3.0 g in the dairy experiment and 33.5, 31.5, 7.0, 15.0, 10.0, and 5.0 g in the meat experiment during age intervals of 0-4, 4-8, 8-12, 12-16, 16-20 and 20-24 weeks, respectively; the estimates of maternal heterosis for body weights and gains ranged from 1.0 to 27.0 %.

Molecular analyses

In rabbits:

Molecular analyses for economic traits to be used in genetic improvement program in rabbits are very limited. In this concept, RAPD technique is one of the most widely used techniques in applications of molecular biology for identifying the markers linked to traits of interest without the necessity for mapping the entire genome (Bardakci, 2001). RAPD marker of OPF09700 explained variation ranged from 10 to 14.7% for rabbit litter weight, gains, pre-weaning mortality and milk yield, while OPF12900 marker explained 14.7 and 16.8% of the variation for body weight at 4 and 8 weeks of age, respectively (Khalil *et al*., 2008). In practice, Microsatellite DNA is currently the most useful marker of choice for a wide range of molecular genetic studies in rabbits. Before building the genetic linkage map for QTLs for important traits in local and synthesized breeds, we must make some preliminary tests for variability within the breed and knowing which markers can be used and will be informative. The markers must be polymorphic to be useful. Actually, there were 305 initial sequences (AJ874368–AJ874672), a set of 183 were cytogenetically mapped in rabbits by Chantry-Darmon *et al.* (2006) and they are referred as INRA microsatellites. The second set were seven microsatellite sequences (D1Utr4, D3Utr2, D5Utr3, D6Utr4, D7Utr4, D12Utr2 and D19Utr2) and this

assigned to chromosomes 1, 3, 5, 6, 7, 12 and 19, respectively (Korstanje *et al.* 2001, 2003). These markers are referred as Utrecht microsatellites. However, the first step of developing the genomic resources of rabbits was taken in August 2004 with the announcement by the National Human Genome Research Institute (USA) of a program for the partial sequencing of the rabbit genome, together with that of eight other mammals (http://www.nih.gov/news/pr/aug2004/nhgri-04.htm).

Till now, marker-assisted selection (MAS) is not generally used in current rabbits' selection programs in developing countries and the recent molecular technologies were used only in these countries to detect the associations between phenotypic traits and genetic markers. Khalil *et al.* (2008) used RAPD markers to search for the linkage between markers and quantitative traits and they used 526 rabbits in this analysis from a sire-granddaughters design in their selection program. From a total of 40 primers (10-mer) used in their study, five primers (OPA12, OPA19, OPA20, OPF09, and OPF12) were able to identify five polymorphic fragments at molecular weights of 1500, 1100, 1200, 700 and 900 bp, respectively, and only three markers of these markers ($OPF12_{900}$, $OPA19_{1100}$, and $OPF09_{700}$) showed significant associations with phenotypic traits, which indicated the presence of linkage between the three markers for litter and lactation traits and body weight at 4 and 8 weeks of age. Recently, El-Zarei (2010) detected five markers to differentiate between individuals for carcass traits and to study the association between these markers and some carcass, tissues composition traits and meat quality traits.

Genomic selection

Applications and Methodology

Implementing genomic selection conceptually proceeds in the following steps:

1. Estimation of the effects of chromosome segments in a reference population.
2. Prediction of genomic EBVs (GEBVs) for animals not in the reference population, for example selection candidates.
3. Three distinct categories of animals (training, validation, and application) are involved in WGS. In practice whole genome selection has been applied successfully to the selection of AI young bulls in the dairy industry.

For analyzing SNPs data, Verbyla *et al.* (2010) reported that at each loci (total number of locus, p) there are three possible combinations of two alleles (e.g. A or B), the homozygote of one allele (AA), the heterozygote (AB) and the homozygote of the other allele (BB). These are then quantitatively represented by 0, 1 and 2 respectively. Subsequently, phenotypic records at each time point were modelled as:

$$\boldsymbol{\gamma = \mu 1_n + \Sigma X_j \beta_j + Zu + e}$$

where y is the vector of phenotypes of the trait being analysed for all n individuals, μ is the mean, 1n is a vector of ones of length n, Xj is a vector of indicator variables representing the genotypes of the jth marker for all individuals (xij=0,1,2), βj is the size of the QTL effect associated with marker j, u is the vector of random polygenic effects of length n (Z is the associated design matrix) and is assumed to be normally distributed, $u \sim N(0, \delta^2 u A)$ where A is the pedigree derived additive genetic relationship matrix and e is the residual error also assumed to be normally distributed, $e \sim N(0, I \delta^2 e)$ where I is the nxn identity matrix. The prior distributions for the variances of the random polygenic effects and the residual were uninformative flat priors of the form $X^{-2}(-2, 0)$. The GEBV at each time point were calculated as:

$$\boldsymbol{GEBV = \hat{u} + X\beta + e}$$

Genomic selection (Advantages)

Whole genome selection (**WGS**) has the following advantages:

It is an approach to use **DNA** markers that are distributed throughout the entire genome. Genes affecting the most economic important traits are distributed throughout the genome and there are relatively few that have large effects with many more genes with progressively smaller effects.

Traditional marker-assisted selection (**MAS**) focuses only on those regions which are relatively certain to influence the trait of interest and leaves most of the genome and much of the genetic variation unaccounted for, while whole genome selection puts the greatest emphasis on those regions with the largest effects (that we can be most certain of) and still accounting appropriately for the more ambiguous genetic variation in the remainder of the genome.

It uses genotypes of thousands of single nucleotide polymorphism markers (**SNP**), like those from the 50,000 (50K) SNP chip, to predict breeding values (EBVs).

It is similar to the marker sets for DNA tests being offered now, but with much more density throughout the genome. Therefore, it allows SNPs with smaller effects on target traits to be used effectively. In theory, this will allow WGS to account for a greater proportion of genetic variation.

The same set of SNP could be used for all traits, because the SNP in the test span the entire genome.

Genomic selection is not a replacement for our current procedures and programs, but it will enhance our current programs by providing more reliable **PTA**s for young animals (especially sires). This will lead to more use of younger AI sires with moderately reliable PTAs and increase genetic progress for production traits as well as for health traits, reproductive traits and longevity.

Genomic selection will change breeding programs in rabbits and other small animals in the near future, already happens in dairy cattle since it has the same reliability for all traits in both sexes and it will shorten the generation intervals.

It improves the possibilities to select for functional traits (e.g. health, fertility) and improves also the selection against unfavorable recessive alleles.

Utilizing the genomic relationships and it provides new tools for risk management in breeding programs

Genomic selection (Disadvantages)

1) As compared to current DNA tests based on dozens to hundreds of SNP, whole genome selection would have greater cost per animal, because it uses the 50K chip.
2) Still need relatives for good EBV's and breeding programs.
3) Animals will be selected when sexually mature.

Updated future research work of genetic improvement programs

In order to produce a new synthetic multipurpose line in Egypt, we have to use three-way crossing among lines synthesized recently in Egypt (APRI, Moshtohor and Alexandria) and to use the genomic selection in such breeding program**.** To achieve this event, APRI bucks will be crossed with Moshtohor does to get the F_1 cross of ½APRI½M, then does of this F_1 cross will be crossed with Alexandria bucks to get ½A¼APRI¼M and then the progeny of this cross will be inter-se mated $(½A¼APRI¼M)^2$ for three generations to produce the multi-purpose line (Figure 1). Across generations, animals will be selected using a BLUP genome methodology.

CONCLUSIONS

1) Specialized maternal or paternal lines were recently developed to be used in Egypt on commercial scale.
2) Synthesizing multi-purpose lines are necessary to be established in the national rabbit industry.
3) The favourable estimates of direct and maternal heterosis reviewed for lactation, growth and carcass traits and heat-stress physiological parameters would be an encouraging factor for the producers in our Arabian area to use crossbred does and dams on commercial scale (Khalil *et al.*, 2002; Al-Saef *et al.*, 2008).
4) Insignificant recombination effects for milk traits and growth traits gave an impression to conclude that crossbred does resulting from crossing V-line with native breeds of rabbits in hot climate countries could be effective to develop multipurpose synthetic lines having more available heterosis to be used in commercial production in hot countries.
5) Till now, marker-assisted selection (MAS) is not generally used in current rabbits' selection programs in developing countries and the recent molecular technologies were used only in these countries to identify the associations between phenotypic traits and genetic markers (Khalil *et al.*, 2008).
6) Genomic selection is much better than classical BLUP if there are no known relatives, and just better if there are (as reported by Legarra *et al.*, 2008).

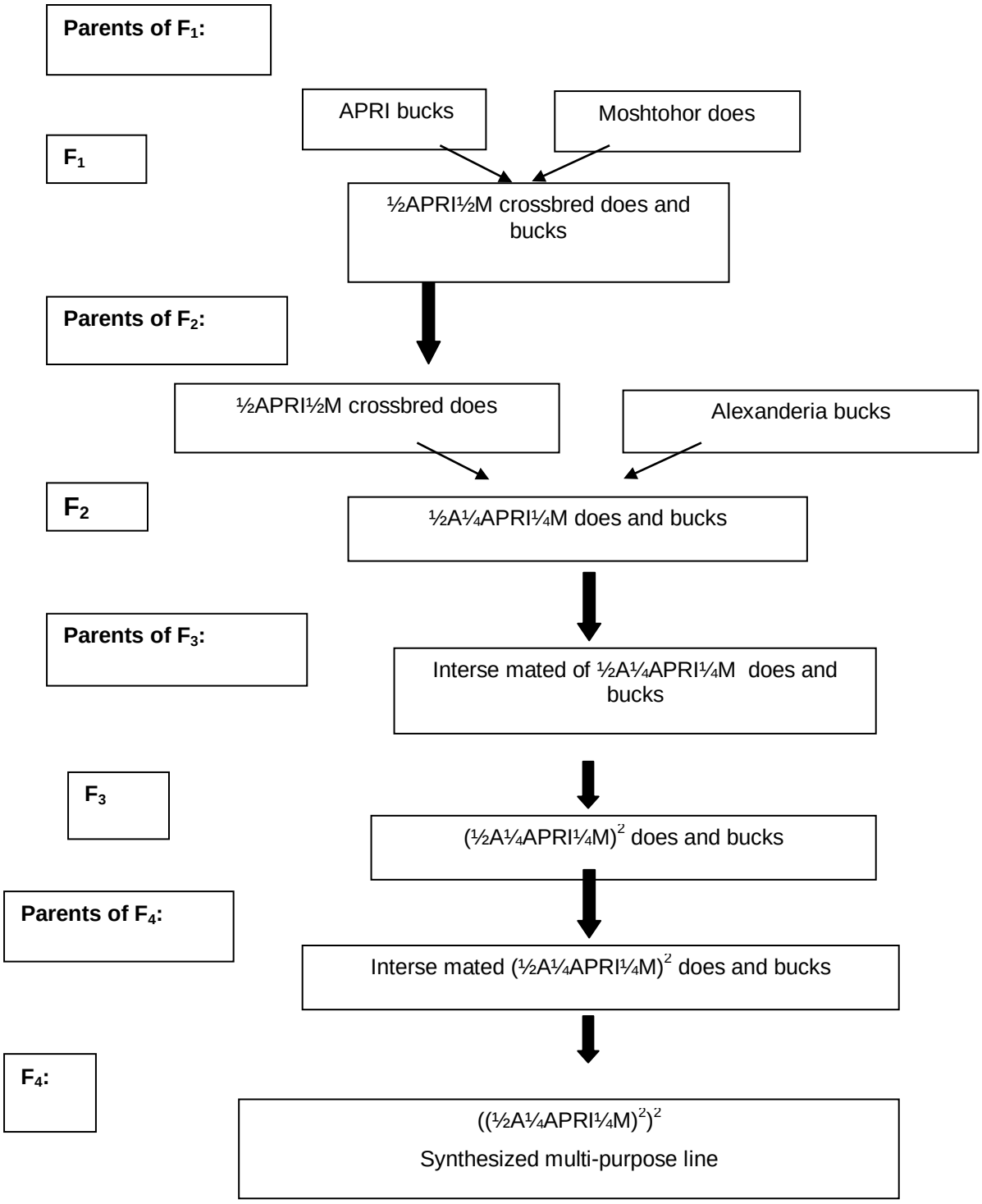

Figure 1: Diagram of three-way crossing to synthese the multi-purpose line.

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Agrobacterium-Mediated Transformation, a Key Player in Advancing Agricultural Biotechnology

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Abstract

No could one have imagined that the plant pathogen, *Agrobacterium tumefaciens* would advance agricultural biotechnology to the extent it enjoys nowadays. The bacterium brought Plant Pathologists to the frontiers of modern biotechnology. Agrobacterium-mediated transformation (AMT) is an integral step in the process of generating transgenic plants. Although other techniques are used to introduce exogenous DNA into plant cells, the wide host range of the bacterium, the ease of the procedure and the ability to carry relatively large fragments of DNA, AMT remains the most efficient and most used methodology. The bacterium which acts as a vehicle harbouring a Ti-plasmid vector, of which a T-DNA segment is transferable to plant cell, was harnessed to introduce and incorporate foreign desirable genes (transgenes) into plant cells in a process termed 'transgenesis'. The likelihood of transgenesis with AMT is much higher than that with other techniques. Transformation does not guarantee transgenesis as transformation is achieved when a transgene is introduced into a cell regardless it stays free or becomes incorporated into the target cell genome, whereas transgenesis is a transformation with successful gene expression of the transgene. Notably, AMT is the only available technique that ensures the occurrence of both, transformation and transgenesis. It has indeed revolutionised genetic engineering in the field of agricultural biotechnology. Through AMT, major achievements were made to improve plants, both quantitatively and qualitatively. Virus-resistant plants, insect-resistant Bt-corn and many other crops, herbicide-tolerant soybean, salt and drought tolerant rice, long shelf Life 'Flavr Savr' tomato, golden rice and blue rose are just a few example of the many achieved. The molecular mechanism involved in generating transgenic plants via AMT will be discussed and a number of major achievements reported.

Keywords: *Agrobacterium*-mediated transformation, Transgenic plants, Agricultural Biotechnology, Transgenesis, Ti-plasmid, T-DNA.

Introduction

Transformation is an essential element in the process of generating transgenic plants by genetic engineering. It involves the introduction of a small piece of DNA representing a desirable gene(s) (conferring one or more valuable agricultural trait) along with other sequences of different purposes such as a promoter, a selectable marker and/or a reporter gene into a target cell. Genes derived from distantly related or totally unrelated species belonging to even different kingdoms such as viruses, phytoplasma, rickettsiae, bacteria, fungi, algae, animals and human that would otherwise be inaccessible can be introduced into plants using genetic engineering techniques. Several methods of transformation to introduce foreign genes into plant cells with the subsequent regeneration of transgenic plants have been devised, but *Agrobacterium*-mediated transformation technique remains the method of choice for most applications.

The bacterium *Agrobacterium tumefaciens* is a soil-borne pathogen causing cancer to many dicotyledonous plants in nature and monocotyledonous plants in the laboratory. The bacterium has a segmented genome of dsDNA made up of a giant cyclic chromosome with three other much smaller ones, a linear chromosome, a cyclic cryptic plasmid and a cyclic Ti-plasmid (Wagih, 2000; Wagih, 2004). The ability of this bacterium to cause cancer is due to the possession of the Ti-plasmid whose name is derived from its role in tumour induction. Losing the Ti-plasmid causes the bacterium to turn avirulent. The Ti-plasmid has two main important segments, T-DNA (transferred DNA) and *vir* region (virulence region). T-DNA contains two types of genes: the oncogenic genes, encoding the enzymes involved in the synthesis of auxins (indole acetic acid, IAA) and cytokinins responsible for tumour formation and the genes encoding the synthesis of one of a group of unusual amino acids called 'opines'. The *vir*-region carries genes involved in the process of T-DNA transfer from the bacterium to the plant cell. Outside the T-DNA, are located the genes for opine catabolism and the genes involved in bacterium-bacterium plasmid conjugative transfer (Zupan and Zambryski,1995). *A. tumefaciens* has thus the exceptional ability to transfer a copy of the T-DNA into host cells where it is then stably integrated into the genome within the nucleus and transcribed there, causing cancer (Nester *et al*., 1984). Tumour cells demonstrate high level of the phytohormones, IAA and

cytokinins beside the synthesis and excretion of an opine (e.g. octopine or nopaline). IAA causes cells to grow larger (hypertrophy) and cytokinins cause them to divide faster (hyperplasia), the two phenomena responsible for tumour formation, whereas opines are consumed by the bacterium as a source of carbon and nitrogen. Further studies have shown that The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the *vir* region and in the bacterial chromosome. Since any foreign DNA, no matter where it comes from, placed in between the T-DNA borders can be transferred with the T-DNA to plant cells, scientists were able to construct the first vector and bacterial strain system for plant transformation by disarming the Ti-plasmid via the removal of oncogenes from the T-DNA and replacing them with the gene(s) of interest (Hooykaas and Shilperoort, 1992; Hamilton, 1997).

Agrobacterium

The genus *Agrobacterium* spp. encompasses five species, namely, *A. tumefaciens*, the causal agent of plant cancer, *A. rhizogenes*, the causal agent of hairy root disease, *A. rubi*, the cause of gall disease on raspberry, *A. vitis*, the cause of galls on grape and few other plants and *A. radiobacter* that is not known to infect any plant. But since *A. tumefaciens* can be converted into *A. rhizogenes*, simply by substituting Ti-plasmid for Ri-plasmid, the term species becomes meaningless. For this, some suggested classifying the genus *Agrobacterium* into 'biovars', rather than species, based on growth and metabolic characteristics (Keane *et al.*, 1970).

A. tumefaciens is a Gram-negative peritrechously flagellated motile, short rod, non-spore forming and soil borne bacterial plant pathogen that causes plant cancer in a large number of plants covering a wide range of more than 130 family of both gymnosperms and dicotyledonous plants of angiosperms. Although monocotyledonous plants are immune to infection, some, including *Asparagus, Narcissus, Chlorophytum, Zea mays, Saccharum officinarum* (Arencibia *et al.*, 1998), *Mosa* spp. And many others could be infected. The bacterium can also experimentally infect some fungi (de Groot *et al.*, 1998) including *Saccharomyces cerevisiae* (Piers *et al.*, 1996), *Agaricus bisporus, Aspergillus niger* and *Colletotricum gloeosporioides, C. lindemuthianum* and *Neurospora crassa* (Allen *et al.*, 2000). The bacterium has exceeded the limit and could infect human cells (Eugene Nester, personal communication; Kunik *et al., 2001*).

***Agrobacterium tumefaciens* Genome**

The genome of the bacterium *A. tumefaciens* (Fig. 1) amounts in size to approximately 5,900 kbp. It consists of four dsDNA chromosomes, differing both in shape and in size. A giant cyclic chromosome of 3,000 kbp long, a surprisingly linear chromosome of 2,100 kbp (approximately two thirds of the giant chromosome, a small cyclic cryptic plasmid of no known function of 550 kbp (i. e. about 1/5 the size of the giant chromosome) and the smallest , yet, the most significant, from the pathological point of view, cyclic plasmid, known as the Ti-plasmid, of a size as small as 250 kbp, (i.e. 1/12 the size of the giant chromosome), although it could reach a size of 800 kbp in some strains.

The presence of both circular and linear chromosomes is rather remarkable, as other bacteria only have either circular chromosomes or linear chromosomes, never both simultaneously.

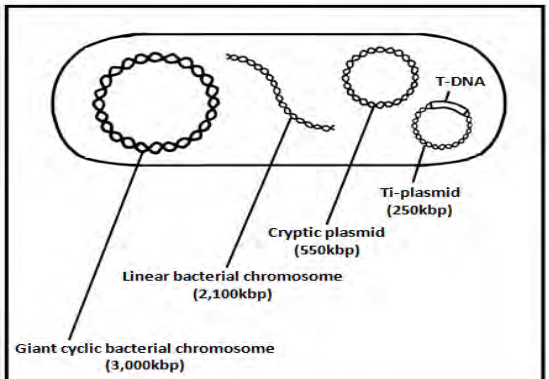

Fig. 1. Diagrammatic sketch of *Agrobacterium tumefaciens* genome. Chromosomes are shown with molecular length in kbp in parentheses.

Ti-Plasmid

The Ti-plasmid is a cyclic, covalently closed, dsDNA molecule (Fig. 2) whose size is in the range of 200 to 8oo kbp.

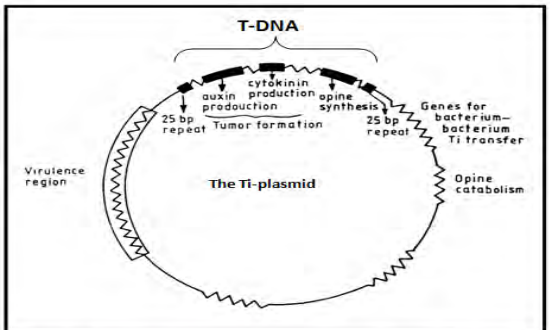

Fig. 2. Diagrammatic drawing of the Ti-plasmid of *Agrobacterium tumefaciens* showing its main regions, the T-DNA (dark bars) with its major genes and flanking border sequences (25bp each) and the virulence (vir-) region. Genes located outside these regions such as those involved in opine (e.g. octopine and nopaline) catabolism are also shown.

It derives its name from the fact that its presence in the cell turns the bacterium into virulent and enables it to induce tumours in the host plant, hence Ti (tumour-inducing) plasmid, whereas its absence causes it to lose virulence and becomes avirulent.

The part of the Ti-plasmid that is responsible for tumour induction and of which a copy is transferred to plant cells is called transferred DNA (T-DNA) (Martineau *et al.*, 1994). It carries at least thirteen genes transcribable into 13 polyadenylated transcripts by independent promoters. Both strands are transcribed. The T-DNA varies in size between about 10 to 30 kbp (i.e. representing 10% of the Ti-plasmid) and is flanked by a right and a left border of 25bp long each (Peralta and Ream, 1985). Some of these genes code for the synthesis of two phytohormones, auxin, indole-3-acetic acid (IAA), and cytokinin whose accumulation causes the cell to grow larger (hypertrophy) and divide faster (hyperplasia) resulting in the formation of tumours and the genes are thus called oncogenic genes. The T-DNA region includes also a gene coding for the synthesis of an enzyme, like *octopine synthase* or *nopaline synthase*, catalysing the synthesis of an unusual amino acid (octopine or nopaline, respectively). Beside these genes, there are other genes controlling the synthesis of some phosphorylated sugars. Interestingly, Ti-plasmid may contain one or more T-DNA.

The transfer of a copy of T-DNA from the bacterium to the target plant cell is under the control of several genes in a distinct region on the Ti-plasmid known as the virulence region (*vir*-region) (Krishnamohan *et al.*, 2001). The *vir* region is a 30 kb fragment representing a regulon organised in six operons (*virA*, *virB*, *virD*, *virG*, *virC* and *virE*) that are essential for the T-DNA transfer (*virA*, *virB*, *virD*, and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995, Jeon et al.., 1998). The T-DNA is transferred and stably maintained and expressed in the transformed cell. Although Ti-plasmid is considered a large plasmid, it is small enough to be easily lost from or gained by the cell containing it, depending on environmental conditions (Fig. 3) (Baron *et al.*, 2001). At a temperature above 28°C, the bacterium loses the plasmid and becomes avirulent. The cell is said to have been cured of the plasmid. Conversely, when a cured (Ti-plasmidless) strain is grown at a temperature below 28°C it acquires the plasmid and acquires with it virulence.

Role of Chromosomal Genes in the Transformation Process

Several genes located on the giant chromosome of *A. tumefaciens* have been shown to play a role in the attachment of the bacterium to the plant cell: the loci *chvA* and *chvB* are involved in the synthesis and excretion of the β -1,2 glucan (Cangelosi *et al.*, 1989); the *chvE* is required for the sugar enhancement of *vir* genes induction and bacterial chemotaxis (Cangelosi *et al.*, 1990, 1991); the *cel* locus is responsible for the synthesis of cellulose fibrils (Matthysse 1983); the *pscA* (*exoC*) locus plays a role in the synthesis of both cyclic glucan and acid succinoglycan (Cangelosi *et al.*, 1991), and the *att* locus is involved in the cell surface proteins (Matthysse, 1987).

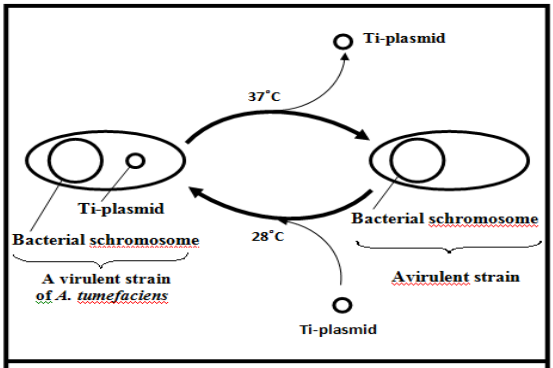

Fig. 3. The loss and gain of the Ti-plasmid in *Agrobacterium tumefaciens* as affected by the ambient temperature.

Mechanism of Transformation & Transgenesis

Transformation requires wounding. Upon wounding, the bacterium enters the host plant through the induced wound and attaches itself to one of the cells lining the wound (Fig. 4). A copy of the T-DNA region of the Ti-plasmid is transferred and inserted, randomly, into one of the chromosomes of the cell genome transforming it into a cancerous cell that autonomously grows into a massive malignant tumour. Wounding causes the release of specific phenolic compounds of which acetoseringone and α-hydroxy acetoseringone (Fig. 5) are the most commonly known compounds. Their role in transgenesis will be discussed soon.

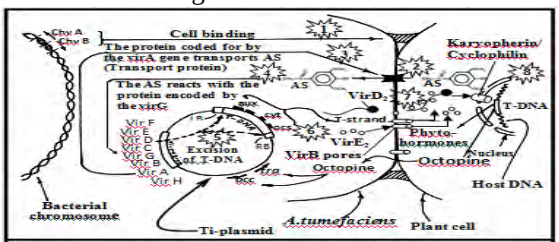

Fig. 4. Summary of the transformation of a plant cell by an octopine strain of *Agrobacterium tumefaciens*, a process that involves 8 events:

1. **Binding of the bacterial cell to a plant cell via a carbohydrate material whose synthesis is controlled by the two chromosomal genes, *chvA* and *chvB*.**
2. **Release of the *vir*-genes-inducer acetosyringone (AS).**
3. **Transport of the inducer AS into the bacterial cell by the transport protein encoded by the *virA* gene.**
4. **Binding of of the inducer with the protein product of the *virG* resulting in the induction of the remaining *vir*-region genes**
5. **The protein product (D2) of the *virD* gene acts as an *endonuclease* and excises the T-strand.**
6. **Transport of the T-strand from the bacterial cell to the plant cell with its 5′ end linked to D2 protein (a product of *virD* gene) and coated with molecules of E2 protein (a product of *virE* gene) forming what is known as the T-complex**
7. **Transport of T-complex takes place through a protein known as B-protein which is coded for by the virB gene.**
8. **The T-complex enters the nucleus through nuclear pores with the help of some host-related proteins known as Importins (e.g. karyopherin alpha and cyclophilin). Once in the nucleus, the T-strand is incorporated into the host genome and upon converting to dsDNA (T-DNA), Phytohormones and the amino acid octopine are produced.**

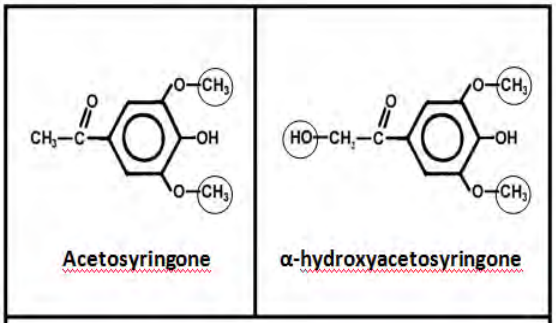

Fig. 5. The molecular structure of acetosyringone and α-hydroxyacetosyringone, the chemical inducers of virulence (*vir*-) genes in *Agrobacterium tumefaciens* which are known to be excreted by dicotyledonous but not by monocotyledonous plants.

The invading bacterial cell binds itself to the target host cell with the help of the gene products of two virulence-related genes, *chvA* and *chvB*, of the giant chromosome. The *virA* gene of the *vir*-region, expresses itself and produces a protein that integrates with the bacterial cell membrane to allow the phenolic compounds (AS and α-HO AS), released by the plant cell, to pass into the bacterial cell.

In coordination with the monosaccharide transporter *chvE* and in the presence of the appropriate phenolic and sugar molecules, *virA* protein autophosphorylates and subsequently phosphorolates the *virG* protein product (Jin *et al.*, 1990). *VirG* protein in the non-phosphorylated form is inactive; however, on phosphorylation, the protein helps activate the transcription of the remaining *vir* genes through interacting with the promoter that controls their expression.

A single strand of the T-DNA region is nicked between nucleotides 3 and 4 of the 25bp right border sequence by the *endonuclease* function of the *virD2* protein, a product of the gene locus virD and a single strand termed the T-strand (Tinland *et al.*,1994; Yusibov *et al.*, 1994; Zupan and Zambryski, 1995) is peeled off with its 5′ -end first and when peeling is almost complete, another nicking takes place at the left border with the help of the same *endonuclease virD2* protein.

The T-strand is transported from the bacterial to the plant cell forming a complex with two proteins, D2 and E2, coded for by the *vir-D* and *vir-E* genes, respectively. The *VirD2* and *VirE2* proteins play a complementary role in *Agrobacterium*-mediated transformation. These two proteins have been proposed to constitute, with the T-strand, a "T-complex" that is the transferred form of the T-DNA (Citovsky *et al.*, 1989, Howard and Citovsky, 1990; Zupan and Zambryski, 1997). A single D2 protein molecule binds covalently to the 5′ -end of the T-strand (Young and Nester,1988) and acts as a pilot protein to guide the T-strand to and through the type IV export apparatus, while many molecules of the E2 protein binds in a non-sequence-specific manner along the length of the T strand to coat it and protect it from *nuclease* degradation (Citovsky *et al.*, 1989; Rossi *et al.*, 1996). It is possible that one function of *VirE2* is to form a pore in the plant cytoplasmic membrane to facilitate the passage of the T-strand. Once in the plant cell, the D2 protein demonstrates another function through the nuclear localisation signal it carries and this is to guide it and the attached T-strand to the plant nucleus (Koukolikova-Nicola and Hohn,1993). E2 protein can also direct the T-strand to the nucleus (Zupan *et al.*, 1996) and alters the random coiling of the Ti-strand into a regular (telephone coil-like) coiling facilitating its passage through the nuclear pores. Upon entering the nucleus, the T-strand is converted into double stranded T-DNA and incorporated into one of the host cell chromosomes. Integration of T-DNA into the host cell chromosomes is associated with the D2 protein. E2 protein was also shown to protect the T-strand in the T-complex from degradation by *nucleases* (Rossi *et al.*, 1996), both in the plant cytoplasm and in the nucleus.

It was shown that the Ti plasmid, rather than chromosomal genes, was the major genetic determinant of host range (Thomashow *et al.*, 1980). Several virulence (*vir*) loci on the Ti plasmid, including *virC* (Yanofsky and Nester, 1986) and *virF* (Regensburg-Tuink and Hooykaas, 1993), were reported to determine the susceptibility of different plant species to *A. tumefaciens*. The *virH* (formerly called *pinF*) locus appeared to be involved in the ability of *Agrobacterium* to transform maize (Jarchow *et al.*, 1991)). Other *vir* genes, including *virG*, contribute to the "hypervirulence" of particular strains (Chen *et al.*, 1991)

Together with the *VirD4* protein, the 11 *VirB* proteins make up a type IV secretion system necessary for transfer of the T-DNA and several other *vir* proteins, including *VirE2* and *VirF* (Vergunst *et al.*, 2000). *VirD4* may serve as a "linker" to promote the interaction of the processed T-DNA/*VirD2* complex with the *VirB*-encoded secretion apparatus (Hamilton *et al.*, 2000). Most *VirB* proteins either form the membrane channel or serve as *ATPases* to provide energy for channel assembly or export processes. Several proteins, including *VirB2*, *VirB5*, and possibly *VirB7*, make up the T-pilus (Sagulenko *et al.*, 2001). *VirB2*, which is processed and cyclised, is the major pilin protein (Lai and Kado, 2000). The pilus may serve as the conduit for T-DNA and *vir* protein transfer, or it may merely function as a "hook" to seize the recipient plant cell and bring the bacterium and plant into close proximity to effect molecular transfer. One aspect of pilus biology that may be important for transformation is its temperature

liability. Although *vir* gene induction is maximal at approximately 25 to 27°C (Jin *et al.*, 1993), the pilus of some, but not all, *Agrobacterium* strains is most stable at lower temperatures (approximately 18 to 20°C) (Baron *et al.*, 2001).

Inserting desirable genes directly into the T-DNA region is not an easy task as no single restriction site of a restriction enzyme was found inside it. For this, several strategies were developed to overcome this difficulty and introduce genes into the T-DNA. The most commonly and preferably used strategy is called the binary-vector system (Bevan, 1984; Hoekema *et al.*,1983; McBride and Summerfelt, 1990; Gleave, 1992; Hellens *et al.*, 2000) in which, the T-DNA and the *vir*-region are separated into two different replicons within the same *Agrobacterium* cell (Fig. 6). The replicon carrying the T-DNA is called the binary vector, whereas that containing the *vir*-region is termed the *vir*-helper.

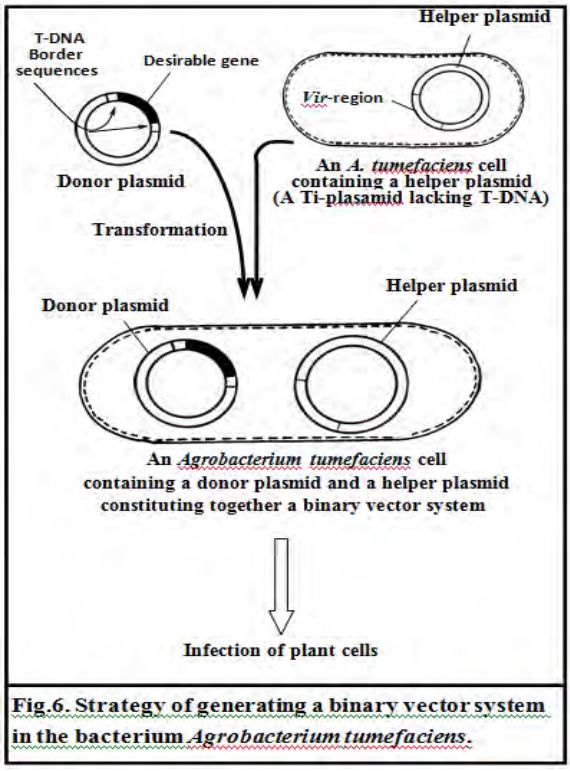

Fig.6. Strategy of generating a binary vector system in the bacterium *Agrobacterium tumefaciens*.

The *vir*-helper plasmid contained a complete or partial deletion of the T-DNA rendering it unable to incite tumours. Under these circumstances, products of the genes constituting the *vir*-region could act in trans on the T-DNA to excise and transfer it to a plant cell. Several *Agrobacterium* strains with disarmed *vir*-helper plasmids have been made available to researchers (Hellens *et al.*, 2000). These strains can be transformed by introducing the binary vector carrying a disarmed T-DNA region in which the gene of interest has been inserted to obtain a binary vector vehicle. Removing the oncogenes responsible for tumour formation and a desirable gene(s) is (are) inserted instead, the disarmed T-DNA is integrated into the plant DNA carrying with it the gene of interest causing no tumour formation. Once the desirable gene (transgene) is stably integrated, it is expressed in a manner like that of host cell genes. Transformation is proven by Southern blotting whereas transgenesis is checked by Northern blotting. This strategy revolutionised the use of *Agrobacterium* for the introduction of desirable genes into plant cells towards the production of transgenic plants.

Practical Approach to Generate Transgenic Plants by *Agrobacterium tumefaciens*

Several methods have been devised to introduce transgenes, via A. timefaciens, into plants for the purpose of generating transgenic plants. These methods vary with the nature of the cells to be transformed and the state they are in whether in suspension or in the form of intact tissue. For this, we will discuss in the following (Fig. 7) one of the most commonly used methodologies.

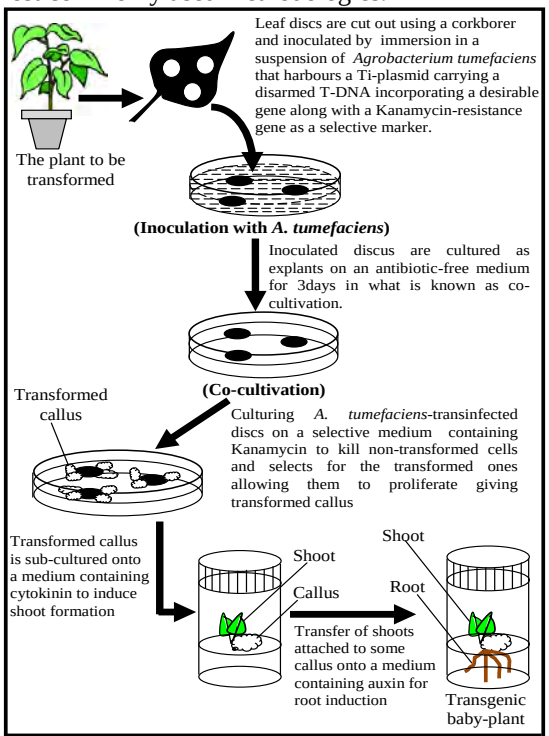

Fig. 7. A common strategy for *Agrobacterium*-mediated transformation and generation of transgenic plants.

Leaf discs are cut out from surface sterilised leaves using a cork borer. Sterilised discs are inoculated by infiltration under vacuum with a suspension of the transforming *Agrobacterium* cells harbouring the Ti-plasmid that carries the gene(s) of interest accompanied with the kanamycin-resistance gene (kan^R) as a selectable marker. Inoculated discs are blotted dry onto sterilised filter paper, co-cultivated onto a suitable tissue culture medium in a Petri plate and incubated under continuous light for 3 days in what is known as the co-cultivation process. Discs are then washed with sterile distilled

water, blotted dry onto sterile filter paper and transferred onto a suitable tissue culture medium containing two antibiotics, carbencillin and kanamycin. Carbencillen is used to kill the bacterial cells remaining attached to leaf cell surface whereas kanamycin selects for transformed cells by killing the non-transformed ones. When transgenic callus developing at the edges of leaf discs reaches a measurable size, it is sub-cultured onto auxin and/or cytokinins for rooting and/or shooting induction. Transgenic baby plants are individually separated, potted in sterile peat moss, covered with transparent plastic bag and kept under fully controlled conditions in a phytotron for acclimatisation. Plastic bags are perforated daily with a few punctures using a pin and bags totally removed before plants are transferred to an insect-proof greenhouse.

Biotechnological Achievements Made via *Agrobacterium*-Mediated Transformation

Agrobacterium-mediated transformation has revolutionised plant genetic engineering and consequently agricultural biotechnology. Several achievements in agricultural biotechnology were made and these included the production of transgenic plants expressing useful agronomic traits. Production of pathogen, viral, bacterial, fungal, nematode and parasitic flowering plant-resistant and abiotic stress-tolerant plants (Wagih, 2004; David *et al*., 2010), insect-resistant crops (Wagih, 2004; Roh *et al*., 2007), herbicide-tolerant soybean (Hinchee *et al*.,1988; Wagih, 2004), salt and drought tolerant rice (Rohila *et al*., 2002; Wagih, 2004; Yang *et al*., 2007), long shelf Life 'Flavr Savr' tomato (Redenbaugh *et al*., 1992; Wagih, 2004). , golden rice (Beyer *et al*., 2002; Wagih, 2004) and blue carnation and rose (Wagih, 2004; Katsumoto *et al*., 2007) are just a few example of the many successes achieved.

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PJan25 vectors series, an enhancement of the gateway vector pgwb533, For broader promoter testing applications

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Abstract

Agrobacterium-mediated transformation of plants has enhanced our ability to progress more rapidly in plant genetic engineering. Development of binary vectors for *Agrobacterium* has played a major role in advancing plant biology. Here, we report new features added to the Gateway vector pGWB533 for promoter testing with the reporter gene encoding β-glucuronidase **(**GUS). The original vector contains spectinomycin for bacterial selection and hygromycin for transformed plant selection. However, some bacterial strains used to transform plants, such as *Agrobacterium rhizogenes* strain K599, have elevated tolerance to spectinomycin, thus making bacterial selection of pGWB533 inefficient with spectinomycin. Although the pGWB533 confers chemical selection for transgenic plants using hygromycin resistance, the plasmid has no visual marker that enables visual selection of transformed plants or transgenic tissue. In this regard, adding a gene to constitutively express green fluorescent protein (eGFP) makes it easier to visually select the transformed tissue and trim out the non-transformed.

In this report we describe a series of vectors. pJan25S, pJan25T and pJan25X, that are enhancements of pGWB533 for promoter testing. All three vectors contain gene encoding eGFP as a visual marker for transformed tissue. However, in pJan25S and pJan25T eGFP is controlled by the RolD promoter for roots specific expression, while it is controlled by the CaMV35S promoter in pJan25X for constitutive expression in all plant tissues. Spectinomycin resistance is incorporated in pJan25S for bacterial selection; however, pJan25T and pJan25X contain the gene encoding tetracycline resistance for bacterial selection. These changes resulted in enhanced vectors which have broader applications in promoter studies where in root tissue or all plant tissue with different bacterial selection.

Key words: Plant genetic engineering, Promoters of gene expression, Pjan25 vector series and gateway vector pgwb533.

Introduction

Many applications in biotechnology require promoters that provide specific temporal and spatial gene expression at a certain level. This can be achieved through the use of specific promoters to express a gene of interest in the correct amount at the specific place and time where it is needed. In this regard, testing promoters to evaluate their temporal and spatial specificity and level of expression is an important area of study in genomics. Many expression vectors with different resistance and reporter genes were developed by Nakagawa (2007) using the Gateway® recombinase cloning system (Invitrogen, Carlsbad, CA). Most of these vectors were derived from pPZP221 (Hajdukiewics 1994) which contained the gene encoding streptomycin/spectinomycin adenylyltransferase *aadA* that confers bacterial resistance to streptomycin. Although, the Gateway® cloning system was used to construct numerous vectors, these vectors cannot be used for all applications that are required for in-depth promoter studies due to certain limitations. For instance, most of the Gateway® vectors use only chemical selection for transformed plants, e.g kanamycin or hygromycin resistance. This requires growing the transformed plants on tissue culture media with the right concentration of chemical and requires sterile technique with all the costs and risks of microbial contamination. In this regard visual selection of transformed plant tissue is much less expensive and easier. For instance, when using *Agrobacterium rhizogenes*-mediated root transformation, some root tissue could be transformed while other tissue is not. However, if the transforming vector contain a gene encoding enhanced green fluorescent protein (eGFP) to transform roots, the transformed roots can be readily distinguished from non-transformed roots by eGFP fluorescence and thus untransformed roots can be easily excised and discarded.

Several research studies require that the transformed and non-transformed plant material to be distinguished. For example, gene silencing constructs were examined to determine if they conferred resistance to nematodes if expressed in soybean roots of composite plants (Klink et al 2009, 2010 and Ibrahim et al. 2011). Non-transformed roots were

visualized using eGFP and non-transformed roots were excised before adding nematodes to the transformed roots. Mature female nematodes were counted thirty five days later. In these cases *Agrobacterium rhizogenes* was used for root transformation and the vector contained the gene encoding eGFP controlled by the *rolD* promoter from *A. rhizogenes* and which is root specific (White et al. 1985; Elmayan and Tepfer 1995).

In this study, the pGWB533 vector (Fig. 1a) was used to develop the pJan25S, pJan25T and pJan25X vectors to be used with *A. rhizogenes* and *A. tumefaciens*-mediated root and whole plant transformation, respectively (Tepfer 1984 and Ko et al 2006). The Gateway (Invitrogen, Carlsbad, CA) compatible pJan25 destination vectors were engineered specifically for *Agrobacterium*-based plant transformation and visualization of transformed tissue and plants.

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Material and methods

Addition of Tetracycline resistance and eGFP

The genes encoding tetracycline resistance (TetR) and green fluorescent protein (eGFP) were amplified from the pRAP17 vector (Klink et al 2009). TetR provides for antibiotic selection of bacteria transformed with the vector, while eGFP provides a visual marker for identifying transformed plant tissue. The gene encoding eGFP was controlled by the rolD promoter and the nos terminator. RolD-eGFP-T35s cassette was amplified using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) from pRAP17 as template (Klink et al, 2009) with cassette-specific primers (Table 1). Amplification reactions were performed according to Williams et al. (1990) in 50 µl. Briefly, each reaction mixture contained lX of 10X High Fidelity PCR Buffer (600 mM Tris-SO4 (pH 8.9), 180 mM ammonium sulfate) , 0.2 mM of 10 µM dNTPs mix (each dATP, dCTP, dGTP and TTP) (Pharmacia), 2 mM of 50 mM MgSO4, 0.2−0.4 μ M of each primer, 50 ng of pRAP 17 DNA, and 1.0 unit of Platinum® Taq DNA Polymerase High Fidelity in a final volume of 50 ul. The PCR program was used as suggested by manufacturer as follows; Initial denaturation: 94°C for2 minutes followed by 35 cycles of: Denature: 94°C for 30 seconds, Anneal: 55°C for 30 seconds, Extend: 68°C for 2 minutes. Final extension for 10 minutes step at 68°C. 10 µl of PCR product was separated by agarose (1 %) gel electrophoresis, stained and loaded in 2 µl EZ-VISION™ ONE (Amresco, USA), at 100 Volts in 1X SB (10mM NaOH solution with boric acid, pH is 8.5) and photographed on a UV transilluminator (Pharmacia) by a Canon S5 digital camera with a UV filter adaptor. A negative control which contained all the necessary PCR components except template DNA was included for PCRs. The RolD-eGFP-T35s cassette was purified using the MinElute PCR Purification Kit (Qiagen, Santa Clarita, CA) following the manufacturer's instructions.

The RolD-eGFP-T35s cassette was engineered into a HindIII site (Invitogen,
arlsbad, CA), following the manufacturer's instructions resulted in pJan25S. The HindIII site lies inside the left and right T-DNA borders which are transferred into plant tissue upon transformation (Fig.1a,b). The TetR cassette was isolated from pRAP17 using BstEII restriction enzyme (Invitogen, Carlsbad, CA), followed by gel purificationusing a Gel Purification Kit (Qiagen, Santa Clarita, CA) following the manufacturer's instructions.

Table 1. The primers pairs used in the isolation of genes used in this study

	Primer name	Sequence
1	**RolD-eGFP-T35s ClaI Forward**	**ATCGATGCATGCCTGCAGGTTAG**
	RolD-eGFP-T35s ClaI Reverse	**ATCGATGCAGGTCACTGGATTTTGG**
2	**CAMV35s[2] HindIII Forward**	CGTCAAGCTTAACATGGTGGAGCACGACAC
	CAMV35S[2] EcoRI Reverse	GAATTCAGAGAGAGACTGGTGATTTCAGCG
3	**FMV Forward plus CACC**	Cacc TTTACAGTAAGAACTGATAAC
	FMV Reverse	Cactccccctctctaaaaaa
4	**GUS Forward**	**AGGAAGTGATGGAGCATCAG**
	GUS Reverse	**CATCAGCACGTTATCGAATCC**
5	**Tet-BF (BSTEII)**	**TCG TGG TCA CCA TCA GCG ATC GGC TCG TT**
	Tet-BR (BSTEII)	**AGC GGT GAC CAT CAA TCG TCA CCC AAA**

The quality and concentration of the purified fragments were measured using the Nanodrop spectrophotometer. The TetR cassette was engineered into a BstEII site of Gateway vector pGWB533 which was digested by the same restriction site. The 5'-phosphates from pGWB533 linear plasmid was removed using Shrimp Alkaline Phosphatase (SAP) at 37 C for one hour to prevent self-ligation of the plasmid. The SAP was inactivated by incubation 65 C for 15 minutes. The BstEII site lies outside the left and right T-DNA borders and will not be transferred to plant tissue (Fig.2b). Adding the TetR cassette to pJan25S resulted in the formation of pJan25T. Replacing the RolD-eGFP-T35s from pJan25T with CaMV35s-eGFP-T35s in the HindIII site resulted in pJan25X. The pJan25X with the CaMV35 promoter driving the eGFP gene expresses the green florescent protein in all the transformed plant tissue for other application.

PCR Amplification for testing positive clones
Separate individual colonies were selected and grown overnight in 5ml LB culture supplemented with tetracycline, 10 µg/mL, for pJan25T and pJan25X or with spectinomycin 100 µg/mL for pJan25S. Cells from overnight cultures were collected by centrifugation and the plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Santa Clarita, CA). All isolated plasmids from each culture were used as a template in a 20 ul PCR reaction to confirm the presence of the all the genes in the plasmid in right orientation using Invitrogen Taq PCR kit following manufacturer instructions (Invitrogen, Carlsbad, CA). Polymerase chain reaction was started with an initial denaturation of 94°C for 5 minutes followed by 25 cycles of denaturation at 94°C for 35 seconds, annealing (based on the primers used, Table1)°C for 35 seconds, and extension at 72°C for 2 minute per kb of PCR product. Final extension of the amplification was for 10 minutes denaturation at 72°C. All PCR reactions were performed using in DNA Engine Tetrad 2 Peltier Thermal Cycler, Bio-Rad, USA.

Transfer FMV promoter from pENTR plasmid to pJan25
The FMV promoter fragment was transferred from pENTR plasmid to the pJan25T , pJanS and pJan25X plasmids using Gateway® LR Clonase™ II Enzyme Mix kit and following the manufacturer instruction (Invitrogen, Carlsbad, CA). All the pJan25 plasmids harboring the isolated FMV fragment were used to transform *E.coli* cells (One Shot® Mach1™-T1R Chemically Competent cells} (Invitrogen, Carlsbad, CA). The transformed cells were grown over night on LB selective media containing 10ug/ul tetracycline for pJan25T and pJan25X, and 100ug/ul for pJan25S. The pJan25 plasmids were isolated from overnight cultures using Spin Miniprep Kit (Qiagen, Santa Clarita, CA) and prepared to transform onion cells using Biolistic gene transfer method**Microprojectile bombardment**
Micro projectile bombardment was conducted using the Biolistic Particle Delivery System (BioRad; Hercules, CA) according to the manufacturer's instructions. The PDS-1000/He instrument was used with gold microprojectiles coated with plasmid 6 ul DNA 1ug/ul. The gold particles were carried on the macro carriers and bombarded into layers of onion cells using rupture disk that withstand pressure 1100 PSI. Each construct were delivered into three replicates.

Fluorescence was monitored using a Nikon SMZ 1500 stereomicroscope. The eGFP filter (Nikon Corporation; Tokyo, Japan). Stereomicroscope images were captured with an Optronics MagnaFire model S99802 CCD camera (Optronics; *Goleta, CA)* Fig 1, b3a and c3a. The functionality of pJan25 was tested using the FMV-sgt promoter driving GUS expression with the uidA reporter gene. Transformed Onion tissue was incubated with xgluc to determine β-glucuronidase activity. GUS activity was revealed with the GUS stain (2 mM 5-bromo-4-chloro-3-indolyl glucuronide, 100 mM potassium phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100) (Jefferson et al. 1987). The colorimetric reaction proceeded by immersion in GUS stain and subsequent vacuum infiltration with 500 ul of GUS stain for 10 minutes. Tissue was subsequently incubated at 37 C overnight to promote development of the GUS stain. These two validation experiments demonstrated activity of both the rolD and CaMV35 promoter, driving eGFP expression, and FMV-sgt ,driving GUS expression, in pJan25.

Results and discussion

Overview of pJan25S, pJan25T and pJan25X
The pJAN25 vectors were developed from the pGWB533 vector (12,977 Pb) which contains the GATEWAY® attR1 and attR2 sites for easy cloning of promoters of interest (Fig1.a). The attR2 site is adjacent to the gene encoding GUS which serves as a reporter gene for promoter activity (Fig1.b) .
The *rolD* promoter-eGFP-T35s cassette was added to pGWB533 to provide visual selection of transformed root material and making a new construct which we called pJan25S (Fig1. b). The pJan25S plasmid provides spectinomycin resistance for bacterial selection (Fig1. e). PCR-based tests demonstrated positive PCR amplification of the eGFP gene in bacterial clone's plasmid DNA (Fig1. c). Yet, the genes in construct have to be tested as transcriptionally competent (Fig1. d). The functionality of the pJan25S were examined using the figwort mosaic virus subgenomic transcript (FMV-sgt) promoter (Bhattacharyya et al. 2002) to drive GUS transcript (Fig1. f&g). Microprojectiles bombardment of onion layers was used for rapid (24 h) visual confirmation that pJan25S constructs had positive eGFP expression and thus were competent for visual screening (Fig1. d).
Agrobacterium rhizogenes (Strain K599) was used for plant transformation and in the production of composite plants with transformed roots (Klink et al 2009, 2010 and Heba 2011). However, strain K599 has an elevated tolerance to spectinomycin, but it is very sensitive to tetracycline. Thus, adding a tetracycline (TetR) resistance gene to pJan25S for bacterial selection resulted in pJan25T which much more efficient for selection (Fig2 a & b). In this regards, tetracycline (TetR) resistance was successfully engineered into a BstEII site that lies outside the left and right borders and produced higher rate of positive clone (Fig2. e). The pJan25T construct were used to transform onion cells (Fig2.d).

To test the functionality of the pJan25T, the figwort mosaic virus subgenomic transcript (FMV-sgt) promoter (Bhattacharyya et al. 2002) was inserted from pENTR plasmid into pJAN25T vectors in attR1attR2 cloning site replacing the chloramphenicol acetyltransferase gene and drive GUS transcript using LR Clonase reaction (Invitrogen, Carlsbad, CA). The eGFP expression and GUS expression were positive in onions cells indicating the functionality of the plasmid (Fig2. d, f). These constructs then were shuttled into *Agrobacterium rhizogenes* K599 and used for root transformation experiments (Fig2.I).

The pJan25X vector was constructed by replacing the *rolD* promoter-eGFP-T35s with CaMV35s-eGFP-T35s in the HindIII site (Fig 3 a&b). The pJan25X vector was tested for Tetracyclin resistance by growing on LB plats contain (10 ug/ul) tetracycline (Fig3. C).The expression of eGFP was tested by shooting the pJan25X plasmids into Onion cells as described in Material and Methods section (Fig 3 d). The functionality of the plasmids were tested by adding the figwort mosaic virus subgenomic transcript (FMV-sgt) promoter (Fig3e) and delivered in onion by gene gun and tested for driving -glucuronidase (GUS) reporter activity (Fig3f)

In summary, The pJan25S vector contains the gene encoding spectinomycin , meanwhile the pJan25T and pJan25X vectors contain the gene encoding tetracycline resistance for bacterial selection and all contain enhanced Green fluorescent protein (eGFP) (Haseloff et al. 1997) for visual screening of the transformed tissue. The eGFP gene product is a visual beacon for screening transformed roots in a non-axenic conditions (Collier et al. 2005) and works particularly well in roots studies (Klink et al. 2008).

All vectors were examined for their functionality using the figwort mosaic virus subgenomic transcript (FMV-sgt) promoter (Bhattacharyya et al. 2002) driving -glucuronidase (GUS) reporter activity. All plasmids were used to transform Soy bean roots (Fig 4 a,b and c) an can be used as a perfect tool for studying active promoter against roots pathogens in plants.

Acknowledgments

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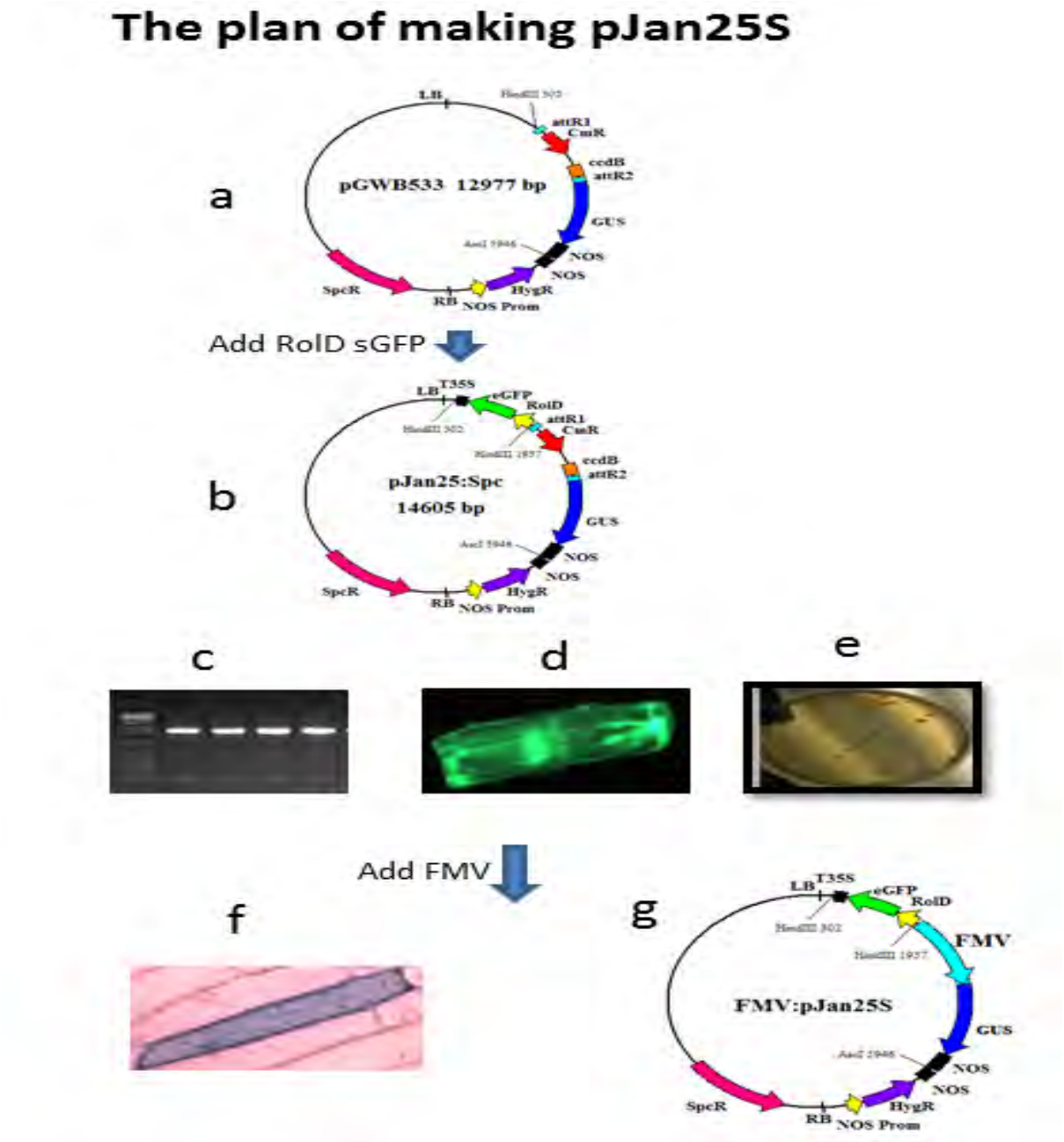

Figure 1. A diagram presenting the modifications and enhancement of the Gateway vector pGWB533 that resulted in pJan25S vector for testing the spatial and temporal expression of promoters. (a) The original feature and structure of the pGWB533 Gateway vector; (b) the construct map of the pJan25S vector that resulted from adding a cassette containing the RolD promoter driving eGFP followed by terminator. (c, d, and e) show the amplicon sizes inserted into the vector, expression of eGFP in a transformed onion cell, and colonies growing on spectinomycin plates respectively. (f) shows Gus activity with the FMV promoter in a cell transformed by pJan25S. (g) Shows the FMV:pJan25S construct map.

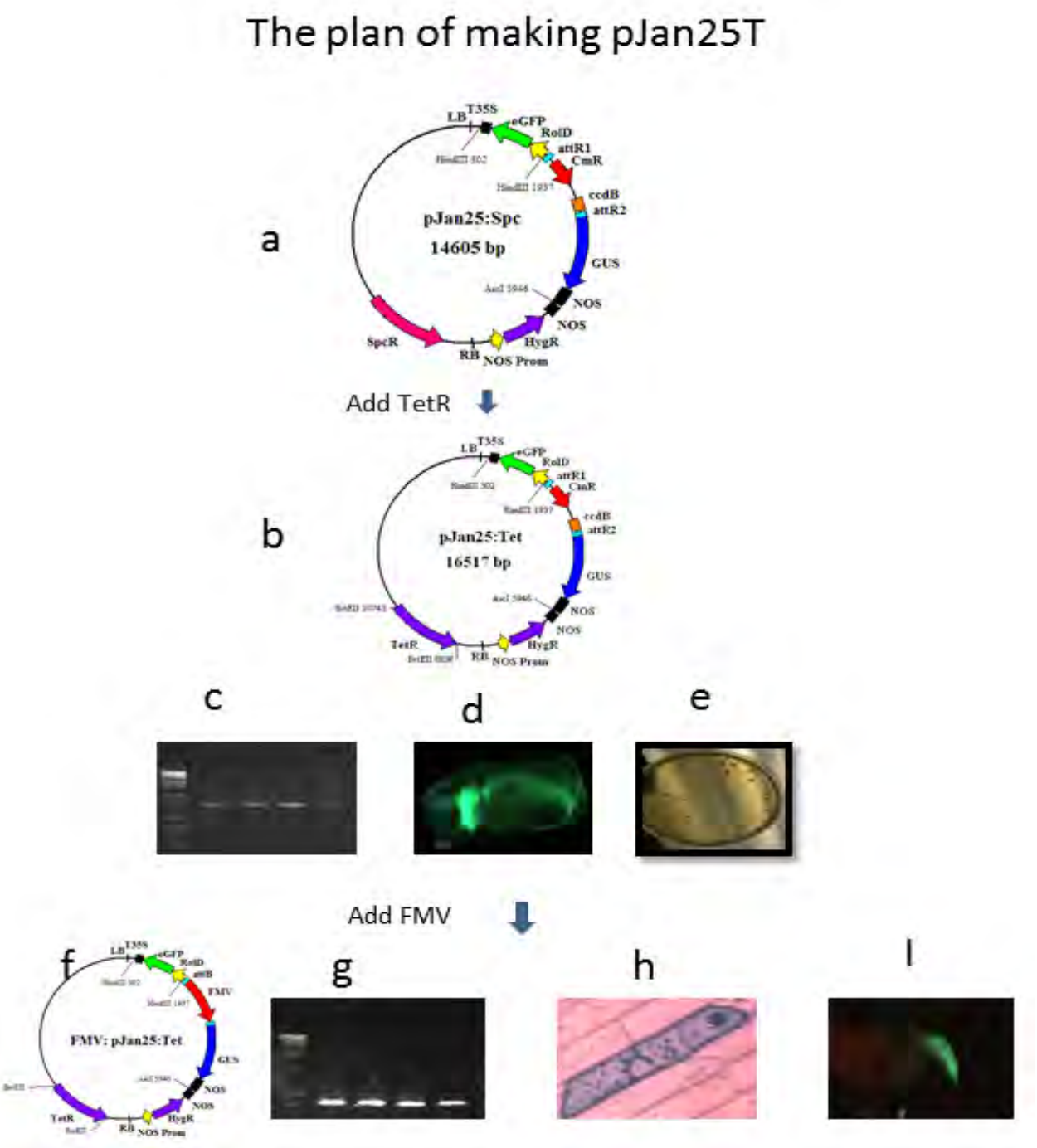

Figure.2 A diagram presenting the modifications of pJan25S that resulted in pJan25T vector for testing the spatial and temporal expression of promoters .(a, b) shows the map of pJan25S before and after adding the tetracycline resistance (tetR) gene which resulted in pJan25T respectively. (C) the tetR amplicon; (d) transformed cell exhibiting fluorescence; (e) colonies growing on the tetracycline plates. (f) map of pJan25T with FMV promoter; (g) FMV amplicon; (h) FMV driving Gus activity in onion cell. (I) The root specific eGFP fluorescence of Jan25 in G. Max roots

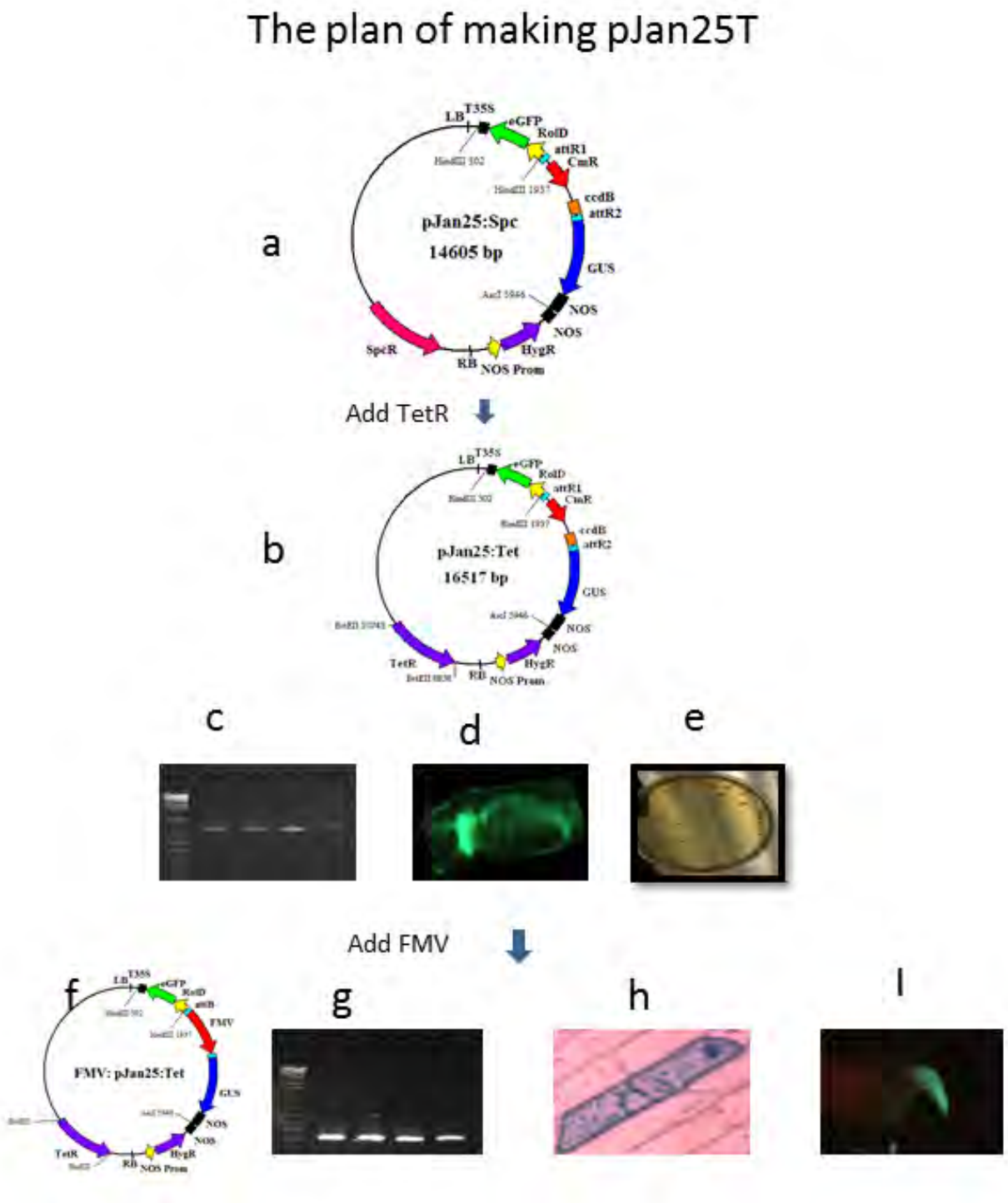

Figure.3 A diagram presenting the modifications of pGWB533 that resulted in pJan25X vector for testing the spatial and temporal expression of promoters (a, b) shows the map of pJan25X after adding the tetracycline resistance (tetR) gene and eGFP driven by CaMV35s promoter respectively. (C) transformed onion cell with pJan25X exhibiting fluorescence; (d) colonies growing on the tetracycline plates. (e) FMV amplicon; (h) FMV driving Gus activity in onion cell.

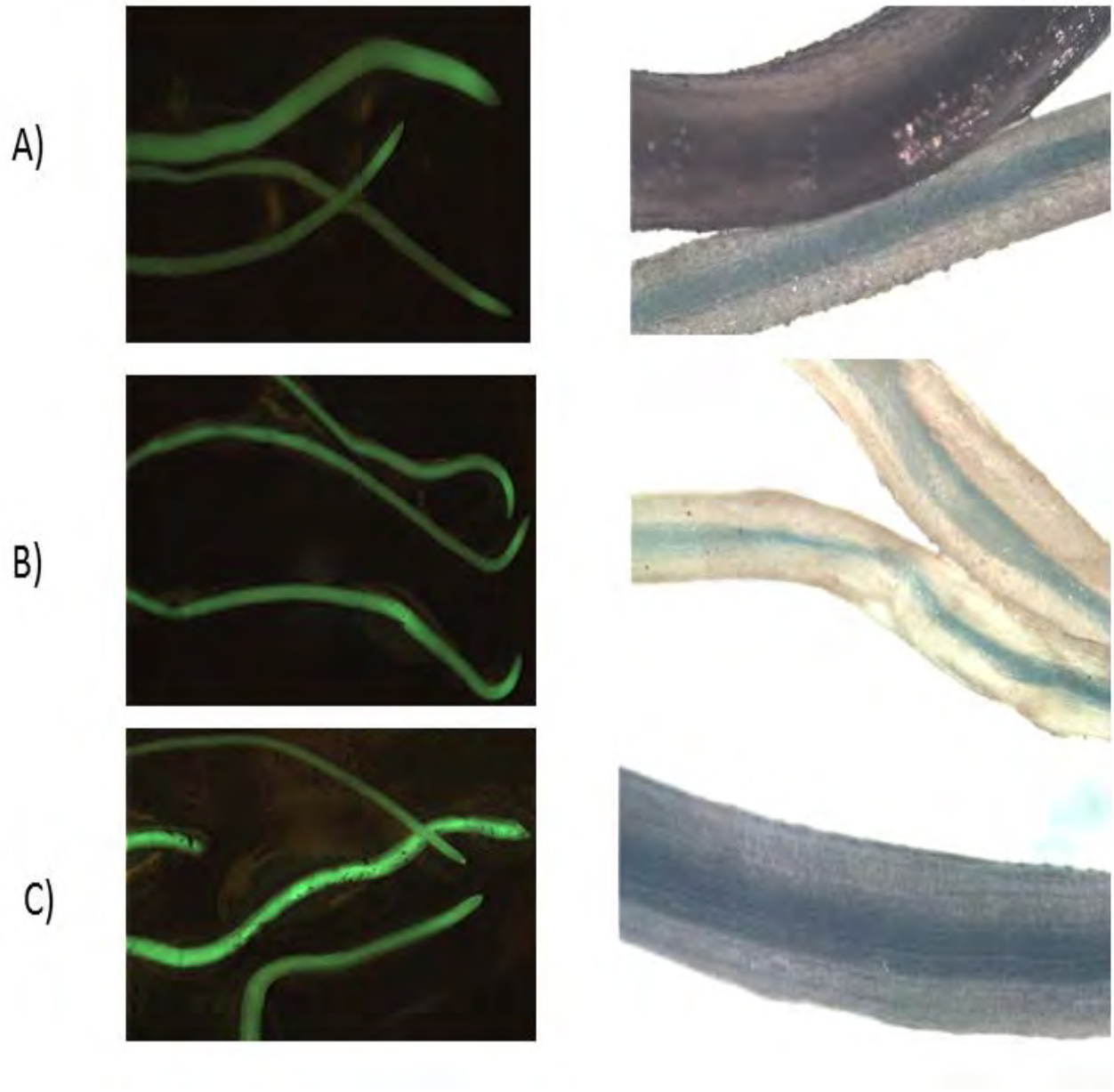

Figure.4. Shows the expression of eGFP and GUS gene in the transformed plant roots with pJan25 constructs. A) Soy bean roots exhibiting fluorescence (left) and GUS staining (right) after transformation with FMV:pJan25S ; B) Soy bean roots exhibiting fluorescence (left) and GUS staining (right) after transformation with FMV:pJan25T ; C)Soy bean roots exhibiting fluorescence (left) and GUS staining (right) after transformation with pJan25X

Nano-biotechnologies and their Agricultural Applications

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Abstract

Recent developments in the field of nanotechnology have paved the way for lots of innovation in a number of industrial and consumer sectors, including Agriculture and Agriculture industry. Whilst, nano-Agriculture includes nanofood, nanopesticides, nano-fertilzers, and nano-biotechnology sectors are relatively new emergent, they are widely expected to grow rapidly in the future. Nowadays, markets in some countries have a number of nano-sized additives and supplements for food and healthfood products, and their number is expected to increase in the coming years. Whilst such developments offer enormous benefits to the food sector, they have also raised a number of issues in relation to consumer safety, environmental impacts, and ethical, policy and regulatory aspects. In this paper, we will go through the definition of nanotechnology, history of evolution, current and future applications, in addition to that, the possibility to characterize materials produced by this technology in addition to the required risk assessment of such nanomaterials

Key Words: Nanotechnology, agricultural applications.

Introduction

"Nanotechnology has given us the tools… to play with the ultimate toy box of nature – atoms and molecules. Everything is made from it… The possibilities to create new things appear limitless."
On Dec. 29, 1959, at the California Institute of Technology, Nobel Laureate Richard R Feynman gave a talk at the annual meeting of the American Physical Society that has become one of the twentieth century's classic science papers, titled "There's Plenty of Room at the Bottom" [Feynmann, 1960]. He presented a technological vision of extreme miniaturization several years before the word "chip" became part of the lexicon. He talked about the problem of manipulating and controlling things on a small scale. Extrapolating from known physical laws, Feynman envisioned a technology using the ultimate toolbox of nature, building nano objects atom by atom or molecule by molecule. Since the 1980s, many inventions and discoveries in the fabrication of nanoobjects have become a testament to his vision.
Nanotechnology is the manipulation or self-assembly of individual atoms, molecules, or molecular clusters into structures to create materials and devices with new or vastly different properties. Nanotechnology can work from the top down (which means reducing the size of the smallest structures to the nanoscale e.g. photonics applications in nanoelectronics and nanoengineering) or the bottom up (which involves manipulating individual atoms and molecules into nanostructures and more closely resembles chemistry or biology). The definition of nanotechnology is based on the prefix "nano" which is from the Greek word meaning "dwarf". In more technical terms, the word "nano" means 10-9, or one billionth of something. For comparison, a virus is roughly 100 nanometres (nm) in size. The word nanotechnology is generally used when referring to materials with the size of 0.1 to 100 nanometers; however it is also inherent that these materials should display different properties from bulk (or micrometric and larger) materials as a result of their size. These differences include physical strength, chemical reactivity, electrical conductance, magnetism, and optical effects [Joseph, 2006].

History

- Democritus in ancient Greece: concept of atom
- Rutherford, 1900: discovery of atomic nucleus
- 1959 Feynman's talk- prospects for miniaturization investigated
- 1968 Alfred Cho and John Arthur of Bell labs invent molecular –beam epitaxy, a technique to deposit single atomic layers on a surface
- 1981 Gerd Binnig and Heinrich Rohrer create the STM which can image single atoms. Nobel prize.
- 1985 Robert Curl, Harold Kroto and Richard Smalley discover buckyballs which are about 1 nm in diameter
- 1986 K. Eric Drexler publishes "Engines of Creation" a futuristic book about nanotech
- 1989 Donald Eiger of IBM writes letters "IBM" using single atoms
- 1991 Sumio Iijima of NEC Japan discovers carbon nanotubes.
- 1993 Warren Robinett of Univ N Carolina and R. Stanley Williams of UCLA devise a virtual reality system connected to an STM that lets users see and touch atoms
- 1998 Delft Univ of Technolgy in Netherlands creates a transistor from a carbon nanotube

- 1999 James Tour, now at Rice U. and Mark Reed of Yale demonstrate that single molecules can act as switches. (snap a wire then put molecule between STM like tips)
- 2000 The Clinton administration announces NNI, the National Nantotechnology Initiative- large funding now available for projects in nanotech.
- 2000 Eigler and other devise a quantum mirage- placing a magnetic atom at the focus of an elliptical ring of atoms creates an mirage atom at the other focus- transmitting info without wires?

Types of Nanomaterials

There are many types of intentionally produced nanomaterials, and a variety of others are expected to appear in the future. For the purpose of this paper, most current nanomaterials could be organized into four types:

Carbon-based materials. These nanomaterials are composed mostly of carbon, most commonly taking the form of a hollow spheres, ellipsoids, or tubes. Spherical and ellipsoidal carbon nanomaterials are referred to as fullerenes, while cylindrical ones are called nanotubes. These particles have many potential applications, including improved films and coatings, stronger and lighter materials, and applications in electronics. Figures 1 shows examples of carbon-based nanomaterials.

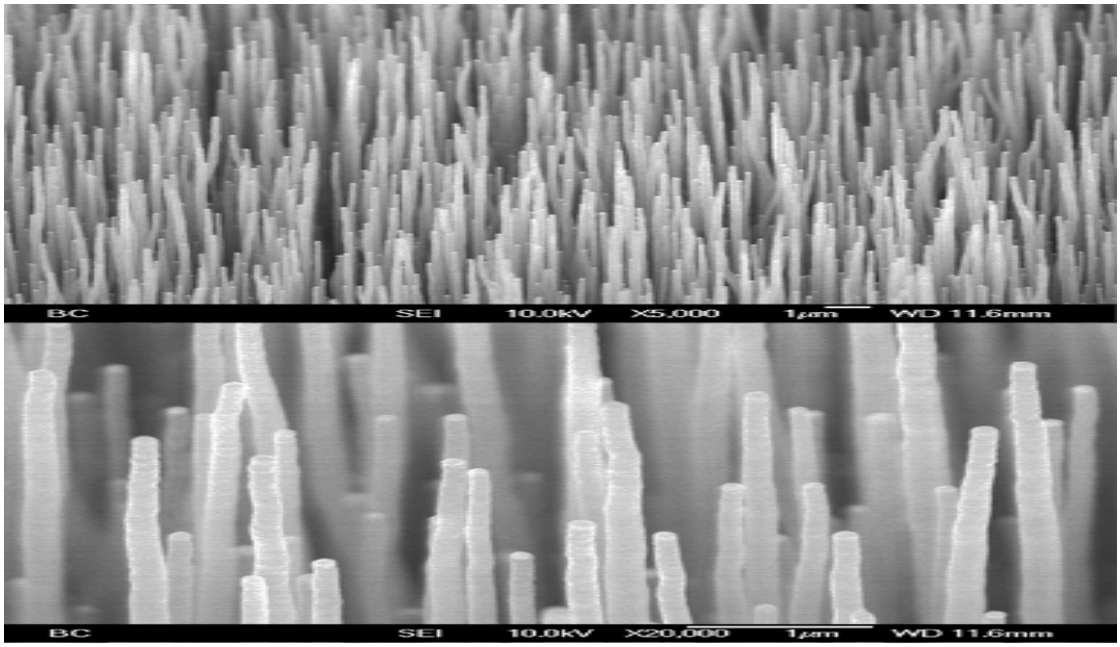

Figure 1. "Forest" of aligned carbon nanotubes. (Image courtesy David Carnahan of NanoLab, Inc.)

Metal-based materials. These nanomaterials include quantum dots, nanogold, nanosilver and metal oxides, such as titanium dioxide. A quantum dot is a closely packed semiconductor crystal comprised of hundreds or thousands of atoms, and whose size is on the order of a few nanometers to a few hundred nanometers. Changing the size of quantum dots changes their optical properties. Figures 2 shows examples of metal-based nanomaterials.

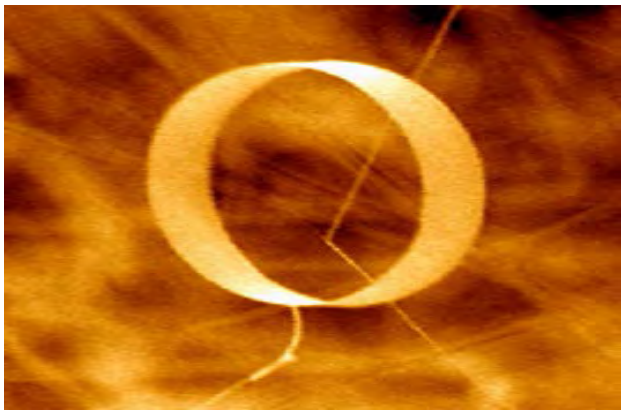

Figure 2. Zinc oxide nanostructure synthesized by a vapor-solid process. (Image courtesy of Prof. Zhong Lin Wang, Georgia Tech)

(1) Dendrimers. These nanomaterials are nanosized polymers built from branched units. The surface of a dendrimer has numerous chain ends, which can be tailored to perform specific chemical functions. This property could also be useful for catalysis. Also, because three-dimensional dendrimers contain interior cavities into which other molecules could be placed, they may be useful for drug delivery. Figure 3 shows an example a dendrimer.

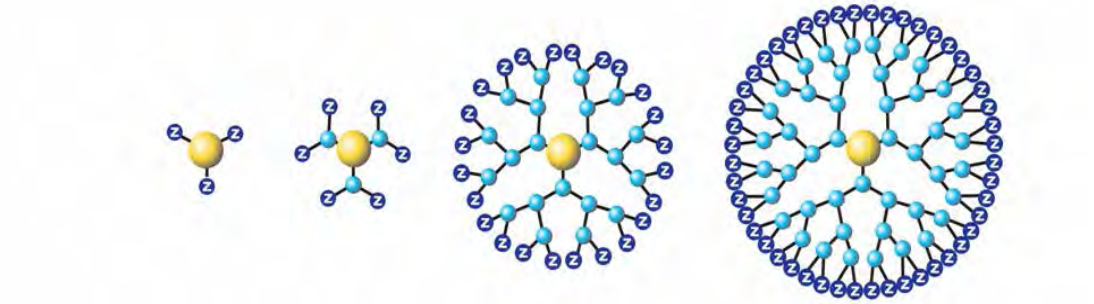

Figure 3. Computer image of generations of a dendrimer. Dendrimers are nanoscale branched polymers that are grown in a stepwise fashion, which allows for precise control of their size. (Image courtesy of Dendritic NanoTechnologies, Inc.)

Composites combine nanoparticles with other nanoparticles or with larger, bulk-type materials. Nanoparticles, such as nanosized clays, are already being added to products ranging from auto parts to packaging materials, to enhance mechanical, thermal, barrier, and flame-retardant properties. Figure 4 shows an example of a composite.

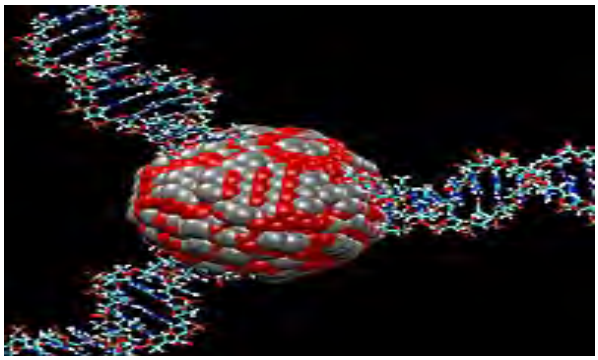

Figure (4). Computer image of a nano-biocomposite. Image of a titanium molecule (center) with DNA strands attached, a bio-inorganic composite. This kind of material has potential for new technologies to treat disease. (Image courtesy of Center for Nanoscale Materials, Argonne National Lab**).**

The unique properties of these various types of intentionally produced nanomaterials give them novel electrical, catalytic, magnetic, mechanical, thermal, or imaging features that are highly desirable for applications in commercial, medical, military, and environmental sectors.

Preparation & Designing of Nanomaterials

Nanotechnology is the manipulation of matter for use in particular applications through certain chemical and / or physical processes to create materials with specific properties. There are both "bottom-up" processes (such as self-assembly) that create nanoscale materials from atoms and molecules, as well as "top-down" processes (such as milling) that create nanoscale materials from their macro-scale counterparts. Figure 5 shows an example of a nanomaterial assembled through "bottom-up" processes. Nanoscale materials that have macro-scale counterparts frequently display different or enhanced properties compared to the macro-scale form.

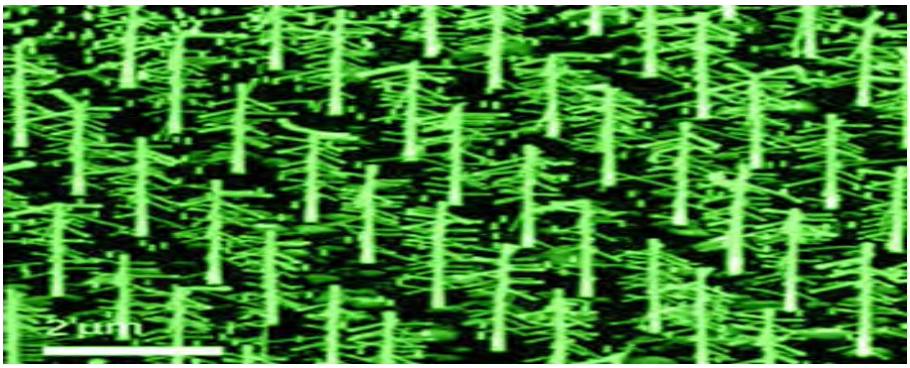

Figure (5). Gallium Phosphide (GaP) Nanotrees. . (Image used by permission, Prof. Lars Samuelson, Lund University, Sweden. [Dick et al. 2004])

Characterization methods of nanomaterials

The fundamental of nanotechnology lies in the fact that properties of materials change dramatically when their size is reduced to the nanometer range. But measuring this nano dimension is not a very easy task. Although research is going on to synthesise nanostructured and nanophasic materials, characterizing these nano sized materials is also an emerging field posing lot of challenges to scientists and technonologists. Thus, nanotechnology has motivated the upsurge in research activities on the discovery and invention of sophisticated nano characterization techniques to allow a better control of morphology, size and dimensions of materials in nano range. The most important characterization techniques used for nanotechnology research are the microscopic techniques [Joshia, 2008].

Microscopic Characterisation of Nanomaterials

Optical microscopes are generally used for observing micron level materials with reasonable resolution. Further magnification cannot be achieved through optical microscopes due to aberrations and limit in wavelength of light. Hence, the imaging techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM/HRTEM), scanning tunneling microscopy (STM), atomic force microscopy (AFM), etc. have been developed to observe the sub- micron size materials. Though the principles of all the techniques are different but one common thing is that they produce a highly magnified image of the surface or the bulk of the sample. Nanomaterials can only be observed through these imaging techniques as human eye as well as optical microscope cannot be used to see dimensions at nano level.

Applications of nanotechnology

Nanotechnology is expected to become very important in various agricultural applications such as food and feed manufacturing, where it is expected that the production of nano food and feeds will be the challenge to solve the starvation problems facing the world in this century. Another promising areas are drug delivery, molecular imaging, biomarkers, and biosensors. Target-specific drug therapy and methods for early diagnosis and therapy of plant diseases are among the priority research areas for application of nanotechnology. Further, nanotechnology could play a very important role in environmental science, for example, via the development and usage of nanospheres to trap heavy metals and toxic metals, and via nanopore materials that can filter out bacteria, viruses, and toxins from food, water, soil and air. That is why a great need for establishing nanotechnology lab and building quality control system specified for nanomaterials enable the scientist and government to be in the first line beside the developed countries. In addition, assessing the potential hazards of this technology's nanoparticles, and the nanomaterials manufactured using the nanoparticles is an emerging area in toxicology and health risk assessment. The development of toxicity data sets and exposure assessments for various nanoparticles and nanomaterials is ongoing as new particles and materials are developed.

Risk Assessment of Nanomaterials

Nanotechnology is expected to become very important in various biomedical applications such as in drug delivery, molecular imaging, biomarkers, and biosensors. Target-specific drug therapy and methods for early diagnosis and therapy of diseases are among the priority research areas for application of nanotechnology. Further, nanotechnology could play a very important role in environmental science, for example, via the development and usage of nanospheres to trap polychlorinated biphenyls and toxic metals, and via nanopore materials that can filter out bacteria, viruses, and toxins from water [Borm, 2002]. That is why assessing the potential hazards of this technology's nanoparticles, and the nanomaterials manufactured using the nanoparticles is an emerging area in toxicology and health risk assessment. The development of toxicity data sets and exposure assessments for various nanoparticles and nanomaterials is ongoing as new particles and materials are developed. A related issue in toxicology and risk assessment is the extent to which nanoparticle toxicity can be extrapolated from existing toxicology databases for particles and fibers. From the toxicological point of view, it is very important to consider the nanomaterial as a new material which must be tested with complete toxicological evaluation even its bulk form has a known studied toxicological data. Other information needs being addressed includes the environmental and biological fate, transport, persistence, and transformation of manufactured nanoparticles, and the recyclability and overall sustainability of manufactured nanomaterials. Outline of the testing and evaluation of new chemicals is illustrated in figure 6.

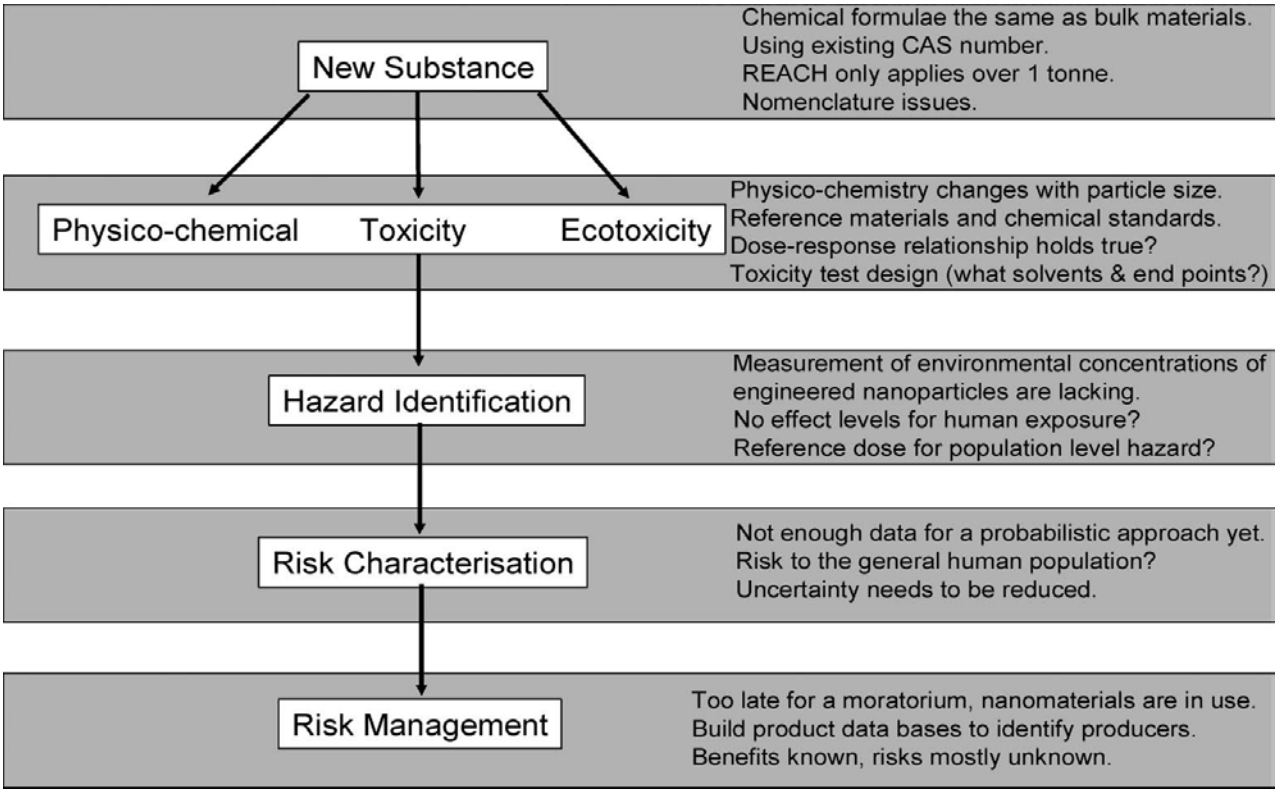

Figure 6. A simplified generic outline of the testing and evaluation of new chemicals

The identification and characterization of chemical substances and materials is an important first step in assessing their risk. Understanding the physical and chemical properties in particular is necessary in the evaluation of hazard (both toxicological and ecological) and exposure (all routes). Chemical properties that are important in the characterization of discrete chemical substances include, but are not limited to, composition, structure, molecular weight, melting point, boiling point, vapor pressure, octanol-water partition coefficient, water solubility, reactivity, and stability. In addition, information on a substance's manufacture and formulation is important in understanding purity, product variability, performance, and use.

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Application of Biotechnologies in Plant Pathology

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Abstract

One of the ultimate aims of agriculture biotechnology is to feed an expanding world population. A recent survey by the Economist shows that the world population has increased by 90% in the past 40 years while food production has increased by only 25%, with the additional 1.5 billion mouths to feed by 2020. Farmers worldwide will have to produce 39% more grain .Genetic engineering has the potential to provide a cornucopia of beneficial plant traits, particularly an enhanced ability to withstand or resist attack by plant pathogens. New approaches to plant disease control are particularly important for pathogens that are difficult to control by existing methods. The percentage of crop losses caused by plant pathogens, insect pests and weeds has steadily increased to 42% worldwide accounting for $500 billion dollars worth of damage. Worldwide pesticides applications costing $26 billion annually are applied to manage pest losses. Genetically engineered plants resistant to plant pathogens can prevent crop losses and reduce pesticide usage. This article provides a current perspective on the major areas of research and application of plant genetic engineering for resistant to plant pathogens.

Key Words: Application, plant pathology, biotechnology, pesticides.

Understanding plant-microbe interaction

Before biotechnologies, plant- microbe interaction considered dark area , it was very difficult to answer many principle questions about pathogen specification on particular hosts and how the plant can resist the pathogens and what kind of molecular aspect underlying vertical or horizontal resistance . Therefore, biotechnologies played very important roles in the following areas:

Identification of genes of pathogenicity of fungi, *Oomycetes*, bacteria, nematodes, viruses….

By the beginning of twenty one century more than 1000 genes of pathogenecity were identified in phytopathogenic fungi and Oomycetes, these genes were classified into different groups according to their roles in fungal-plant microbe interaction and they identified as:

- Genes of fungal enzymes e.g pectinases, cellulases, hemicellulases, proteases etc….
- Genes of toxins
- Genes of the types of secretion
- Genes adapt the pathogen to interact with host cell environment

And many other genes, biotechnology proved that any directed mutation of any of them resulted in loss of pathogenicity.

Until recently, phenotypic conversation of the bacteria *Ralestonia solanacearum* from saprophytic stage to pathogenic stage was one of the most obscure topics of plant pathology, now thanks to biotechnology, the role of transcription factors in the conservation of *PhcA* gene become clear.

Agrobacterium tumefaciens, the crown gall pathogen of many host plants has become one of the most promising agents in all aspects of biotechnology. Its plamid Ti is the best agent in caring many genes to many living things including plants monocot or dicot, yeasts, fungi, and even human cells. Now still clear its role in inducing tumors in infected plants and how many *vir* genes involved in transportation of Ti region of Ti plasmid.

Biotechnology plays very important role in modeling quorum sensing and type III secretion system in bacterial plant pathogens and determining their genes involved in all parts of secretion needle.

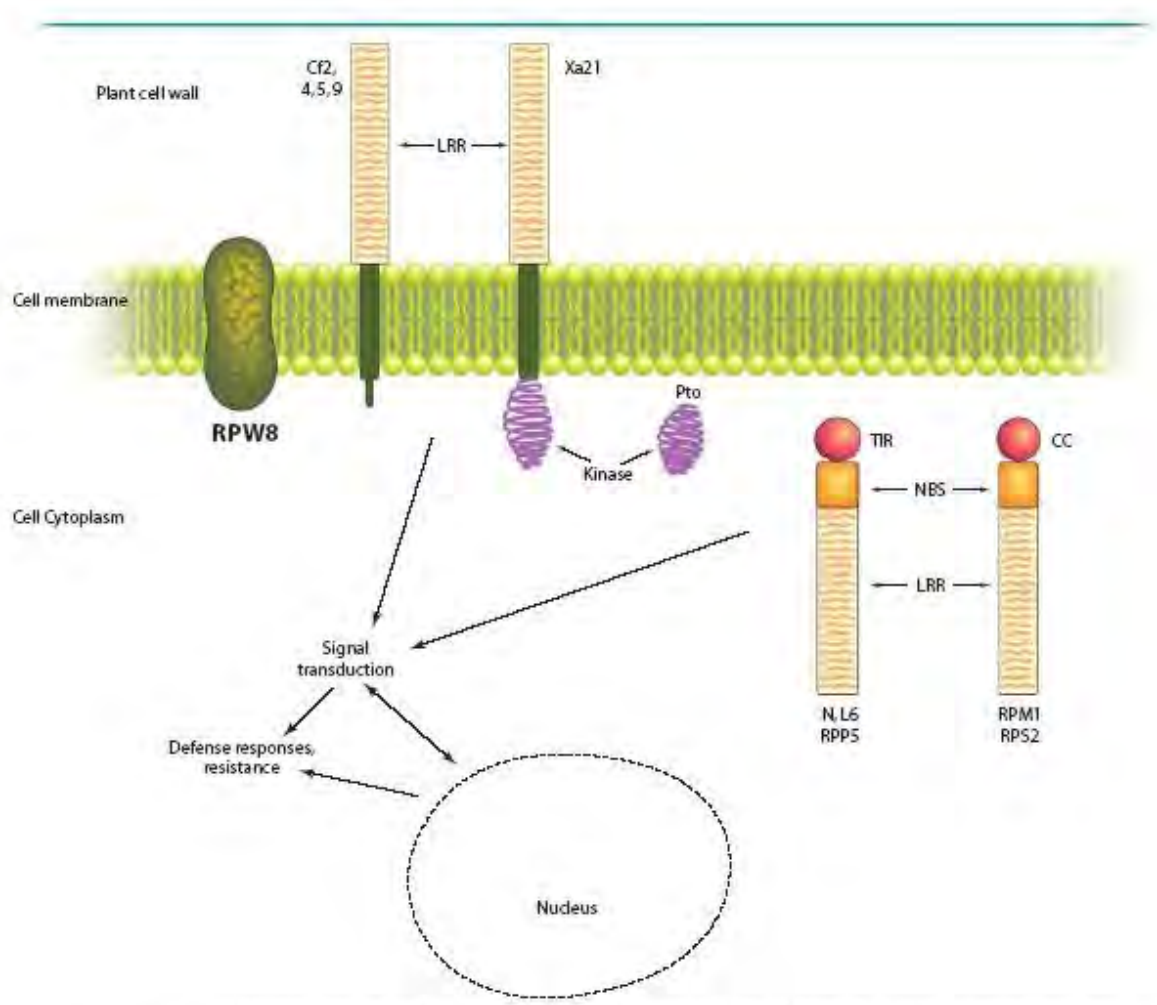
Genetics of the interaction between phytopathogenic nematodes and their hosts still obscure. Biotechnology contributed to the understanding of some genes of their pathogenicity on host plants especially in their feeding sites in plant root and how they up regulate genes of actin and mucin microfibers of plants to serve mitotic division of cell nucleus for the establishment of feeding site.

Mechanism of plant virus evolution and identification of genetics of bottlenecks become one of the promising fields of biotechnology. *Rep*, coat protein and translocation genes playing the essential role in virus pathogenicity, extensively studied in many laboratories all over the world.

Identification of resistance genes (R genes) and their role in plant disease resistance

Thousands years ago, farmers noticed the presence of differences within their plant population in their fields toward diseases affecting their crop. The majority of plants heavily suffered from infection, and few plants showed different degrees of resistance. In fact, farmers tended to select resistant plant for further cultivation and hence those earlier technologists have changed the genetic structure of

plant population, and thus without realizing, they contributed in changing microbial population.
During the 1930th Flor has started his experiments on the genetics of line resistance toward the rust fungus *Melampsora lini*, he has reached to very important conclusion: resistant trait is dominant and pathogenicity trait is recessive. His conclusion nominated as gene-for-gene concept.
During 1963 van der Plank has published his book: Plant Diseases. In this book he differentiated between two types of resistant: Flor's concept (vertical resistance) controlled by single or oligogenes and horizontal resistance controlled by many genes (polygenic resistance).
Since genome project has appeared for the presence in his directions: functional genetics and positional genetics, R genes of plants and their function become in the focus of attention in many laboratories all over the world.
Biotechnologists have proved that R genes encoded for receptor sites localize in plant cellular membrane and/ or within cytoplasm and recognize pathogen elicitors led to stimulation of genes of resistant factors.



Schematic diagram of the structure and cell location of the six types of R-coded receptor proteins. Three types have transmembranous domains, while the other three are membrane-associated cytoplasmic proteins. LRR, leucine-rich repeats; NBS, nucleotide-binding site. TIR, Toll–interleukin-1 resistance receptor domain; CC, coiled coil with leucine zipper domain. Genes listed are tomato *Cf*-2, -4, -5, -9, rice Xa21, tomato Pto, tobacco N, flax L6, Arabidopsis RPM1, RPS2, RPP5, and the Arabidopsis broad-spectrum gene RPW8.

Most isolated R genes encode proteins possessing a leucine-rich-repeat (LRR) domain, of which the majority also contains a nucleotide-binding site (NBS) domain. It was proved the presence of similarity and/or domain homology between plant R protein and animal immunity proteins.

Molecular biology technique postulated the presence of two basic strategies for an R protein to recognize pathogen effectors, which called avirulence factor: Avr: direct physical interaction and indirect interaction via association with other host protein targeted by the Avr factor. Direct R-Avr recognition

leads to high genetic diversity at paired R and Avr loci due to balancing selection.

What about horizontal resistance, the modern advancement in biotechnological equipments provided scientists by amazing techniques to study plant genomics and identification of many genes encoded in the same time. These equipments include two-dimension electrophoreses, capillary electrophoreses and microarrays. Nowadays we become familiar with many aspects of horizontal resistance.

Molecular diagnostic of plant pathogens

Conventional methods of plant pathogen identification often depended on identification of disease symptoms, isolation and culturing of the organism, and identification by morphology and biochemical testes. The major limitations of these culture based morphological approaches, however are the reliance on the ability of the organism to be cultured, the time consuming nature and requirement of extensive taxonomic expertise.

Furthermore, diagnosis of plant diseases can be even more difficult with asymptomatically infected propagative materials such as tree grafting stocks or potato tubers. The use of molecular methods can circumvent many of these shortcomings. Accordingly, there have been significant developments in the area of molecular detection of plant pathogens in the last three decades. The advent of antibody based detection, the monoclonal antibodies and the enzyme linked Immunosorbent assay (ELISA), was an important turning point in virology and bacteriology. Then came the DNA based technologies, such as the polymerase chain reaction (PCR) which revolutionized molecular diagnostics and biological science. In the last decade, the ranges of targets that can be diagnosed using diagnostic PCR have grown tremendously. The very flexibility and application specific variations in the basic theme of the system have allowed the development of many PCR variants adapted to wide range of applications. Furthermore, diagnostic PCR has been greatly improved by the introduction of the second generation PCR, known as the Real Time PCR where closed-tube fluorescence detection and quantification during PCR amplification (in real time) is possible eliminating the need for laborious post-PCR sample processing steps which greatly reduces the risk of carryover contamination. Using Real Time PCR, it is possible not only to detect the presence or absence of the target pathogen, but it is also possible to quantify the amount present in the sample allowing the quantitative assessment of the number of the pathogen in the sample. Enumerating the pathogen upon detection is crucial to estimate the potential risks with respect to disease development and provides a useful basis for disease management decision.

Crops can be attacked by many pathogens which, in addition, often occur in complexes. Therefore, many disease diagnostic applications require simultaneous detection and quantification of several targets. Methodological limitations, however, are in many cases the reason for developing simplex or assays designed for only few targets. The DNA Microarray technology, originally designed to study gene expression and generate single nucleotide polymorphism (SNP) profiles, is currently a new and emerging pathogen diagnostic technology, which in theory, offers a platform for unlimited multiplexing capability. It is viewed as a technology that fundamentally alters molecular diagnostics. The fast growing databases generated by genomics and biosystematics research provides unique opportunity for the design of more versatile, high-throughput, sensitive and specific molecular assays which will address the major limitation of the current technologies and benefit plant pathology. Nowadays, it is available accurate techniques for diagnostics of:

- Soil borne fungal pathogens
- Phytopathogenic bacteria
- Plant parasitic nematodes
- Plant viruses
- Phytoplasms
- Plant viroides

Enhance resistance of plants to pathogens

As mentioned before, plants either under field conditions or under protected cultivation suffer from many diseases. Management of such diseases require quick decision in the appropriate time, and any delaying in application the suitable method of plant protection lead to tremendous effects. Therefore, cultivation of resistant crops considers the best method of protection against diseases.

To reach this point, there are two directions: classical breeding or genetic modification of plants (GM plants). There are many directions for engineered plants to defend themselves against the pathogens, these include: pathogen derived resistance, *R* genes derived resistance, factors of resistant genes and antimicrobial proteins derived resistance.

Pathogen derived resistance

Plants can be protected from diseases with transgenes that are derived from the pathogens themselves. For example, plant viral transgenes can protect plants from infection by the virus from which the transgene was derived. Many examples of this type of resistance became available as potato plants resistant to *potato leaf role virus* which transgene by coat protein gene of the same virus. This plants become resistant to many viruses infect potatoes and belong to the same serological type. Another example is *Papaya ring spot virus* which caused huge damage to Papaya trees and all methods of management failed in protection of trees from virus infections.

Transgene Papaya plants by coat protein gene of the same virus protect trees from further infection by this virus. *Rep* gene was also used as transgenic.

Enhancing a plant's resistance with genes from the plant kingdom (R genes)

Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Not all pathogens can attack all plants and a single plant is not susceptible to the whole plethora of plant pathogenic fungi, viruses, bacteria or nematodes. Recombinant DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms . What is useful in one plant/pathogen system may be transferred to another, increasing the recipient plant's ability to defend itself from a previously uncontrollable pathogen. Furthermore, biotechnology facilitates the discovery and elucidation of the molecular interactions between plants and pathogens. Understanding the molecular basis of plant-pathogen interactions increases our ability to deploy selected plant resistance genes from virtually any plant. A short description of the use of plant genes to fight against pathogens within the framework of genetic engineering and plant transgenic research is given below

Plant intrinsic responses that can be engineered to attain a wider, more durable resistance include the Hypersensitive Response (HR) and Systemic Acquired Resistance (SAR) although these phenomena are complex and our knowledge of them incomplete, this is an area of enormous promise in plant protection. Ideally a plant could be engineered to show an incompatible reaction with an invading pathogen that leads to localized cell death through HR by transforming it with an appropriate plant resistance gene or an elicitor molecule. Furthermore, the same plant could be engineered so that SAR is expressed even in the absence of a pathogen. For example, the over-expression of the transcriptional regulator Npr1 in transgenic *Arabidopsis thaliana* enhanced the plant's resistance level against a diverse array of pathogens. Plants potentially could be engineered to change a previously compatible reaction with a pathogen (disease) to an incompatible reaction or HR (localized cell death, no disease). This approach would provide the transgenic plant with a first level of pathogen control. A second infection control point would be provided by a systemic acquired resistance state (SAR) that is already functioning before the physical presence of the pathogen. Although we have yet to apply this sophisticated approach to bolster plant resistance networks, individual components of such systems are being tried with different degrees of success Papaya trees transgenic with coat protein of papaya ring spot virus (in the middle) and non-transgenic ones.

Pathogenesis Related (PR) proteins, for example, are a group of diverse proteins whose accumulation is triggered by pathogen attack or abiotic stress. In a sense, PR proteins constitute a point where the various response networks intersect by reacting with different inducers such as salicylic acid, jasmonic acid, systemin, and ethylene. PR proteins have been classified into 12 major groups or families. Some of them show antifungal activity. The functions of most PR proteins remain a mystery but some of them are known to be b -1,3-glucanases (PR-2), chitinases (PR-3) or fungal membrane permeablizers (PR-5) . In theory, the constitutive expression of PR proteins, either singly or combined, might confer decreased susceptibility to a specific group of pathogens.

In a different approach, other researchers have used various single compounds that are part of more

complex networks aimed at fighting against plant pathogens. Phytoalexins are low-molecular weight compounds of a non-proteinaceous nature with antimicrobial and antifungal activity produced by plants after exposure to microorganisms. In tobacco, the expression of stilbene synthase from grapevine leads to the production of the phytoalexin resveratrol that reduces by half the number of plants infected by the gray mold pathogen *Botrytis cinerea*. The production of active oxygen species like superoxide anions, hydroxy radicals and hydrogen peroxide, H_2O_2, have been observed in many plant-pathogen interactions and are known to play an important role in plant defense. Plants have been engineered to continuously produce active oxygen species. For example, expression of a defective calmodulin gene or less active catalase in transgenic tobacco plants led to increased accumulation of H_2O_2 and to an activated expression of PR proteins. Plant lectin genes have been engineered into recipient plants to prevent infection by pathogenic nematodes and defensin genes have been cloned to deter fungal attacks.

Finally, pathosystem-specific plant resistance genes, *e.g. Pto* , etc, have been used as transgenes to confer resistance in different plants. Briefly, a gene that confers resistance to a certain pathogen in plant species A is identified, cloned and transformed into plant species B. Plant species B, the recipient genotype, by virtue of the new gene acquired by transformation, becomes resistant to the same pathogen plant species A inherently is resistant to. Unfortunately, in some cases the gene separated from its original genetic background is not able to confer resistance. In other words, the pathway that makes the resistance gene work properly in plant species A may not be complete or functional in plant species B. However, there is usually a way to engineer the downstream responses triggered by those genes. Plant pathologists are using this 'obstacle' (*i.e.* absence of the complete regulatory pathway allowing a foreign gene to function in the transgenic plant) to fuel their efforts to obtain greater insights into how plant-pathogen interactions work at the molecular level. A more refined knowledge of plant-pathogen interactions is expected to lead to more consistent, accurate and successful resistance engineering strategies.

Antimicrobial protein

One approach to improve a plant's defense against a particular pathogen that has been made possible by genetic engineering is to use genes found in fungi, insects, and animals. Antimicrobial proteins, peptides, and lysozymes that naturally occur in insects, plant animals and humans are now a potential source of plant resistance The production of active oxygen species like superoxide anions, hydroxy radicals and hydrogen peroxide, H_2O_2, have been observed in many plant-pathogen interactions and are known to play an important role in plant defense. Plants have been engineered to continuously produce active oxygen species. In transgenic potatoes containing a H_2O_2-generating glucose oxidase gene from the fungus *Aspergillus niger*, the resulting apoplastic accumulation of peroxide ions enhanced the plants resistance to *Phytophtora infestans*, late blight; *Verticillium dahliae*, Verticillium wilt; and *Alternaria solani*, early blight (Wu et al. 1997). Lytic peptides are small proteins with an amphipathic a-helical structure which makes pores in membranes resulting in the lysis, for example, of the bacterial cell membrane. Cecropins are antibacterial lytic peptides native to the hemolymph of *Hyalophora cecropia*, the giant silk moth. Transgenic tobacco plants expressing cecropins have increased resistance to *Pseudomonas syringae* pv. *tabaci*, the cause of tobacco wildfire, a devastating disease that is difficult to control Bacterial blackleg of potato caused by *Erwinia carotovora* subsp. *atroseptica* can result in 30% yield reduction and 25% loss in storage even though chemical treatments and breeding for resistance are practiced. Synthetic lytic peptide analogs, Shiva-1 and SB-37, produced from transgenes in potato plants reduce bacterial infection caused by *E. carotovora* subsp. *atroseptica* in transgenic potato plants. Fire blight, a bacterial disease of apple caused by *E. amylovora* is hard to control because of limited effectiveness of antibiotic sprays, the development of antibiotic-resistant bacterial populations in orchards, and lack of commercially acceptable fire blight resistant varieties. Transgenic apple expressing the SB-37 lytic peptide analog showed increased resistance to *E. amylovora* in field tests.

Attacins are another group of antibacterial proteins produced by *H. cecropia* pupae. The mechanisms of antibacterial activity of this protein are to inhibit the synthesis of the outer membrane protein in gram negative bacteria . Attacin expressed in transgenic potato enhanced its resistance to bacterial infection by *E. carotovora* subsp. *atrospetica*. Transgenic pear and apple expressing attacin genes have significantly enhanced resistance to *E. amylovora* in *in vitro* and growth chamber test. In field tests, reduction of fire blight disease has been observed in transgenic apples expressing attacin genes .Transgenic apple expressing attacin targeted to the intercellular space, where *E. amylovora* multiplies before infection, has significantly reduced fire blight, even in apple plants with low attacin production levels (K Lysozymes are enzymes that hydrolyze the peptidoglycan layer of the bacterial cell wall. Hen egg-white lysozyme (HEWL), T4 lysozyme (T4L), and human lysozyme genes have been cloned and transferred to enhance plant bacterial or fungal resistance. These lysozyme genes have been used to confer disease resistance against plant pathogenic bacteria in transgenic tobacco plants. T4L, from T4-bacteriophage, also has been reported to enhance resistance in transgenic

potato against *E. carotovora*, which causes bacterial soft rot. Transgenic apple plants with the T4L gene showed significant resistance to fire blight infection. Human lysozyme transgenes have conferred disease resistance in tobacco through inhibition of fungal and bacterial growth, suggesting the possible use of the human lysozyme gene for controlling plant diseases.
The fungus *Trichoderma harzianum* is a biological control agent that has antagonistic activity against phytopathogenic fungi. The mechanism of this activity is to inhibit spore germination and germ-tube elongation, and to degrade the tips of fungal hyphae. To achieve this, *Trichoderma* produces enzymes that catalyze the hydrolysis of chitin in the fungal cell wall. Chitinases can be classified as endochitinase, exochitinase (N-acetyl-b -D-glucosaminidase), and chitobiosidase. Transgenic potato plants expressing the endochitinase gene showed resistance to the pathogens *A. solani* (early blight) and *B. cinerea* (gray mold). *Venturia inaequalis*, the fungus causing apple scab, infects apple leaves and fruits causing reductions in fruit productivity, marketability, and shelf life. Multiple applications of fungicides are relied upon by apple growers almost exclusively to control this disease during the growing season. Transgenic 'McIntosh' apple trees expressing either the endo- or exochitinase gene or both genes have increased resistance to apple scab. These results suggest the potential broad usage of chitinase transgenes to control fungal diseases of plants.

Plantibodies

One of the most remarkable aspects of recombinant DNA technology is when a protein belonging to the exclusive realm of animals can be successfully used in plants to help them fight against pathogens that are difficult to control. The expression of viral- or nematode-specific antibodies *in planta* and hence, the term plantibody, is a promising new avenue for controlling plant pathogens. This unconventional method of pathogen control relies on cloning the variable parts of the light (VL) and the (VH) heavy chains of an antibody molecule linked to a carrier peptide; the expression of the plantibodies in the transgenic plant can lead to their accumulation in the plant cell cytosol. These plantibodies will specifically interact with the intended target inactivating its biological function. So far, two clever strategies have been devised against two important plant pathogens: Tomato Spotted Wilt Virus (TSWV) and root-knot nematodes *Meloidogyne* spp. (Baum *et al.* 1996).
In the case of TSWV, the production of secretory monoclonal antibodies from the corresponding cloned mammalian gene will allow the engineered plant to inactivate the N, G1/G2 and NSm proteins of the virus. TSWV is a destructive pathogen of tomato crops and no reliable method of control is available. In the case of root-knot nematodes a cellulase from *M. incognita*, and other *Meloidogyne* species, in addition to other stylet secreted proteins have been selected as targets for this strategy. The plant parasitic nematode's cellulases are important in the initial steps of pathogenesis. The rationale of selecting cellulases as the target of plantibodies is that upon contact between the anti-cellulase plantibody and the nematode cellulase, the migration of the nematode inside the plant will be stopped or diminished. If successful, this strategy will allow farmers to avoid using highly toxic nematicides, soil sterilants or fumigants. Other useful targets under analysis are the proteins involved in the initiation of the cell cycle that leads to the generation of giant cells that support the feeding nematodes in infected roots

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Application of Biotechnologies in Food/Feed Safety Assessment – Risk assessment approach

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Abstract

Recently, the global area of transgenic crops has markedly risen.In the last two decades,governments have been planning strategies and protocols for safety assessment of food and feed genetically modified (GM). Evaluation of food safety should be taken on a case-by-case analysis depending on the specific traits of the modified crops and the changes introduced by the genetic modification, using for this the concept of substantial equivalence.
Criteria and measurable properties to assess harm for human health and environmental safety are listed, and the possible consequences are evaluated in terms of significance.Finally, a mapping of the possible risks deriving from GMO release is reported, focusing on gene transfer to related species, horizontal gene transfer, direct and indirect effects on non-target organisms, development of resistance in target organisms, and effects on biodiversity.

Key words: GM crops, risk assessment, food, feed, environmental, gene transfere.

Aspects, tools and uses of biotechnology:

The world's population is estimated to double by the year 2033. This poses a huge challenge to agricultural systems. Meanwhile, traditional farming equipment and practices are reaching their limits of effectiveness in increasing agricultural productivity. As countries develop, people are also demanding more and better food. These pressures are multiplied by shrinking farmland, rising costs and shortage of farm workers.

Food biotechnology (or genetically modification) offers an additional method to improve the sustainability of existing farmlands and to improve the quality of our food supply. The processing of food and food ingredients using biotechnology provides a wide variety of fermented foods and food ingredients that are extensively used.

Biotechnology is a set of tools that utilize living organisms or parts of organismsto make or modify products, to improve plants or animals for agriculture, or toengineer microorganisms for specific purposes. Biotechnologytools may include cell and tissue culture, embryotransplantation, microbial fermentation, and genetic engineering. Scientists usethese tools to make new food products, improve plants and animals and to find out how living systems interact,such as plants with insects and soil organisms. (Thieman and Palladino, 2008)

Biotechnology has applications in four major areas: health care (medical), crop production and agriculture, non-food (industrial) uses of crops and other products (e.g. biodegradable plastics, vegetable oil, biofuels), and environmental uses.

Green biotechnology is the branch of biotechnology applied to agricultural processes. An example would be the designing of transgenic plants to grow under specific environments in the presence (or absence) of chemicals.(U.S. Department of State International Information Programs, 2002)

The primary aim of modern biotechnology is to make a living cell perform a specific useful task in a predictable and controllable way. The task could be to ferment soya beans to make soya sauce or to breed a plant that has a higher yield or increased resistance to insect attacks (Thieman and Palladino, 2008).

Whether a living cell will perform these tasks is determined by its genetic make-up that is by the instructions contained in the collection of chemical messages found within its genes. These genes are passed on from one generation to the next so that offspring inherit a range of individual traits from their parents.

In 1953, scientists discovered that deoxyribonucleic acid (DNA) is found in all living things and that a gene is a segment of DNA that has a specific sequence, or code, of chemicals. This code determines various characteristics or traits such as eye or hair color. In 1973, scientists identified a way to isolate genes and by the 1980s, they had developed the tools necessary to transfer genes (and therefore traits) from one organism to another.

With the discovery of enzymes that could be used to cut or remove a gene segment from a chain of DNA at a specific site along the strand, scientists were able to introduce new instructions that would cause cells to

produce needed chemicals, carry out useful processes or give an organism desirable characteristics. This technique is called "recombinant DNA" (rDNA) technology. In addition to transferring genes between species, it is possible to eliminate undesirable traits by switching off the genes responsible for these traits. For example, this technology has been used to switch off the gene responsible for softening in tomatoes. In the future, it may even be possible to remove the proteins that may cause allergic reactions from foods such as peanuts and milk (Thieman and Palladino, 2008).

Impacts of using plant biotechnology

Traditional plant breeding techniques using the controlled pollination of plants have limitations. Firstly, sexual crosses can only occur within the same or related species. This limits the genetic sources breeders can depend upon to enhance desirable characteristics of plants.

Secondly, when two whole plants are crossed, each having some 100,000 genes or so, all the genes from both plants get jumbled up. This presents a problem as the plant offspring may express both desirable and undesirable traits of the parent plants. Because of this, breeders must spend years "back crossing" the jumbled up plants with the plant they started with, again and again, to slowly breed out the tens of thousands of genes they do not want. Traditional plant breeding takes time, sometimes as long as 10 to 12 years.

Plant biotechnology is an extension of traditional plant breeding with one important difference. Instead of mixing hundreds of thousands of genes to improve a crop plant, modern breeders can use biotechnology to select a specific trait from any plant, microbe or animal and move it into the genetic code of another plant. This is possible because of the similarity of all living things at the DNA level. After the gene has been transferred, the newly modified plant exhibits specific modifications rather than the extensive changes that occur with traditional breeding.

There are many positive targets that may be gained through using biotechnology in plants:

Increasing the crop yield:

Using the techniques of modern biotechnology (genetic engineering), genes may be transferred to a highly developed crop variety to impart a new character that would increase its yield. However, many of the genetic characteristics associated with yield (e.g., enhanced growth) are controlled by a large number of genes, each of which has a minimal effect on the overall yield, therefore, much scientific work to be done in this area (Springham *et al.*, 1999 and Krattiger, 2000).

Reduced vulnerability of crops to environmental stresses

Crops containing genes that will enable them to withstand biotic and abiotic stresses may be developed. For example, drought and excessively salty soil are two important limiting factors in crop productivity. Biotechnologists are studying plants that can cope with these extreme conditions in the hope of finding the genes that enable them to do so and eventually transferring these genes to the more desirable crops (Sara Abdulla, 1999).

Increased nutritional qualities

Proteins in foods may be modified to increase their nutritional qualities. Proteins in legumes and cereals may be transformed to provide the amino acids needed by human beings for a balanced diet. A good example is the production of Golden rice which is produced through genetic engineering to biosynthesize beta-carotene, a precursor of pro-vitamin A in the edible parts of rice (Springhamet al.,1999)..

Improved taste, texture or appearance of food

Modern biotechnology can be used to slow down the process of spoilage so that fruit can ripen longer on the plant and then be transported to the consumer with a still reasonable shelf life. This alters the taste, texture and appearance of the fruit. More importantly, it could expand the market for farmers in developing countries due to the reduction in spoilage.

The first genetically modified food product was a tomato which was transformed to delay itsripening, also bread stays fresher longer by adding an enzyme called maltogenic amylase to the flour. Assuming that 10–15% of bread is thrown away as stale, if it could be made to stay fresh another 5–7 days then perhaps 2 million tons of flour per year would be saved (Springham*et al.*, 1999).

Reduced dependence on fertilizers, pesticides and other agrochemicals

Most of the current commercial applications of modern biotechnology in agriculture are on reducing the dependence of farmers on agrochemicals. For example, Bacillus thuringiensis (Bt) is a soil bacterium that produces a protein with insecticidal qualities. Traditionally, a fermentation process has been used to produce an insecticidal spray from these bacteria. In this form, the Bt toxin occurs as an inactive protoxin, which requires digestion by an insect to be effective. There are several Bt toxins and

each one is specific to certain target insects. Crop plants have now been engineered to contain and express the genes for Bt toxin, which they produce in its active form. When a susceptible insect ingests the transgenic crop cultivar expressing the Bt protein, it stops feeding and soon thereafter dies as a result of the Bt toxin binding to its gut wall. Bt corn is now commercially available in a number of countries to control corn borer (a lepidopteran insect), which is otherwise controlled by spraying (a more difficult process).

Crops have also been genetically engineered to acquire tolerance to broad-spectrum herbicide. The lack of herbicides with broad-spectrum activity and no crop injury was a consistent limitation in crop-weed management. Multiple applications of numerous herbicides were routinely used to control a wide range of weed species detrimental to agronomic crops. Weed management tended to rely on pre-emergence—that is, herbicide applications were sprayed in response to expected weed infestations rather than in response to actual weeds present. Mechanical cultivation and hand weeding were often necessary to control weeds not controlled by herbicide applications. The introduction of herbicide-tolerant crops has the potential of reducing the number of herbicide active ingredients used for weed management, reducing the number of herbicide applications made during a season, and increasing yield due to improved weed management and less crop injury (Gianessi*et al.,* 2002).

Production of novel substances in crop plants

Biotechnology is being applied for novel uses other than food. For example, oilseed can be modified to produce fatty acids for detergents, substitute fuels and petrochemicals. Potatoes, tomatoes, ricetobacco, lettuce, safflowers, and other plants have been genetically engineered to produce insulin and certain vaccines. If future clinical trials prove successful, the advantages of edible vaccines would be enormous, especially for developing countries. The transgenic plants may be grown locally and cheaply. Homegrown vaccines would also avoid logistical and economic problems posed by having to transport traditional preparations over long distances and keeping them cold while in transit. And since they are edible, they will not need syringes, which are not only an additional expense in the traditional vaccine preparations but also a source of infections if contaminated.In the case of insulin grown in transgenic plants, it is well-established that the gastrointestinal system breaks the protein down therefore this could not currently be administered as an edible protein. However, it might be produced at significantly lower cost than insulin produced in costly bioreactors. For example,

Impacts of using animal biotechnology

In animals, biotechnology techniques are being used to improve genetics and for pharmaceutical or industrial applications. Molecular biology techniques can help drive breeding programs by directing selection of superior animals. Animal cloning, through somatic cell nuclear transfer (SCNT), allows for genetic replication of selected animals. Genetic engineering, using recombinant DNA, alters the genetic makeup of the animal for selected purposes, including producing therapeutic proteins in cows and goats (Eenennaam, 2006).

Impacts of using microbial biotechnology:

Biotechnology may be used in the field of microbiology for production of enhanced bacteria and fungi which can positively affect the production. (Diaz, 2008).

a) Protection of plants from frost damage via engineered symbiotic bacteria

One of the earliest examples of successful modification of a symbiotic bacterium was performed in Pseudomonas syringae, which is found at high concentrations on the leaves of many plants. Many strains of this bacterium produce an ice nucleation protein that is apparently located on the surface of the bacteria cell, and the presence of this protein cause the formation of ice at temperature only little below 00 C, inflicting significant frost damage on important crop plants & so facilitating invasion of plant tissues by the bacteria.(Diaz, 2008).

b) Use of nitrogen fixing bacteria to improve crop yields

All animals, plants & most bacteria depend on the availability in their environment of some form of "combined nitrogen" nitrate (No3), or such nitrogen containing organic compounds as amino acids. The huge amounts of N_2 that exist in the atmosphere are unavailable to the biological world except through the process of nitrogen fixation.

The biological process of nitrogen fixation does not require the consumption of fossil fuels or electricity does not produce environmental pollution. Thus increasing the extent of biological nitrogen fixation has been an important goal for biotechnology. According to one estimate, the biological process fixes times more nitrogen (24 x 10 17 tons/year) than is converted into chemical fertilizers by industrial processes. Thus even a small improvement in

exploiting the biological process would have been a global impact.

The biological process of nitrogen fixation is complex and consumes a large number of ATP, molecules, because the enzymes involved in it must overcome the same huge activation energy barriers that face the chemical synthesis of ammonia. Two enzymes are required: component I (nitrogenase) & component II (nitrogenasereductase). After component II is reduced by a strong biological reductant, 16 molecules of ATP are hydrolysed to accomplish the reduction of component I. Reduced component II alone is not capable of overcoming the activation energy barrier. Reduced component I finally reduced N_2 to two molecules of NH_3. Nitrogen fixation is strongly reductive reaction, and the enzymes involved are usually irreversibly inactivated when they are exposed to oxygen (Martins, 2008).

The species of Closiridium & Klebsilla fix nitrogen only under anaerobic conditions. Other free living bacteria, however, can fix nitrogen even under aerobic conditions. The cyanobacteria, which carry out oxygen - evolving photosynthesis, perform nitrogen fixation only in specialized cells called heterocysts, which do not produce oxygen. Azotobactor consumes oxygen at an extremely high rate, and in doing so apparently protects its nitrogen fixing machinery. Another group of bacteria fix nitrogen only when they are in a symbiotic nitrogen fixer is Rhizobium, which invades the root tissues of leguminous plants, such as alfalfa, pea, clover and soyabean and lives in intracellular vacuoles. The vacuoles become filled with an oxygen-binding protein produced by the plants. This creates an environment low in free oxygen where Rhizobium is able to carry out nitrogen fixation (Diaz, 2008).

Bioremediation and biodegradation

Biotechnology is being used to engineer and adapt organisms especially microorganisms in an effort to find sustainable ways to clean up contaminated environments. The elimination of a wide range of pollutants and wastes from the environment is an absolute requirement to promote a sustainable development of our society with low environmental impact. Biological processes play a major role in the removal of contaminants and biotechnology is taking advantage of the astonishing catabolic versatility of microorganisms to degrade/convert such compounds. New methodological breakthroughs in-sequencing, genomics, proteomics, bioinformatics and imaging are producing vast amounts of information to help in this subject such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters, nitro-aromatic compounds, industrial solvents, pesticides and metals (Martins, 2008).

GM crops in the horizon

Studies of the genetically engineered crops currently on the market indicate that such cropshave contributed to enhancing global agricultural sustainability specially in the reductions of insecticides in the environment (Qaim and Zilberman, 2003, Huang *et al.,* 2005), improved soil qualityand reduced erosion (Committee on the Impact of Biotechnology on Farm-Level Economics and Sustainability and National Research Council, 2010), prevention of the destruction of the Hawaiianpapaya industry (Tripathi *et al.,* 2006), enhancedhealth benefits to farmers and families as a result of reducedexposure to harsh chemicals (Huang *et al.,* 2002, 2005), economic benefits to local communities (Qaim *et al.,* 2010), enhanced biodiversity of beneficial insects (Cattaneo *et al*., 2006), reduction in the number of pestoutbreaks on neighboring farms growing non-geneticallyengineered crops (Hutchison *et al.,* 2010) and increasedprofits to farmers (Tabashnik, 2010). Geneticallyengineered crops have also dramatically increased crop yields—.30% in some farming communities (Qaim *et al.,* 2010). As has been well-documented for Bt cotton in Arizona, the ability to combine innovations infarming practice with the planting of genetically engineeredseed has had a huge positive benefit/cost ratio,far beyond what could be achieved by innovating farmingpractices or planting genetically engineered crops alone (Ronald, 2011). The benefit/cost ratio of Bt crops is the highest for anyagricultural innovation in the past 100 years. There are dozens of useful genetically engineeredtraits in the pipeline, including nitrogen use efficiency. Success of crops enhancedfor this efficiency would reduce water eutrophicationcaused by nitrogenous compounds in fertilizersand greenhouse gas emissions resulting from the energyrequired to chemically synthesize fertilizers. The USDA Animal and Plant Health InspectionService has developed a transgenic plum variety, the"HoneySweet," which is resistant to Plum Pox, a plantdisease that infects plum and other stone fruit trees, includingpeach, nectarine, plum, apricot, and cherries.Although Plum Pox is very rare in the United States,and its outbreaks are immediately eradicated, the Honey-Sweet variety was developed as a precautionary measureto avoid a major disruption in the availability of plums,prunes, and other stone fruits should Plum Pox becomewidespread as is already the case in Europe (Ronald, 2011).

Other promising applications of genetic engineeringare those that affect staple food crops. For

example, riceis grown in 114 countries on six of the seven continents.

In countries where rice is the staple food, it isfrequently the basic ingredient of every meal. Thus,even modest changes in tolerance to environmentalstress or enhanced nutrition in rice can have a largeimpact in the lives of the poor.

With regard to nutritional enhancements, some effortshave focused on vitamin deficiencies. Vitamin A deficiencyis a public health problem in 100 countries,especially in Africa and Southeast Asia, affecting youngchildren and pregnant women the most (Golden Rice Project, 2010). Worldwide, 124 million children are estimated to be vitamin A-deficient. Many of these childrengo blind or become ill from diarrhea, and nearly 8million preschool-age children die each year as the result of this deficiency. Researchers estimate that 6000 childrenand young mothers die every day from vitamin Adeficiency-related problems. The World Health Organization estimates that improved vitaminA nutritional status could prevent the deaths of1.3–2.5 million late-infancy and preschool-age children each year (Ronald, 2011)

To combat vitamin A deficiency, the World Health Organization has proposed an arsenal of nutritional "well-being weapons," including a combination of breast feeding and vitamin A supplementation, coupled with long-term solutions, such as promoting vitaminA-rich diets and food fortification. In response tothis challenge, a group of Rockefeller Foundation-supported scientists decided to try to fortify rice plantswith higher levels of carotenoids, which are precursorsto vitamin A. Using genetic engineering, they introduceda gene from daffodils (which make carotenoids,the pigment that gives the flower its yellow color) andtwo genes from a bacterium into rice (Ronald, 2011)

The resulting genetically engineered golden and carotenoid-rich rice plants were named "Golden Rice. "Results from human feeding studies indicate that the carotenoids in the second generation of Golden Rice (called Golden Rice-2) can be properly metabolized intothe vitamin A that is needed by children. One 8-ounce cup of cooked Golden Rice-2 provides_450mg of retinol, which is equivalent to 50–60% ofthe adult Recommended Dietary Allowance of vitamin A (Ronald, 2011)

Other studies support the idea that widespread consumption of Golden Rice would reduce vitamin A deficiency, saving thousands of lives. The positiveeffects of Golden Rice are predicted to be most pronounced in the lowest income groups at a fraction ofthe cost of the current supplementation programs. If predictions prove accurate, this relatively low-tech, sustainable, publicly funded, people-centered effort will complement other approaches, such as the development of home gardens with vitamin A-rich crops, such as carrots and pumpkins.

In a sense, the resulting nutritionally enhanced rice issimilar to vitamin D-enriched milk - except the processes different. Vitamin A fortification of rice is also similarto adding iodine to salt, a process credited with drastically reducing iodine-deficiency disorders in infants.Worldwide, iodine deficiency affects 2 billion peopleand is the leading preventable cause of mental retardation.The benefits of iodized salt are particularly apparentin Kazakhstan where local food supplies seldomcontain sufficient iodine and where fortified salt wasinitially viewed with suspicion. Campaigns by the governmentand nonprofit organizations to educate thepublic about fortified salt required both money andpolitical leadership, but they eventually succeeded. Today, 94% of households in Kazakhstan use iodizedsalt, and the United Nations is expected to certify thecountry officially free of iodine-deficiency disorders (Ronald, 2011)

The development of genetically engineered crops thatare tolerant of environmental stresses is also predicted tobe broadly beneficial. Such crops are expected toenhance local food security, an issue of importance especially for farmers in poorer nations that have limited access to markets and are now often dependent on others for their staple foods (Royal Society, 2009).

The development of submergence tolerant rice (Sub1rice), through a non-genetically engineered process thatinvolved gene cloning and precision breeding, demonstrates the power of genetics to improve tolerance toenvironmental stresses such as flooding, which is a majorconstraint to rice production in South and Southeast Asia. In Bangladesh and India, 4 million tonsof rice, enough to feed 30 million people, are lost eachyear to flooding. Planting of Sub1 rice has resulted inthree- to fourfold yield increases in farmers' fields duringfloods compared to conventional varieties. Although theSub1 rice varieties provided an excellent immediate solutionfor most of the submergence-prone areas, a higherand wider range of tolerance is required for severe conditionsand longer periods of flooding. With increasingglobal warming, unusually heavy rainfall patterns arepredicted for rain-fed as well as irrigated agriculturalsystems. For these reasons, we and others have identifiedadditional genes that improve tolerance. Such genesmay be useful for the development of "Sub1plus" varieties.

In Africa, three-quarters of the world's severe droughtshave occurred over the past 10 years. The

introduction ofgenetically engineered drought-tolerant corn, themost importantAfricanstaple food crop, ispredicted to dramaticallyincrease yields for poor farmers. Drought-tolerant cornwill be broadly beneficial across almost any non-irrigatedagricultural situation and in any management system. Drought-tolerance technologies are likely to benefit other-agricultural crops for both developed and developingcountries.

In addition to environmental stresses, plant diseasesalso threaten global agricultural production (Borlaug, 2008). For example, an epidemic of stem rust threatenswheat, a crop that provides 20% of the food calories forthe world's people. Because fungal spores travel in thewind, the infection spreads quickly. Stem rust has causedmajor famines since the beginning of history. In North America, huge grain losses occurred in 1903 and 1905 and from 1950 to 1954. During the 1950s, Norman Borlaugand other scientists developed high-yielding wheatvarieties that were resistant to stem rust and other diseases.These improved seeds not only enabled farmersaround the world to hold stem rust at bay for .50 yearsbut also allowed for greater and more dependable yields.However, new strains of stem rust, called Ug99 becausethey were discovered in Uganda in 1999, are much moredangerous than those that destroyed as much as 20% ofthe American wheat crop 50 years ago. Effective resistancedoes not exist in American wheat and barley varieties,but recently resistance was identified in Africanvarieties and molecular markers mapped to facilitate introgressionof the trait using marker-assisted selection[64].Bananas and plantains are the world's fourth mostimportant food crop after rice, wheat, and maize. Approximately one-third of the bananas produced globallyare grown in sub-Saharan Africa, where the cropprovides .25% of the food energy requirements for.100 million people in East Africa alone. Banana Xanthomonaswilt disease, caused by the Gram-negative bacterium Xanthomonas vasicolapv. musacearum, is a major threat to banana productivity in eastern Africa. Cavendishbanana, which represents 99% of export bananas, isthreatened by a virulent form of the soil-borne fungus Fusarium oxysporum called Tropical Race Four. The fungal leaf spot disease Black Sigatoka,caused by the ascomycete Mycosphaerellafijiensis, hasspread to banana plantations throughout the tropicsand is increasingly resistant to chemical control (Marin *et al.,* 2003). Research to develop new methods to controlthese diseases of banana is underway in several laboratories (Ronald, 2011).

Present knowledge on GM safety

Worldwide, there is a range of perspectives within non-governmental organizations on the safety of GM foods. For example, the US pro-GM group AgBioWorld has argued that GM foods have been proven safe,[52] while other pressure groups and consumer rights groups, such as the Organic Consumers Association, and Greenpeace (Organization for Economic Co-operation and Development, 2009) claim the long-term health risks which GM could pose, or the environmental risks associated with GM, have not yet been adequately investigated. In Japan, Consumers Union of Japan are opposed to GMO foods. They also claim that truly independent research in these areas is systematically blocked by the GM corporations which own the GM seeds and reference materials.

The European Commission Directorate-General for Research and Innovation 2010 report on GMOs noted that "The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research, and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se more risky than e.g. conventional plant breeding technologies (EU, 2010-b). A 2008 review published by the Royal Society of Medicine noted that GM foods have been eaten by millions of people worldwide for over 15 years, with no reports of ill effects (Key *et al.,* 2008). Similarly a 2004 report from the US National Academies of Sciences stated: "To date, no adverse health effects attributed to genetic engineering have been documented in the human population (NRC, 2004) A 2004 review of feeding trials in the *Italian Journal of Animal Science* found no differences among animals eating genetically modified plants (Aumaitre, 2004). A 2005 review in *Archives of Animal Nutrition* concluded that first-generation genetically modified foods had been found to be similar in nutrition and safety to non-GM foods, but noted that second-generation foods with "significant changes in constituents" would be more difficult to test, and would require further animal studies (Flachowsky*et al.*, 2005). However, a 2009 review in *Nutrition Reviews* found that although most studies concluded that GM foods do not differ in nutrition or cause any detectable toxic effects in animals, some studies did report adverse changes at a cellular level caused by some GM foods, concluding that "More scientific effort and investigation is needed to ensure that consumption of GM foods is not likely to provoke any form of health problem (Magaña-Gómez and de la Barca, 2009).

A review published in 2009 by Dona and Arvanitoyannis concluded that "results of most studies with GM foods indicate that they may cause some common toxic effects such as hepatic, pancreatic, renal, or reproductive effects and may alter the hematological, biochemical, and immunologic parameters (Dona, 2009 and Dona and Arvanitoyannis, 2009). However responses to this review in 2009 and 2010 note that the Dona and Arvanitoyannis concentrated on articles with an anti-GM bias that have been refuted by scientists in peer-reviewed articles elsewhere - for example the 35S promoter, stability of transgenes, antibiotic marker genes and the claims for toxic effects of GM foods. (Amman, 2009 a-b and Craig, 2010). In 2007, a review by Domingo of the toxicity by searching in the Publimed database using 12 search terms, cited 68 references, found that the "number of references" on the safety of GM/transgenic crops was "surprisingly limited" and questioned whether the safety of genetically modified food has been demonstrated (Domingo 2007). However, an article in 2007 by Vain found 692 research studies focusing on GM crop and food safety and identified a strong increase in the publication of such articles in recent years. Vain commented that the multi-disciplinarian nature of GM research complicates the retrieval of GM studies and requires using many search terms (he used more than 300) and multiple databases.

Risk assessments

Risk assessment can be defined as “a process of evaluation including the identification of the likelihood and severity of an adverse effect(s)/ event(s) occurring to man or the environment following exposure under defined conditions to a risk source(s)” (Report of the Scientific Steering Committee’s Working Group, 2000) A risk assessment comprises hazard identification, hazard characterization, exposure assessment and risk characterization. A hazard is the potential of a risk source to cause an adverse effect. The sequential steps in risk assessment of GMOs identify characteristics which may cause adverse effects, evaluate their potential consequence, assess the likelihood of occurrence and estimate the risk posed by each identified characteristic of the GMOs (Commission Decision, 2002).

The risk assessment strategy seeks to identify appropriate methodologies and approaches to compare the GM crop or product with its non-GM counterparts. This comparison is the starting point of the safety assessment which then focuses on any intended or unintended differences identified. It is obvious that the insertion of genes and other associated DNA from a donor organism into the host will result in a plant that is not completely identical to the parent. The risk assessment process therefore concentrates on the outcomes of the transformation process. To this end the concepts of substantial equivalence were developed by the OECD(OECD, 1993-a and OECD, 1993-b) and further elaborated by WHO/FAO (FAO/WHO, 2000) for the assessment of the environmental safety of GMOs and the safety of genetically modified foods/feeds, respectively.

Concept of substantial equivalence

The starting point for the safety assessment of genetically engineered food products is to assess if the food is "substantially equivalent" to its natural counterpart (Organization for Economic Co-operation and Development, 2009).

The "substantial equivalence" concept was proposed by the FAO in 1993 and endorsed by the FAO and WHO in early 1996 as a means to reassure consumers by obtaining official approval for genetically modified foods. The testing normally take years before a product gains approval for marketing. The adoption of the concept of substantial equivalence permitted marketing of new foods without any safety or toxicology tests as long as they were not grossly different in chemical composition to foods already on the market (Brunner and Mayer, 1999).

"Substantial equivalence embodies the concept that if a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety (i.e., the food or food component can be concluded to be as safe as the conventional food or food component) (FAO/WHO, 1996). The rationale for this approach is that it would be impossible to test all the new crop varieties every year for food safety. To decide if a modified product is substantially equivalent, the product is tested for unexpected changes in a limited set of components such as toxins, nutrients or allergens that are present in the unmodified food. If these tests show no significant difference between the modified and unmodified products, then no further food safety testing is required.

However, if the product has no natural equivalent, or shows significant differences from the unmodified food, then further safety testing is carried out (Organization for Economic Co-operation and Development, 2009 and Kok and Kuiper, 2003):

Food/feed safety assessment

In addition to the molecular characterization of the genetically modified plants and the necessary comparative compositional data to assess the extent of equivalence, further information is needed for the safety assessment of material intended for use as human food or animal feed.

Product Specification

Specification of the origin and the composition of the GM plant and GM food/feed is needed to ensure the identity between the product tested/evaluated and the product to be marketed. In the design of the specification, parameters most relevant for the characterization of the product from a safety and nutritional point of view should be considered. Information on the availability of specified reference material should be submitted.

Effect of the production process

For processed foods/feeds derived from GM sources a description of the production process should be provided which should comprise a general outline of the processing steps and a detailed description of the conditions applied (description of physical, chemical and biochemical parameters). It is important to assess if, and to what extent,the processing steps lead to the concentration or to the elimination, denaturation
and/or degradation of DNA and the novel protein(s)9 in the final product.

Anticipated intake/extent of use

An estimate of the expected intake is necessary for the safety evaluation of GM
food/feed and to evaluate nutritional significance. Information should be provided on the intended function, the dietary role of the product, and the expected level of use. On the basis of the available consumption data, the anticipated average and maximum intake of the GM food should be estimated. If possible, particular sections of the population with an expected high exposure should be identified and this should be considered within the risk assessment. Any assumptions made in the exposure assessment should be described.

The concentrations of the new gene products and constituents produced, or modified by the intended genetic modification (e.g. due to changes in metabolic pathways) in those parts of the GM plant intended for food or feed use, should be determined by appropriate methods. Expected exposure to these constituents should be estimated taking into account the influences of processing, storage and expected treatment of the food/feed in question.

Toxicology

The toxicological requirements for food and feed derived from GM plants must be
considered on a case-by-case basis and will be determined by the outcome of the assessment of the biological significance of any differences identified between the GM product and its conventional counterpart. This would not only include studies on newly expressed proteins but also the consequences of any genetic modification (e.g. gene silencing or over-expression of an endogenous gene). In principle, the safety assessment must consider the presence of proteins expressed as result of the genetic modification, the potential presence of other novel constituents and/or possible changes in the level of natural constituents beyond normal variation. These potential deviations from the conventional counterparts require different toxicological approaches. There may be circumstances, when the notifier considers that a decision on safety can be taken without conducting some of the tests recommended in this chapter and/or that other tests are more appropriate. In such cases the notifier must state the reasons for not submitting the required studies or for carrying out studies other than those mentioned below. Those toxicological studies which are carried out should be conducted using internationally agreed protocols. Test methods described by the OECD (OECD, 2002) or inthe most up to date European Commission Directives on dangerous substances are recommended(EC, 2002-a). Use of any methods that differ from such protocols should bejustified. Studies should be carried out according to the principles of Good Laboratory Practice (GLP) described in Council Directive 87/18/EEC and accompanied by a statement of GLP-compliance (EU, 1986). Any adverse effect noted in individuals exposed professionally should be submitted by the notifier.

Testing of novel proteins

To demonstrate the safety of novel proteins the following information is needed:

A molecular, biochemical and functional characterisation of the novel protein including the determination of the primary sequence and the molecular weight, studies on post-translational modifications and a description of the function are needed. In the case of novel enzymes, information on the principal and subsidiary enzyme activities is needed including the temperature and pH range for optimum activity, substrate specificity, and possible reaction products.

A search for homology to proteins known to cause adverse effects, e.g. protein toxins, should be conducted. A search for homology to proteins exerting a normal metabolic or structural function can also contribute valuable information. The database(s) used to carry out the search should be specified. The stability of the protein under conditions that represent processing, storage andexpected treatment of the food/feed in which it is present should be studied. Theinfluences of temperature and pH changes should normally be examined and potentialmodification(s) of the proteins (e.g. denaturation) and/or stable protein fragmentsgenerated through such treatments should be characterised.

Data concerning the resistance of the novel protein to proteolytic enzymes (e.g. pepsin) should be obtained, e.g. by in vitro investigations using appropriate and validated tests. Stable breakdown products should be characterized and evaluated with regard to the hazards linked to their biological activity. The studies required to investigate the toxicity of the novel protein should be selected on a case-by-case basis, depending upon the knowledge available with respect to the protein's function/activity and any history of its prior human consumption.

In the case of novel proteins with insufficient data base and, in particular, if the available data suggest the existence of any cause for concern, a repeated dose study should be performed, using laboratory animals capable of reacting rapidly to any induced physiological or metabolic disturbances, for example, young rapidly growing animals. Normally, this is a 28-day oral toxicity study according to OECD guideline 407 (1995)10. Additional targeted investigations should be conducted if the novel protein is suspected to act on specific organs or tissues including interactions with receptors of the endocrine, reproduction or nervous system.

If the notifier considers that a decision on safety can be taken without conducting a repeated dosing study or that other tests are more appropriate, the notifier must state the reasons for this. It is essential that the tested protein is equivalent to the novel protein as it is expressed in the GM plant. If, due to the lack of sufficient amount of test materials (e.g. plant proteins), a protein is used which was produced by micro-organisms, the structural and functional equivalence of the microbial substitute to the novel plant protein has to be demonstrated. For example, comparisons of the molecular weight, the isoelectric point, amino acid sequence**,** post-translational modification, immunological reactivity and, in the case of enzymes, the enzymatic activity, are needed to provide evidence for the equivalence.

Testing of novel constituents other than proteins (novel metabolites)

Other identified novel constituents other than proteins should be evaluated according to the traditional toxicological approach on a case-by-case basis. For establishing their safety, information analogous to that described in the “Guidance on submissions forfood additive evaluations by the Scientific Committee on Foods” (Scientific Committee on Food, 2001) is needed.

This implies the submission of information on a core set of studies and the consideration of whether any other type of study might also be appropriate. Normally, the core set includes information on metabolism/toxicokinetics, subchronic toxicity, genotoxicity, chronic toxicity/carcinogenicity and reproduction and developmental toxicity.

Information on natural food constituents

Natural food constituents comprise a large variety of substances: macro- and micronutrients, secondary plant metabolites as well as natural toxicants and antinutritional factors. If the content of such natural food constituents is increased beyond the natural variation, a detailed safety assessment based on the knowledge of the physiological function and/or toxic properties of these constituents should be submitted. The result of this assessment would determine if and to what extent toxicological tests are required.

Testing of the whole GM food/feed

If the composition is modified substantially, or if there are any uncertainties on the equivalence to a traditional counterpart, not only novel constituents, but also the whole GM food/feed should be tested. For foods and products that can be used for both food and feed purposes, the testing programme should include at least a 90-day feeding study in rodents. Special attention must be paid to the selection of doses and the avoidance of problems of nutritional imbalance. Additional toxicological studies may also be necessary, depending on the potential exposure, the nature and extent of deviation from traditional counterparts and the findings of the feeding study. For feed use only, where the modification is expected to substantially change composition and/or bioavailability then a suitable comparator for feeding studies is unlikely to be available. In these cases feeding studies with the more important target species should be made to demonstrate wholesomeness for the animal.

Allergenicity

Allergy is an adverse reaction (in this context to foods) which, by definition, is immune-mediated and particularly involves IgE antibodies. This section deals with the risks to genetically predisposed (i.e. atopic) individuals when exposed to foods derived from GM plants with regard to sensitisation or to elicitation of an allergic reaction. The potential allergenicity of a protein is not a completely predictable parameter and will depend upon the genetic diversity and variability of specific IgE response infood additive evaluations by the Scientific Committee on Foods" (Scientific Committee on Food, 2001) is needed. This implies the submission of information on a core set of studies and the consideration of whether any other type of study might also be appropriate. Normally, the core set includes information on metabolism/toxicokinetics, subchronic toxicity, genotoxicity, chronic toxicity/ carcinogenicity and reproduction and developmental toxicity.

Information on natural food constituents

Natural food constituents comprise a large variety of substances: macro- and micronutrients, secondary plant metabolites as well as natural toxicants and antinutritional factors. If the content of such natural food constituents is increased beyond the natural variation, a detailed safety assessment based on the knowledge of the physiological function and/or toxic properties of these constituents should be submitted. The result of this assessment would determine if and to what extent toxicological tests are required.

Testing of the whole GM food/feed

If the composition is modified substantially, or if there are any uncertainties on theequivalence to a traditional counterpart, not only novel constituents, but also the whole GM food/feed should be tested. For foods and products that can be used for both food and feed purposes, the testing programme should include at least a 90-day feeding study in rodents. Special attention must be paid to the selection of doses and the avoidance of problems of nutritional imbalance. Additional toxicological studies may also be necessary, depending on the potential exposure, the nature and extent of deviation from traditional counterparts and the findings of the feeding study. For feed use only, where the modification is expected to substantially change composition and/or bioavailability then a suitable comparator for feeding studies is unlikely to be available. In these cases feeding studies with the more important target species should be made to demonstrate wholesomeness for the animal.

Allergenicity

Allergy is an adverse reaction (in this context to foods) which, by definition, is immune-mediated and particularly involves IgE antibodies. This section deals with the risks to genetically predisposed (i.e. atopic) individuals when exposed to foods derived from GM plants with regard to sensitisation or to elicitation of an allergic reaction. The potential allergenicity of a protein is not a completely predictable parameter and will depend upon the genetic diversity and variability of specific IgE response infrom several steps in the risk assessment process, a cumulative body of evidence which minimises any uncertainty with regard to the protein in question. The intrinsic allergenicity of the foreign protein(s) encoded by the introduced gene(s) must clearly be considered. Moreover, the consequences of any possible unintended effects of the genetic modification on the endogenous allergenic potential of the plant or plant product should also be considered on a case-by-case basis. For example, unintended qualitative or quantitative changes could occur in the pattern of allergenic proteins naturally present in the conventional plant or product.

Assessment of allergenicity of the newly expressed protein

In line with the recommendations of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001 and the Codex ad hoc Intergovernmental Task Force on Foods Derived from Biotechnology (Codex, 2002), an integrated, stepwise, case-by-case approach, as described below, should be used in the assessment of possible allergenicity of newly expressed proteins. In every case a search for sequence homologies and/or structural similarities between the expressed protein and known allergens should be made as the first step in the assessment. Identification of potential linear IgE binding epitopes should be conducted by a search for homologous peptidic fragments in the amino acid sequence of the protein. The size of the contiguous identical or chemically similar amino acid search should be based on a scientifically justified rationale in order to minimise the potential for false negative or false positive results. If the source of the introduced gene is considered allergenic, but no sequence homology to a known allergen is demonstrated, specific serum screening of the

expressed protein should then be undertaken with appropriate sera from patients allergic to the source material using relevant validated immunochemical tests. If the source is not known to be allergenic but if a sequence homology to a known allergen is demonstrated, the specific serum screening should be

conducted with sera from patients sensitised to this allergen.

If the source of the gene/protein is not known to be commonly allergenic and no sequence homology to a known allergen is demonstrated, or if the result of the specific serum screening of a newly expressed protein from a source known to be allergenic is equivocal, additional tests should be performed. These include pepsin resistance tests or targeted serum screening.

Other analyses

Stability to digestion by proteolytic enzymes has long been considered a characteristicof allergenic proteins. Although it has now been established that no bsolutecorrelation exists (Fu et al., 2002), resistance of proteins to pepsin digestion is still proposedas an additional criterion to be considered in an overall risk assessement. In the caseof resistance of a protein to degradation in the presence of pepsin under appropriate conditions, further analysis should be conducted to determine the likelihood of thenewly expressed protein being allergenic. It will also be useful to compare intact, pepsin digested and heat denatured proteins for IgE binding. Targeted serum screening, as proposed in the FAO/WHO expert consultation (FAO/WHO, 2001) aims to assess the capacity of the newly expressed protein to bind to IgE in sera of individuals with clinically-validated allergic responses to categories of foods broadly related to the gene source. If no relevant serum is available the expressed protein should be analysed for evidence of cross-reactivity and/or sensitising potential using other tests such as appropriate animal models or search for T-cell epitopes, structural motifs, etc. Complementary data on the biological origin and function and structural features of the newly expressed protein may also be provided in order to increase the body of facts to support a conclusion.

Assessment of allergenicity of the whole GM plant or crop

If the host of the introduced gene is known to be allergenic, any potential change in theallergenicity of the whole GM food/feed should be tested by comparison of the allergen repertoire with that of the conventional non-GM variety**.** It should be pointed out that these approaches should be applied on a case-by-case basis depending on the available information on the allergenic potential of the source and/or the host.

Data on the prevalence of occupational allergy in workers or in farmers who have significant exposure to GM plant and crops or to the airborne allergens they may contain will provide useful information for the risk assessment process.

Nutritional assessment of GM food

The development of GM foods has the potential to improve the nutritional status of individuals and populations and provide products with enhanced functionality. GM foods also have the potential to introduce nutritional imbalances as a result of both expected and unexpected alterations in nutrients and other food components. The nutritional evaluation of GM foods should consider:

_ nutrient composition

_ biological efficacy of nutrient components in the foods,

_ assessment of dietary intake and nutritional impact

When substantial equivalence to an existing food is demonstrated, the only further nutritional assessment will deal with the impact of the introduction of the GM food on general human dietary intake patterns. Information on the anticipated intake/extent of use of the GM food will be required and the nutritional consequences should be assessed at average and at upper levels of daily intake. The influences of non-nutrient components of the GM food should also be considered.

Specific additional requirements should be applied to those GM foods aimed at modifying nutritional quality. In this case additional detailed studies on specific metabolites, tailored according to the genetic modification(s), would be required. The introduction of a significant nutritional change in a food may require post-market assessment to determine if the overall diet has been altered and to what degree .

Nutritional assessment of GM feed

In cases where composition of the GM plant differs significantly from the non-GM counterpart, a full range of physiological-nutritional studies should be carried out on a case-by-case basis with representative target animals. These studies could include digestibility, balance experiments or the determination of the nutritive value. For feeds, it is recommended that comparative growth studies are conducted with a fast growing livestock species such as the broiler chick. Because of their rapid weight gain, broilers are particularly sensitive to the presence of toxic elements in their feed. Studies of this type are, however, limited to those materials suitable for inclusion in broiler diets and which can be nutritionally matched to a suitable control diet. For feedstuffs intended only for aquaculture, extrapolating results from a growth study made with a fish species such as the catfish, may be preferable to an extrapolation from results obtained with broilers. Similarly, in the presence of a known toxicant, feeding studies may be restricted only to

those livestock known to tolerate the compound. For example, gossypol prevents the use of cottonseed meal in animals other than ruminants. In this case milk production parameters, also recognised as relatively sensitive indicators of body condition, might substitute for growth rate.

Animal products

The safety of products derived from animals fed a diet containing GM plant material and consumed by humans should be considered. However, it is considered unlikely that a potential gene transfer from GM plant material to animal cells will result in the expression of heterologous proteins which might be present in animal products or that intact newly expressed proteins will be absorbed. Consequently, it is not considered

necessary to test routinely for the presence of introduced genes or their products unless their characteristics suggest cause for concern.

Proteins introduced into the GM plant and known to modify plant metabolism may alter the nature or concentration of metabolites which may have toxicological implications for the animal and/or consumers of animal products. In such cases further studies should be performed with respect to the toxicological implications for the animal and/or consumers of animal products. Guidance on these issues is provided in the relevant sections of this document.

Environmental and ecological issues:

Potential gene escape and super-weeds

There is a belief among some opponents of genetic engineering technology that transgenic crops might cross-pollinate with related weeds, possibly resulting in "super-weeds" that become more difficult to control. One concern is that pollen transfer from glyphosate-resistant crops to related weeds can confer resistance to glyphosate. While the chance of this happening, although extremely small, is not inconceivable, resistance to a specific herbicide does not mean that the plant is resistant to other herbicides, so affected weeds could still be controlled with other products.

Some people are worried that genetic engineering could conceivably improve a plant's ability to "escape" into the wild and produce ecological imbalances or disasters. Most crop plants have significant limitations in their growth and seed dispersal habits that prevent them from surviving long without constant nurture by humans, and they are thus unlikely to thrive in the wild as weeds (NRC, 2002 and Wieczorck, 2003)

Impacts on "nontarget" species

Some environmentalists maintain that once transgenic crops have been released into the environment, they could have unforeseen and undesirable effects. Although transgenic crops are rigorously tested before being made commercially available, not every potential impact can be foreseen. *Bt*corn, for instance, produces a very specific pesticide intended to kill only pests that feed on the corn. In 1999, however, researchers at Cornell University found that pollen from *Bt*corn could kill caterpillars of the harmless Monarch butterfly. When they fed Monarch caterpillars milkweed dusted with *Bt*corn pollen in the laboratory, half of the larvae died. But follow-up field studies showed that under real-life conditions Monarch butterfly caterpillars are highly unlikely to come into contact with pollen from *Bt*corn that has drifted onto milkweed leaves—or to eat enough of it to harm them (Gianessi *et al*., 2002, Wieczorck, 2003)

Insecticide resistance

Another concern related to the potential impact of agricultural biotechnology on the environment involves the question of whether insect pests could develop resistance to crop-protection features of transgenic crops. There is fear that large-scale adoption of *Bt*crops will result in rapid build-up of resistance in pest populations. Insects possess a remarkable capacity to adapt to selective pressures, but to date, despite widespread planting of *Bt*crops, no *Bt*tolerance in targeted insect pests has been detected (NRC, 2002 and Wieczorck, 2003).

Loss of biodiversity

Many environmentalists, including farmers, are very concerned about the loss of biodiversity in our natural environment. Increased adoption of conventionally bred crops raised similar concerns in the past century, which led to extensive efforts to collect and store seeds of as many varieties as possible of all major crops. These "heritage" collections in the USA and elsewhere are maintained and used by plant breeders. Modern biotechnology has dramatically increased our knowledge of how genes express themselves and highlighted the importance of preserving genetic material, and agricultural biotechnologists also want to make sure that we maintain the pool of genetic diversity of crop plants needed for the future. While transgenic crops help ensure a reliable supply of basic foodstuffs, U.S. markets for specialty crop varieties and locally grown produce appear to be expanding rather than diminishing. Thus the use of genetically modified crops is unlikely to negatively impact biodiversity **(**Wieczorck, 2003 and EU, 2004).

Social issues

Labeling

Some consumer groups argue that foods derived from genetically engineered crops should carry a special label. In the USA, these foods currently must be labeled only if they are nutritionally different from a conventional food (Wieczorck, 2003 and EU, 2008).

"Terminator" technology

Most farmers in the USA and elsewhere buy fresh seeds each season, particularly of such crops as corn, green peppers, and tomatoes. Anyone growing hybrid varieties must buy new seeds annually, because seeds from last year's hybrids grown on the farm will not produce plants identical to the parent. For this same reason—to avoid random genetic diversity due to open pollination-farmers do not plant mango, avocado, or macadamia from seed, instead, they clone individual plants of known quality through techniques such as grafting.

In developing countries, many farmers who are not growing hybrids save harvested seeds for replanting the next year's crop. A technology has been developed that might be used to prevent purchasers of transgenic crop seeds from saving and replanting them. Such "terminator" seeds are genetically engineered, along with other improvements more acceptable to farmers, to produce plants with seeds that have poor germination. This forces farmers who otherwise save seed to purchase it if they wish to use these improved commercial varieties. And, in the USA, the crops engineered with various characters are sold alongside non-transgenic alternatives for which growers also typically purchase seeds annually.

Despite these mitigating circumstances, this is serious issue among organic growers and in developing countries, where the practice of saving seeds is the norm for farmers who are not growing hybrid crops. Inclusion of "terminator" genes means that these farmers cannot take advantage of improvements brought about by genetic engineering without being brought into the economic cycle that profits the seed companies. Without profit incentive, however, these companies are unlikely to invest in improving crops. This issue is analogous to that faced by pharmaceutical companies developing new medications against human diseases. Clearly, it is a difficult and divisive social issue (Wieczorck, 2003 and EU, 2010-a).

Conclusions

For hundreds of years, farmers have relied on conventional breeding to enhance agricultural production. Without the development of high-yielding crop varieties over recent decades, two to four times more land would have been needed almost allover the world to produce the same amount of food. Looking ahead, without additional yield increases, maintaining current per capita food consumption will necessitate a near doubling of the world's cropland area by 2050. Because substantial greenhouse gases are emitted from agricultural systems, and because the net effect of higher yields is a dramatic reduction in carbon emissions, development and deployment of high-yielding varieties will be a critical component of a future sustainable agriculture. Thus, a key challenge is to raise global yields without further eroding the environment. Recent reports on food security emphasize the gains that can be made by bringing existing agronomic and food science technology and know-how to people who do not yet have it.

The need to explore the genetic variability in the existing food crops should be highlighted to develop new genetic approaches that can be used to enhance more ecologically sound farming practices. In many parts of the world, building local educational, technical, and research capacity food processing capability, storage capacity, and other aspects of agribusiness, as well as rural transportation and water and communications infrastructure must be undertaken.

The many trade, subsidy, intellectual property, and regulatory issues that interfere with trade and inhibit the use of technology must also be addressed to assure adequate food availability to all. Application of the relevant official safety assessment procedures and following the international guidelines before marketing of genetically engineered crops should be addressed. Despite the complexity of many of these interrelated issues, it is hard to avoid the conclusion that ecological farming practices using genetically engineered seed will play an increasingly important role in a future sustainable agriculture.

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Applications and Overviews for a Bioinformatics Program in Molecular Biology Protocols

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Abstract

Computational molecular biology is expanding fast. Better algorithms are constantly being designed, and new subfields are emerging even as I write this subject. Bioinformatics is the application of information technology to the field of molecular biology .The term *Bioinformatics* was coined by Paulien Hogeweg in 1978 for the study of informatic processes in biotic systems .Bioinformatics nowadays entails the creation and advancement of databases, algorithms, computational and statistical techniques, and theory to solve formal and practical problems arising from the management and analysis of biological data. Thus, the use of computational methods to mine the biological information in many fields became a fundamental need. Subsequently, several programs for molecular biology, i.e. computer algorithms to analyze proteins, genes, as well as international databases already exist, which facilitate the understanding and ease studying of molecular biology. On the other hand, many protocols for different molecular biology techniques are available including, e.g. genomics, proteomics, electrophoresis and molecular markers. These protocols are available in a static form such as text books. However, there is a lack in basic protocols software, which could help researchers and provide an efficient tool for utilizing the molecular biology protocols. Such simple programs could reduce the time necessitate for hand calculations, and make the research work manageable and effortless. Therefore, the program described here was developed principally as a tool to assist researchers working in molecular biology. Khirshyat was developed by the author since 2010 and tested by several users to check its simplicity and error-free calculations. The program includes some important protocols for molecular biology with some modifications, which could be used with different plant species. All calculations and technical features included in the program have been checked to avoid the common programming errors. Since the program released online, it seems that increasingly being used by a growing number of students and researchers worldwide who utilize some of its features. An overview of the program, its importance and some of its features are described here.

Key words: Bioinformatics, Molecular Biology, Khirshyat submenus**.**

Program design, download and setup

It is not too easy to find program for a particular task, especially nowadays, scientists are using a computer to help them in their scientific research. If a researcher wants specific program but he cannot find what he wants, subsequently, he has to design and write them using a programming language. Several encoding languages are available, varying in their easiness and utilization. Microsoft® Visual Basic 6, as a one of these languages, was used in designing Khirshyat 1.0. Due to it is simplicity and easiness to learn and allow advanced programmers to create powerful windows applications. On the other hand, a photo editing software tool is required to produce an interactive and smart interfaces. Adobe® Photoshop® CS 8 was used for this task in producing a backgrounds and graphic designs which provides well designed visual interface making the program enjoyable. Khirshyat runs under Windows® operating system, stands on itself and does not need any supporting programs.

The program can be easily downloaded from the Assiut University web-site or by direct request from the author. It is easy to install this micro-program as any other software by clicking on the “Set up” icon and following the coming menu instructions. The program includes a simple manual and "Read me" file for installation help.

Khirshyat submenus

Khirshyat comes with 25 submenus, in which the user can find some of the most common protocols used in molecular biology. Molecular markers (MM) (i.e. amplified fragment length polymorphism (AFLP), sequence related amplified polymorphism (SRAP) and randomly amplified polymorphic DNA (RAPD)), PCR optimization, oligonucleotide properties, calculations of concentration and quantity using MW of most common used chemicals, plant DNA extraction, DNA concentration and purity, Primer preparation, polyacrylamide gel electrophoresis and silver nitrate staining protocols. It accelerates the process in preparation stocks, buffers, solutions as well as provides important tips and general information with simple illustrations about the most common protocols, which help the user understanding different molecular biology techniques.

Calculations for PCR preparations

Khirshyat offers a dynamic PCR-MIX preparation tool for AFLP, SRAP and RAPD molecular markers according to their protocols, in which the user can modify the stock concentration of the PCR components (*i.e.* dNTPs, $MgCl_2$, Primers,… etc.) to a desired concentration. It enables the user to change the total volume and calculate the PCR-components volumes for any number of samples. Moreover, in the case of trying different primers or different DNA samples, these components can be avoided in calculations to be added separately. Users are abele to save or print the thermocycler's program for these molecular markers, which includes its different steps, temperatures and number of cycles. In addition, the program includes a "PCR-optimization" submenu (Fig. 1) which can serve for any PCR-based molecular marker, the user needs just to fill stock and final concentrations according to any protocol, then the program gives all calculations in a proper volumes.

Oligonucleotide properties

The program, in addition, provides ' Oligonucleotide' submenu (Fig. 2), in which the basic properties of any DNA sequence can be calculated by pasting or entering the sequence followed by clicking 'Calculate'. The program will use the currently entered sequence to calculate the length, molecular weight, %C+G content and the melting temperature (Tm) for the inserted sequence. The calculation of melting temperature for any primer sequence will give a good starting point for determining appropriate annealing temperatures for PCR, RT-PCR, hybridization and primer extension procedures. The equations used to calculate the melting temperature depend on the sequence's length. Any number of oligonucleotide can be analyzed, by clicking 'Next' all properties of the first sequence will be stored, and another sequence can be entered etc., all entered sequences along with their properties can be saved and/or printed.

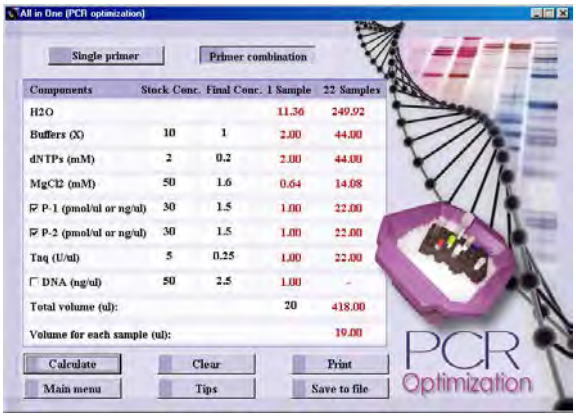

Fig. 1. **PCR optimization submenu**: serves for any PCR-based molecular marker technique.

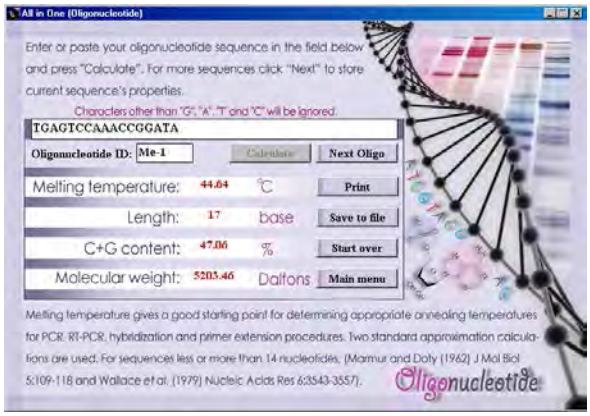

Fig. 2. Oligonucleotide submenu: helps to get information of any oligonucleotide.

Chemicals' concentration and quantity

"Molarity" submenu facilitates users to calculate quantity of chemical (e.g. mg) using its concentration (e.g. mM) and molecular weight in a certain volume, or to calculate concentration using quantity and molecular weight, as well. A list of most common chemicals used in molecular biology and tissue culture is included. In addition, the user can write the molecular weight directly if the chemical is not provided in the list. **Figure 3** showed the interface of molarity submenu.

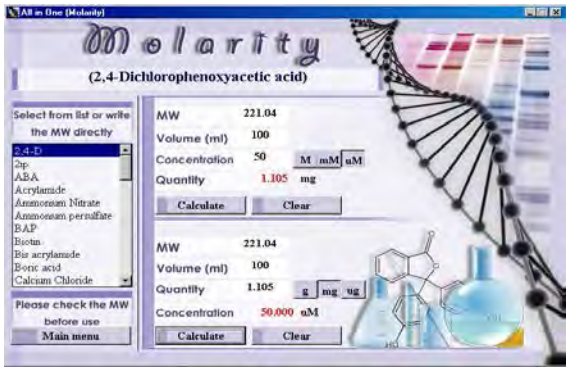

Fig. 3. **Molarity submenu:** for calculations of chemicals' concentration and quantity.

DNA Extraction, concentration and purity

A submenu of DNA extraction stocks (i.e. the main stocks, extraction buffer and TE buffer) preparation is included, in which the user can easily get amounts and volumes of different buffers in a simple way. Program provides some important tips, which should be considered to avoid doing mistakes during the preparation of different solutions. It includes calculations of DNA concentration and purity using the spectrophotometer data (Fig. 4). DNA samples should be read, at least, at two wavelengths, 260_{nm} and 280_{nm} to get information about its concentration and purity. However, it is recommended to read, additionally, at 230, 270 and 320_{nm}. The program provides three options to feed the data of spectrophotometer, one, two, or three readings for each sample, and the average of the replicates will

be directly calculated. It gives a brief report for sample analysis (i.e. purity: either sample is free from phenols, protein, RNA, or organic compounds as well as its concentration). A sample sheet is provided, in which the results and reports can be stored, to enable the user calculating desired concentrations of DNA samples for further work.

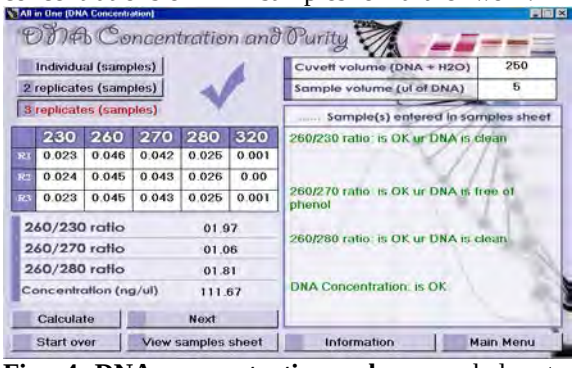

Fig. 4 DNA concentration submenu: helps to calculate DNA concentration and purity.

Primer preparation

Program offers "Primer preparation" submenu (Fig. 5), which enables the user to calculate primer stock dilutions and required concentrations in proper volumes using the primer's micrograms or nanomols information given on primer's vial. The user can make calculations of any number of primers, by clicking 'Next' all information will be stored and another primer can be entered, etc. then, all stored information can be saved or printed.

Polyacrylamide gel electrophoresis

"Electrophoresis" submenu helps the user to prepare the polyacrylamide main stock, the work stock and TBE buffer, used in polyacrylamide gel electrophoresis (**Fig. 6**). The user can determine the required concentration and a volume of acrylamide stock. Depending on gel size, user can enter the volume that he wishes to prepare, and the program will directly calculate the volume and amounts of other components. Moreover, the program provides submenu in which the user easily figure out how to calculate and prepare the loading dyes, with some useful tips.

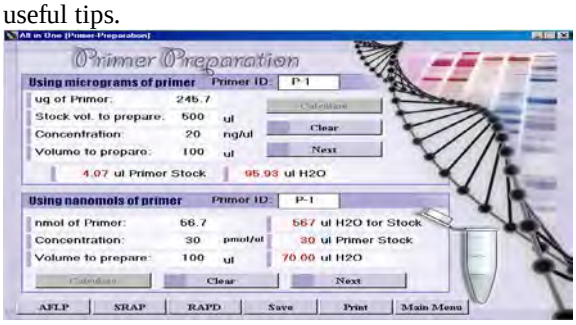

Fig. 5 Primer preparation submenu: serves for primer preparation using its micrograms or nanomols.

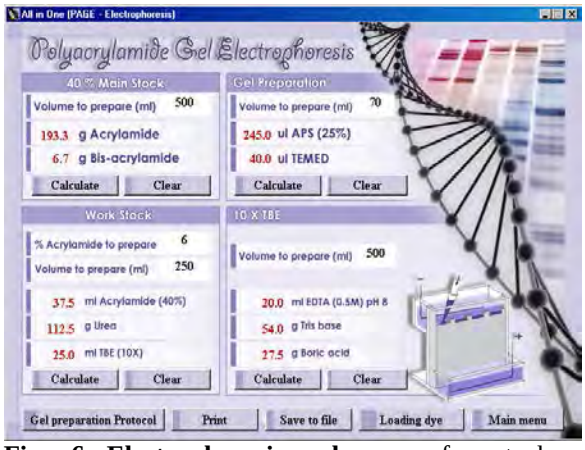

Fig. 6 Electrophoresis submenu: for stocks, solutions preparation and polyacrylamide gel electrophoresis protocol.

Time manager

Khirshyat comes with an alarmed countdown timer, which can be work as a stopwatch, in which the user can select the sound file to alert him and a determined number of minutes. This timer can help managing the time during doing experiments.

Prospective for future enhancement

In near future, the program might be developed to be a comprehensive one by including more protocols and recent ones, e.g. RNA, proteins, isozymes and other recent molecular markers; and a new main menu for tissue culture protocols, including; preparation of the most common used media with different protocols (e.g. micropropagation, somatic embryogenesis, protoplast fusion, cell suspension,…..etc) for important plant species. As well as, more useful illustrations and animated tutorial help might be included.

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Multi-faceted safety assessment of transgenic potatoes expressing a potentially allergenic protease inhibitor from tomato

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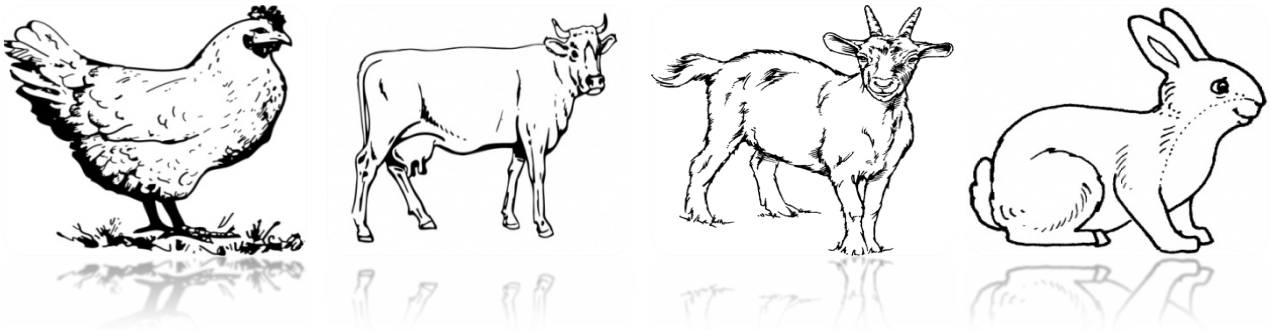
Abstract:

Transgenic potato (Solanum tuberosum L.) lines expressing the broad-spectrum protease inhibitor, tomato cathepsin D inhibitor (CDI), were devised as a 'protein-friendly' platform to produce clinically-useful recombinant proteins. Here we looked for the possible unintended impacts of CDI expression on host tubers, using a classical compositional analysis and a complementary 2-DE analysis of the tuber proteome. 2-D immunodetection assays were then performed for the potentially allergenic, Kunitz-type homologues of CDI found naturally tubers, followed by in vitro protein digestibility assays on raw and processed potatoes treated with simulated human gastric and intestinal fluids. Sera from human patients allergic to soybean proteins were used, finally, to investigate the crossreactivity between soybean trypsin inhibitor and tomato CDI. Overall, our data indicate substantial equivalence between CDI-expressing and control tubers, and a high digestibility of endogenous Kunitz inhibitors in cooked or processed potatoes. The negligible impact of CDI on tuber composition and Kunitz inhibitor content suggests that CDI-expressing potato lines are as safe as their conventional, non-modified counterpart.

Key words: Multi-faceted safety assessment, transgenic potatoes, allergenic protease inhibitor, tomato

I. Contributed Papers

1. Animal Biotechnology



Evaluation of semen characteristics in a project of synthesizing new line of rabbits in egypt

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Abstract

A total number of 30 bucks in three breed groups of rabbits named Gabali, V-line and Moshtohor line (M-line, as new synthetic line 50% Gabali and 50% V-line)), 10 bucks from each breed group were evaluated in project of synthesizing new rabbit line. Traits of ejaculate volume (EV), semen pH, mass motility (MM), sperm cell concentration (SC), individual motility (IM), live sperm per ejaculate (LS), and sperm abnormalities per ejaculate (SA) were studied. Significant differences for EV, SC, IM, MM, LS, and SA in Gabali, V-line and M-line were detected being 0.60, 0.66 and 0.72 ml for EV, 405.83, 474.48 and 456.1 $x10^6$ for SC, 46.95, 48.63, and 54.82% for IM, 2.26, 2.22 and 2.41 grads for MM, 80.63, 82.38, and 81.99% for LS, 11.79, 12.92, and 12.09% for SA, respectively. No significant differences for semen pH grad due to the effect of breed group. Significant differences for semen pH, EV, SC, IM, MM, LS, and SA in autumn, winter, spring and summer seasons were detected being 7.70, 7.74, 7.79 and 7.75 grad for semen pH, 0.67, 0.65, 0.68 and 0.45 ml for EV, 444.37, 447.72, 446.93 and 442.86 $x10^6$ for SC, 54.53, 57.09, 48.77, and 40.12% for IM, 2.57, 2.73, 2.16 and 1.74 grads for MM, 81.21, 83.24, 84.02 and 77.54% for LS, 11.66, 11.02, 11.48, and 14.92% for SA, respectively. The highest values in semen pH, EV, SC, IM, MM, LS, and SA detected to be semen pH (7.88) appeared during the summer with the M-line, 0.7 ml for EV appeared during the autumn with the V-line, 474.13 $x10^6$ for SC was recorded during the spring with the M-line, 58% for IM was recorded during the autumn with M-Line, 2.81 for MM was recorded during the winter with the Gabali, 87.47% for LS was recorded during the spring with V-line, 16.96% for SA was recorded during the summer with V-line, respectively. Gabali breed had superiority in SC and MM in winter, but V-line had superiority in EV and MM in autumn; SC and LS in spring; and MM in winter. However, M-line had superiority in MM and IM in autumn, semen pH and SC in spring and MM in winter. It could be concluded that the M-line, followed by V-line of buck rabbits could be used as effective indicators for good semen quality. Based on heterosis percentages, it is concluded that M-line bucks had superiority in EV (14.3%), semen pH (0.13%), SC (3.6%), MM (7.6%), IM (14.7%), LS (0.6%) and SA (-2.14%) over the two foundations, which might due to dominance and/or over-dominance genes action at different loci on chromosomes.

Key Words: Rabbits, Gabali, Semen, sperm cell, dominance, over- dominance.

Introduction

Rabbit bucks are considered the basis of the reproductive success in the rabbit production especially when artificial insemination is performed as a routine in the rabbitry (Al-Sobayil and Khalil, 2002). Therefore, crossing between rabbit breeds may be required essentially to show heterotic effects on most economic productive and reproductive parameters. M-Line was founded in 2006 (Iraqi et al., 2010) as a synthesizing line between the Egyptian Sinai Gabali (50%), and the V-Line (50%), (Estany et al., 1989). The procedure of foundation began by mating V line does to Sinai Gabali bucks and it was followed by three generations of "inter se" mating. Afterwards the line has been selected to increase litter weight at weaning and individual weight at 56 day. Several methods have been used for routine analysis of mammalian semen, each describing specific quality criteria of the sperm. Semen analysis is the initial step in the evaluation of male reproductive performance. Standard semen analysis including sperm concentration, motility and morphology is widely used as a fundamental indicator of male fertility. Also, Castellini and Lattaiolip (1999) found that sperm number and number of motile sperms are positively associated with both fertilization rate and embryonic quality. Yousef et al. (2001) reported that it is possible to select bucks as sires for breeding programs depending on number of motile sperm per ejaculate, but not percentage of progressive motility per sires. Semen characteristics vary among seasons. Such variations may be attributed to ambient temperature and length of photoperiod (Amin, *et al.* (1987). Increasing ambient temperature adversely affects semen quality, as well as, by reducing the ability of leydig and sertoli cells to respond to LH and the diameter of the semniferous tubules (El-Sherbiny, 1987). The disorders caused by high ambient temperature are amplified with the increase of relative humidity (Marai *et al*., 2010). Semen evaluation must provide information on the fertilizing ability of spermatozoa. The most relevant

parameters correlated with the fertility rate are the number of spermatozoa inseminated and their motility, although the use of a single attribute is not sufficiently accurate to predict the fertilizing ability of the semen (François *et al.*, 2010). The studies of Marai *et al.* (2010) on rabbits showed that semen-ejaculate volume decreased with the elevation of temperature. On the other hand Tawfeek *et al.* (1994) reported no effect of heat elevation on the semen-ejaculate volume in rabbits. Seleem (2005) stated that the sperm motility is one of the most important characteristics for semen quality. The differences among breeds in sperm motility may be due to the variations in pituitary gland activity that can affect the secretion of luteinizing hormone which affects the secretion of testosterone from the interstitial tissue of the testes. There is a significant positive correlation between the sperm motility and fertility in rabbits. Ayyat and El-Aasar (2008) reported that the sperm motility of buck semen decreased significantly ($p<0.01$) in summer season compared to in winter. There was a higher increase in the estimate of the sperm motility from 64 to 78% in all genetic groups, a finding which might be explained on the fact that semen was collected from bucks in their reproductive seasons. The highest percentage of dead sperms (75%) was recorded during summer and the lowest (24.17%) was obtained during spring season (Tharwat *et al.*, 2004). Khalid and Al-Seaf (2007) found that bucks resulted from crossing V-line with Saudi rabbits seemed to be associated with an improvement in the percentage of sperm livability. The relationship between semen quality and fertility keep in mind the sperm cell concentration as one of the main parameters that indirectly define semen quality (Hagen *et al.*, 2002). The objective of this study was to investigate some semen characteristics for bucks of rabbits in Gabali, V-line and M-line (as new synthetic line, 50% Gabali and 50% V-line) within different seasons of production.

Materials and methods

The number of bucks used in this experiment was ten bucks from the Gabali breed, V-line and M-line rabbits, and all animals were apparently healthy and were about 7 – 12 months old with the average weight of 3.3±0.25 Kg. All bucks in the study were housed in individual cages and trained for artificial seamen collection using the artificial vagina. A female rabbit was used as a teaser. After three weeks of training period, semen was collected in the morning from each buck two ejaculate per month according to Breeder man et al. (1964). Each ejaculate was evaluated manually and examined under the microscope. Semen volume was measured per ml by using a graduated tube directly in the collection. If the ejaculates contain gel excretion, it will be measured and removed (El-Sherbiny, 1987). Initial pH of the semen samples was determined just after collection using comparative paper ranging from 6.0 to 8.1 with 0.3 grades (Whatman pH Indicator paper; Whatman Limited Maidstone, England). Mass motility: A drop of freshly collected semen was placed on a slide kept near body temperature (37-38°C) and was examined under low magnification (X120), motility of semen samples were rated according to the vigor of the motility of sperms (El-Sherbiny, 1987). Percentage of progressive motility of spermatozoa: Immediately after each collection, it was assessed by microscopic examination by placing a small drop of fresh semen on a clean warm glass slide (37- 38°C), diluted with two drops of warm 0.9% NaCl and covered with a cover slip. Examination is made under the high power (x400) according to Soad et al. (1996). Sperm cell concentration ($x10^6$/ml): Semen was diluted 200 times with 0.9% NaCl and one drop eosin, sperm cells concentration in millimeter was estimated haemocytometrically. Examination was made under the high power magnification (X675). Percentages of live spermatozoa (LS %): Differentiation between live and dead spermatozoa was assessed by eosin - nigrosin stain technique. Duplicates smears were made and a total of 200 spermatozoa were counted per each slid using the oil sader (X1350). Live spermatozoa were unstained while dead spermatozoa were stained according to Dott and Faster (1972). Percentage of normal spermatozoa: One smear from each ejaculate was stained with eosin-nigrosin stain and a total of 100 sperms were examined randomly. The percentage of normal sperm was estimated as follows according to Khalifa (1977):

Normal sperm (%) = 100 – Abnormal sperm

Statistical analysis

Data of semen characteristics were analyzed using SAS software, SAS (2004) based on the following model**:**

$$Y_{ijk} = \mu + B_i + S_j + (BS)_{ij} + e_{ijk}$$

Where: Y_{ijk} = the k^{th} observation on the rabbit's buck; μ = common mean; B_i = the fixed effect of the i^{th} breed group; S_j = the fixed effect of the j^{th} season; $(BS)_{ij}$ = the fixed effect of the interaction between the i^{th} breed group and the j^{th} season; and e_{ijk}= the random error associated with the individual observation. The data measured as percentages were subjected to arcsine transformation to approximate normal distribution before being analyzed. Tests of significance for the differences between means were carried out according to Duncan (1955).

Results and discussions

Ejaculate volume

Results presented in Tables 1 and 2 show that there are highly significant differences ($p<0.001$) among the three studied breeds in the ejaculate volume. Means of the ejaculate volume were 0.60, 0.66 and 0.72 ml for the Gabali, V-line and M-line,

respectively. From these results it could point out the superiority of males of Moshtohor line over those the two foundations. Another point of view, the Gabali bucks showed the smallest average of ejaculate volume. This may be due to the Gabali breed has low activity in accessory sex galnds in response testosterone hormone in comparison with the other two breed groups. Results of the present study are in agreement with those reported by Tharwat *et al.* (2004), and Tawfeek *et al.* (2010) who found that, breed have significant effect on ejaculate volume. Conversely, these results disagreed with findings of Seleem (2005) and El-Sbeiy *et al.* (2008) who found that, breed have non-significant effect on ejaculate volume. The variation in the ejaculate volume between breeds may be due to differences in the rate of the accessory sex glands activity in response testosterone hormone (Abd EL-Ghaffar, 1992 and EL-Kamash *et al.,* 2000). It was found that means of ejaculate volume did not showing significant differences due to season effect. Means of the ejaculate volume were 0.67, 0.65, 0.68 and 0.45 ml for the autumn, winter, spring and summer seasons, respectively. Results obtained are in agreement with Seleem (2005) who found that, season have non-significant effect on ejaculate volume. Tharwat *et al.* (1994) found that the greatest ejaculate volume (0.5 ml) was obtained in spring and the smallest (0.2 ml) was obtained in summer. The decrease in the ejaculate volume may be attributed to the effect of high temperature on the level testosterone production and/or secretion which is decreased by increasing ambient the temperature. Ayyat and El-Aasar (2008) reported that during summer, the ejaculate volume of buck semen decreased significantly ($p<0.01$) than in winter. It was shown that the highest ejaculate volume (0.7 ml) was recorded during the autumn season with the V-line, while the lowest one (0.47 ml) was estimated during the summer season with the Gabali breed (Table 1).

Semen pH

Means of semen pH as affected by breed group showed non-significant differences, as well as season effect showed also non-significant differences (Table 1). These means mounted 7.76, 7.72 and 7.75 in Gabali, V-line and M-line; 7.70, 7.74, 7.79 and 7.75 in autumn, winter, spring and summer seasons, respectively. The interaction between breed group and season effects has no significant affect on the pH of semen's buck. It was found that the highest pH value (7.88) appeared during the spring season for M-line, while the lowest value (7.62) appeared during the autumn season with M-line. These differences may be attributed to the differences in environmental temperature among season. The measurements of semen pH is of great importance because any semen extender used should be of approximate similar pH value as semen or should act as a buffer against excessive acidity or alkalinity and also, it acts as an indicator for the normal accessory glands secretion and the livability of spermatozoa (Abd EL-Ghaffar, 1992). So, pH is considered a good indicator for semen quality.

Sperm cell concentration ($x10^6$)

Means of sperm cell concentration ($x10^6$) as affected by breed group of the buck were 405.83, 474.48 and 456.11 for Gabali, V-line and M-line, respectively (Table 1). Analysis of variance for data obtained reviled highly significant differences ($P<0.0001$) among the three breed groups and the superiority of V-line and M-line against the Gabali breed was dearly evident (Table 2). The variation in the sperm cell concentration may be due to difference within the breeds, age, environmental conditions, testosterone level, the body size and testicular weight (Abd EL-Ghaffar, 1992). These findings are in agreement with those of Al-Sobayil and Khalil (2002) and Seleem (2005). Most of these studies showed significant effects of breeds on sperm-cell concentration per ml.

Table 1. Least square means[+] and standard errors for factors affecting ejaculate volume (ml), semen pH and sperm cell concentration ($x10^6$) in rabbit bucks.

Classification	No. of Obs.	Ejaculate volume, ml	Semen pH	Concentration, $x10^6$
Breed group (B):				
Gabali	240	0.60 ± 0.01^{b}	7.76 ± 0.03	405.83 ± 4.2^{c}
V-line	240	0.66 ± 0.01^{a}	7.72 ± 0.03	474.48 ± 4.3^{a}
M-line	240	0.72 ± 0.01^{a}	7.75 ± 0.03	456.11 ± 4.2^{b}
Heterosis (%)[++]		14.30	0.13	3.60
Season (S):				
Autumn	180	0.67 ± 0.01	7.70 ± 0.03	444.37 ± 4.6^{a}
Winter	180	0.65 ± 0.01	7.74 ± 0.03	447.72 ± 5.6^{a}
Spring	180	0.68 ± 0.01	7.79 ± 0.03	446.93 ± 5.2^{a}
Summer	180	0.45 ± 0.01	7.75 ± 0.03	442.86 ± 3.8^{b}
Interaction of (BxS):				
Gabali x autumn	60	0.62 ± 0.02^{bcd}	7.70 ± 0.05	461.38 ± 11.54^{ab}
Gabali x winter	60	0.61 ± 0.02^{cd}	7.77 ± 0.05	478.75 ± 9.17^{a}

Table 1. cont.

Gabali x spring	60	0.62±0.02^{bcd}	7.72±0.06	423.72±8.94^{cd}
Gabali x	60	0.47±0.02^{e}	7.75±0.06	403.05±8.66^{de}
V-line x autumn	60	0.70±0.02^{a}	7.66±0.06	470.18±9.10^{a}
V-line x winter	60	0.67±0.01^{b}	7.76±0.04	442.17±8.86^{ab}
V-line x spring	60	0.66±0.02^{bc}	7.77±0.06	474.91±9.61^{a}
V-line x	60	0.49±0.02^{e}	7.71±0.06	393.38±7.44^{e}
M-line x	60	0.67±0.02^{bc}	7.62±0.04	459.80±6.79^{ab}
M-line x winter	60	0.67±0.01^{bc}	7.71±0.04	461.10±6.09^{ab}
M-line x spring	60	0.67±0.02^{a}	7.88±0.06	474.13±6.53^{a}
M-line x	60	0.56±0.02^{d}	7.80±0.08	400.15±8.40^{de}

$^{+}$ Means with different superscript in the same column within each effect are significantly different at ($p<0.05$).
$^{++}$ Heterosis percentages were computed as: {(average of M-line – (average of the Gabali and V-line)) / (average of the Gabali and V-line)} x 100.

Table 2. F-ratios of least square analysis for factors affecting semen pH, ejaculate volume (ml) and sperm cell concentration ($x10^6$) in rabbit bucks.

S .O. V	d.f.	Ejaculate volume, ml	Semen pH	Concentration, $x10^6$
Breed group (B)	2	18.95****	0.41$^{n.s}$	85.05****
Season (S)	3	0.61$^{n.s}$	1.17$^{n.s}$	3.03**
Interaction of (BxS)	6	2.66**	1.70$^{n.s}$	1.68*
Error d.f.	708	4.43	1.42	19.68
Error MS		0.105	0.24	32.92

n.s = non significant, * = $p<0.05$** = $p<0.01$ and ****= $p<0.0001$.

Results obtained in (Table 1) showed highly significant differences ($p<0.01$) in the sperm cell concentration ($x10^6$) of buck semen among the four seasons. The sperm cell concentration means were 444.37, 447.72, 446.93 and 442.86 for autumn, winter, spring and summer seasons, respectively. The lower value of sperm cell concentration ($x10^6$) associated with the hot climate could be attributed to the decline in levels of testosterone and gonadotrophins essential for maintaining the testicular sperm producing potential (Ayyat and El-Aasar, 2008). These results are in agreement with findings of El-Sherbiny (1987) and Daader *et al. (*1999) who revealed that total-sperm output values were significantly higher in winter than in summer. Zeidan *et al*. (1997) stated that the decrease in sperm cell concentration might be attributed to the degeneration of germinal epithelium and atrophy of the semniferous tubules. High ambient temperature was found to affect the total sperm production (Ahmed *et al*., 2006). This could be attributed to a decrease in the activity of sertoli cells which in turn affects daily spermatogenesis. Data presented in Table 1 show that the highest sperm cell concentration (474.13) recorded during the spring season with the M-line, while the lowest average (393.38) was recorded during the summer season with the V-line, respectively. This reflects the superiority of the crossbred line of Moshtohor by 3.6% over the two foundations indicating good semen quality of Moshtohor bucks that have indirect relationship with the buck fertility. The relationship between semen quality and fertility keep in mind the sperm cell concentration as one of the main parameters that indirectly define semen quality (Hagen *et al.*, 2002). Mass motility score (0-5): Data obtained in (Table 4) showed highly significant differences ($p<0.01$) due to breed group effect with means of 2.26, 2.22 and 2.41 in Gabali, V-line and M-line, respectively. This indicates that superiority of the crossbred line of Moshtohor by 7.6% over the two foundations. These findings are in agreement with those of Khalil (1999) and EL-Kamash et al. (2000), who concluded that sperm motility differs between different breeds of rabbits. These differences in sperm motility may be due to the variations in pituitary gland activity that can affect the secretion of luteinizing hormone (LH) which affects the secretion of testosterone from the interstitial tissue (Leydig cells) of the testes (Seleem, 2005). Data concerning the effect of season on mass motility means presented in Table 3 revealed that average of mass motility mounted 2.57, 2.73, 2.16 and 1.74 for autumn, winter, spring and summer seasons, respectively. It is showed highly significant differences ($p<0.01$) due to the effect of seasons (Table 4). The highest means were recorded in autumn and winter while the lowest ones (1.74) were in summer season which could be due to high environmental temperature. These findings are in agreement with those of Tharwat *et al.* (1994) and El-Maghawry and Soliman (2002) who reported that

hot environment in summer have adverse effect on mass and progressive sperm motility. Results obtained in Table 3 show that the highest mass motility average in summer season was recorded for M-line (1.88, $p<0.05$), followed by Gabali breed (1.73), while the lowest ones was recorded for V-line (1.61). This indicates that Moshtohor line has more adaptation ($p<0.05$) with hot climate conditions in summer than the two foundations for mass motility of sperm. This could be due to genetic progress by crossing Gabali with V-line (Khalil, 1996). Individual motility (%): Advanced motility illustrates the sperm activity degree and its importance for passing throws the oviduct and completing fertilization (EL-Darawany and EL-Sayiad, 1995). Results obtained showed highly significant differences ($p<0.01$) due to breed group effect (Table 4) having means of 46.95, 48.63, and 54.82% for Gabali, V-line and M-line, respectively (Table 3). This reflect the superiority of the crossbred line (Moshtohor) over the other breeds by 14.7% which indicate good advanced motility of Moshtohor bucks that have direct relationship with the buck fertility. Many workers noticed that breed has significant effect on the individual motility of sperms (Tharwat et al., 2004 and Seleem, 2005). Mass and individual sperm motilities in Gabali (1.44 and 50%, resp.) were lower than those of New Zealand White (2.82% and 55%, resp.) rabbits (Khalil, 1999). Results obtained allowed that average of individual motility mounted 54.53, 57.09, 48.77, and 40.12% for autumn, winter, spring and summer seasons, respectively, showed highly significant differences ($p<0.01$) among the four seasons (Table 4). The best means were recorded in autumn, winter while the lowest values were recorded during the summer season that could be due to high environmental temperature. These findings are in agreement with those of Amin et al. (1987) who found that, season have significant effect on individual sperm motility. The individual sperm motility of buck semen was noticed to be significantly ($p<0.05$) lowered (37.1%) during the period of high ambient temperature than those (81.1%) during the period of low ambient temperature as reported by Ahmed *et al*. (2006). Ayyat and El-Aasar (2008) reported that during summer, the sperm motility of buck semen decreased significantly ($p<0.01$) than in winter season. Results in Table 3 showed that the highest individual motility average in summer season was recorded for M-line (44.58%), followed by V-line (39.78%), while the lowest ones was recorded for Gabali breed (33.96%). This indicates that Moshtohor line has more adaptation ($p<0.05$) with hot climate conditions in summer than the two foundations for individaul motility of sperm. This could be due to genetic progress by crossing Gabali with V-line (Khalil, 1996). Live sperm per ejaculate: It was found that means of live sperm per ejaculate were 80.63, 82.38, and 81.99% for Gabali, V-line and M-line, respectively (Table 3). This reflects the superiority of the crossbred line of Moshtohor by 0.6% over the two foundations. Such breed differences in live sperm percentage was also reported by EL-Sherbiny (1987). Khalil (1996) reported that the percentage of dead sperm was lower in Gabali (21%) than that in New Zealand White (24%) rabbits. Khalid *et al*. (2009) found that bucks resulted from crossing V-line with Saudi rabbits seemed to be associated with an improvement in the percentage of sperm livability. Results obtained in Table 3&4 showed that average of live sperm per ejaculate were 81.21, 83.24, 84.02 and 77.54% for autumn, winter, spring and summer seasons, respectively which showed highly significant differences ($p<0.01$) due to the effect of four seasons. The highest means were recorded in spring and winter while the lowest value (77.54%) was recorded during the summer season due to the negative and deleterious effects of high environmental temperature. These results in agreement with the finding of El-Maghawry and Soliman (2002), they attributed the low percentage of dead sperms during spring under Egyptian condition to the suitable environmental conditions. The percentage of dead spermatozoa was significantly ($p<0.05$) higher during the period of high ambient temperature (Ahmed *et al.*, 2006). The highest live sperm per ejaculate (87.47%) was recorded during the spring season with V-line, while the lowest (75.94%) was recorded during the summer season with the V-line also. These results show that V-line bucks were more sensitive to the increase of ambient temperature. On the other hand, line sperm in summer season was recorded for M-line (79.0%), followed by Gabali breed (76.87%) and the lowest one recorded for V-line (75.94%). This indicates that Moshtohor line has more adaptation ($p<0.05$) with hot climate conditions in summer than the two foundations for live sperm. This could be due to genetic progress by crossing Gabali with V-line (Khalil, 1996). Sperm abnormalities per ejaculate percentage: Significant differences ($p<0.0001$) was observed, among breed group effects with means of 11.79, 12.92 and 12.09% for Gabali, V-line and M-line, respectively. This reflects the superiority of the crossbred line of Moshtohor by -2.14% for sperm abnormalities over than the two foundations. The percentage of sperm abnormalities per ejaculate are in positive relationship with the sperm cell concentration as shown the smallest concentration in Gabali bucks semen correlated with the smallest percent of abnormal sperms. It is important to notice that the presence of large number of abnormal spermatozoa in semen seem to decrease its fertility. This fact is in agreement with that of Afifi (1997) who revealed that percentage of abnormal sperm in Gabali (20%) was lower than that in New Zealand White (22%) and Baladi (23%) but higher than that in Californian (18%) and Giza White (17%) rabbits. Tawfeek *et al.* (2010) found significant differences in

percentages of normal spermatozoa due to breeds, while other investigations revealed non-significant differences in percentages of normal spermatozoa among different breeds of rabbits (El-Darawany and EL-Sayied, 1995 and Daader *et al.,* 1999). Average of sperm abnormalities (%) mounted 11.66, 11.02, 11.48, and 14.92 % for autumn, winter, spring and summer seasons, respectively, showed highly significant differences ($p<0.01$) among the four seasons (Table, 4). Increasing of sperm abnormalities per ejaculate was found in summer (14.92%) which was higher than the other seasons this may be attributed to the negative and deleterious effects of high environmental temperature during the summer season. During summer, the percentage of abnormal spermatozoa of buck semen increased significantly ($p<0.01$) than in winter (Seleem, 2005). The interaction between breed and season affected average of the sperm abnormalities per ejaculate. The highest sperm abnormalities per ejaculate average 16.96% was recorded during the summer season with V-line, while the lowest average 10.67% was recorded during the spring season with the Gabali breed. This result show that V-line bucks were more sensitive to the increase in ambient temperature and should to be reared in optimum conditions and to be avoided of heat stress while Gabali (as a local breed) is more adapted to hot climate conditions but still in demand of genetic selection and improvement of managerial procedures.

Conclusion

The M-line and V-line of buck rabbits could be used as effective indicators for good semen quality. Based on heterosis percentages, it is recommended that the M-line bucks had superiority in all the studied semen characteristics which might due to dominance and/or over-dominance genes action at different loci on chromosomes.

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Table (3). Least square means[+] and standard errors for factors affecting mass motility, grade, individual sperm motility, live sperm/ejaculate %, and sperm abnormalities /ejaculate % in rabbit bucks.

Classification	No. of Observation	Mass motility, grade	Individual sperm motility, %	Live sperm/ ejaculate,%	Sperm abnormalities/ ejaculate,%
Breed group (B):					
Gabali	240	2.26 ± 0.04^{b}	46.95 ± 0.7^{b}	80.63 ± 0.25^{c}	11.79 ± 0.14^{b}
V-line	240	2.22 ± 0.04^{b}	48.63 ± 0.7^{b}	82.38 ± 0.26^{a}	12.92 ± 0.14^{a}
M-Line	240	2.41 ± 0.04^{a}	54.82 ± 0.7^{a}	81.99 ± 0.25^{b}	12.09 ± 0.14^{b}
Heterosis (%)[++]		7.6	14.7	0.6	-2.14
Season (S):					
Autumn	180	2.57 ± 0.04^{a}	54.53 ± 0.8^{a}	81.21 ± 0.27^{b}	11.66 ± 0.15^{b}
Winter	180	2.73 ± 0.04^{a}	57.09 ± 0.7^{a}	83.24 ± 0.23^{a}	11.02 ± 0.12^{c}
Spring	180	2.16 ± 0.05^{b}	48.77 ± 0.9^{b}	84.02 ± 0.31^{a}	11.48 ± 0.17^{bc}
Summer	180	1.74 ± 0.06^{c}	40.12 ± 1.0^{c}	77.54 ± 0.34^{c}	14.92 ± 0.18^{a}
Interaction of (B*S):					
Gabali x autumn	60	2.26 ± 0.08^{b}	55.02 ± 1.47^{b}	81.52 ± 0.48^{cd}	11.17 ± 0.26^{bef}
Gabali x winter	60	2.81 ± 0.07^{a}	57.75 ± 1.37^{ab}	82.23 ± 0.44^{c}	10.71 ± 0.24^{f}
Gabali x spring	60	2.25 ± 0.09^{b}	41.02 ± 1.64^{d}	81.91 ± 0.53^{cd}	10.67 ± 0.29^{f}
Gabali x	60	1.73 ± 0.09^{cd}	33.96 ± 1.59^{e}	76.87 ± 0.52^{f}	14.52 ± 0.28^{b}
V-line x autumn	60	2.69 ± 0.09^{a}	48.59 ± 1.67^{c}	81.67 ± 0.55^{cd}	11.81 ± 0.30^{d}
V-line x winter	60	2.63 ± 0.07^{a}	55.52 ± 1.19^{ab}	84.46 ± 0.39^{b}	11.44 ± 0.41^{def}
V-line x spring	60	1.97 ± 0.10^{c}	48.62 ± 1.76^{c}	87.47 ± 0.58^{a}	11.76 ± 0.32^{de}
V-line x summer	60	1.61 ± 0.09^{d}	39.78 ± 1.69^{d}	75.94 ± 0.56^{f}	16.96 ± 0.30^{a}
M-line x autumn	60	2.76 ± 0.07^{a}	58.00 ± 1.24^{a}	80.46 ± 0.41^{de}	12.01 ± 0.22^{d}
M-line x winter	60	2.76 ± 0.06^{a}	57.90 ± 1.12^{ab}	83.03 ± 0.36^{c}	10.92 ± 0.20^{ef}
M-line x spring	60	2.26 ± 0.09^{b}	56.67 ± 1.62^{ab}	82.68 ± 0.53^{c}	11.92 ± 0.29^{d}
M-line x	60	1.88 ± 0.10^{c}	44.58 ± 2.11^{cd}	79.80 ± 0.69^{e}	13.55 ± 0.38^{c}

[+] Means with different superscript in the same column within each effect are significantly different at ($p<0.05$).

[++] Heterosis percentages were computed as: {(average of M-line – (average of the Gabali and V-line)) / (average of the Gabali and V-line)} x 100.

Table (4). F- ratios of least square analysis for factors affecting mass motility, grade, individual sperm motility, live sperm/ejaculate %, and sperm abnormalities/ejaculate % in rabbit bucks.

S .O. V	d.f.	Mass motility, grade	Individual sperm motility, %	Live sperm/ ejaculate, %	Sperm abnormalities/ ejaculate, %
Breed group (B)	2	4.97***	28.23***	11.70***	16.87****
Season (S)	3	70.80****	67.08****	81.87****	102.82****
Interaction of (BxS)	6	3.84****	8.35****	11.72****	6.09****
Error d.f.	708				
Error MS		0.54	159.29	17.17	5.26

*** = p<0.001 and ****= p<0.0001.

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Evaluation of doe traits in a project of synthesizing new line of rabbits in Egypt

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Abstract

The experimental work of this study was carried out within a project of "improvement of rabbit lines" with the cooperation of the Spanish agency of international cooperation through four successive seasons from 2007 to 2008 in the rabbitry of the Department of Animal Production, Faculty of Agriculture, Benha University, Egypt. A total number of 45 does in three breed groups of Gabali, V-line and Moshtohor line (M-line) rabbits. Three does from each breed group were chosen randomly and sacrificed at 12 day of gestation (after palpation and pregnancy assurance). The studied were ovulation rates (OR), number of implantation sites/doe (NIS), number of viable embryos/doe (NVE), implantation rate (IR), number of corpora lutea (NCL), weight of corpora lutea (WCL), weight of lutein tissue (WLT), weight of ovary (WO), early embryonic mortality (EEM) % and some histological studies in uterine horn. Obtained results showed significant differences in OR, NIS, NVE, IR, NCL, WCL and WLT between the Gabali, V-line and M-line being 8.67, 8.1 and 9.2 for OR; 7.36, 7.43 and 8.33 for NIS; 5.02, 6.67 and 7.13 for NVE; 84.89%, 91.72% and 90.54% for IR; 12.9, 14.1 and 13.6 mg for WCL; and 111.84, 114.21 and 125.12 mg for WLT, respectively. Average of the early embryonic mortality was 20.69, 9.12 and 10.87% in Gabali, V-line and M-line, respectively. However no significant difference was found in weight of ovary due to the effect of breed group. The mucosal layer of uterus was lined by columnar epithelium with underlying connective tissue of the lamina propria which was rich by dilated blood capillaries in V-line and M-line than in Gabali breed. The muscular layer was thicker in M-line does than in both Gabali and V-line. It is concluded that doe rabbits of M-line had superiority in OR (9.7%), INS (12.6%), NVE (22.0%), IR (2.5%), NCL (9.7%), WCL (0.74%), WLT (10.7%), WO (-1.2%) and EEM (-27.1%) over of the two foundations.

Key Words: Doe and histological traits, rabbits, heterosis for doe traits, synthetic line of Moshtohor.

Introduction

One of the most interesting principles in the rabbit management is the selection for the suitable breeds or lines using in crossbreeding programs to maximize meat production. Selection on uterine capacity could be a more efficient way for improving litter size, although more research is needed to confirm this possibility. The Gabali local breed living in north Sinai are well adapted to the Egyptian conditions (Iraqi *et al.*, 2008). The Spanish V-line exhibits an outstanding maternal ability as related to behavior, fecundity and lactation (Estany *et al.*, 1989 and Garcia *et al.*, 2000). Moshtohor line (M-line) was founded in 2006 (Iraqi et al., 2010 and Youssef *et al.*, 2008) as a synthesizing line between the Egyptian Sinai Gabali (50%), (Afifi, 2002) and the V-line (50%), (Estany et al., 1989). The procedure of foundation began by mating V line does to Sinai Gabali bucks and it was followed by three generations of "inter-se" mating. Afterwards the line has been selected to increase litter weight at weaning and individual weight at 56 day. The main objective of the crossbred is to produce larger numbers of rapidly growing rabbits than those could be produced by pure breeding (El-Maghawry et al., 1999). There is a high variation in the reproductive performance of does in Egypt, which could be attributed to management decision in terms of post-partum mating, remating schedule... etc (Khalil, 1993). Argente *et al.* (2003) found that the high line showed higher ovarian weight (0.08 g, $p<0.05$) linked to a higher ovulation rate. Also the number of implanted embryos and live fetuses, fetal survival, and uterine weight and length were positively associated and explained most of the observed variation. Litter traits are usually regarded as the best estimate of number and weight of young produced by the doe since they constitute functions of all pre-weaning effects. Litter weight at weaning, as a composite trait, reflects the contribution of fertility, maternal behavior, milk production, pre-weaning growth and survival (Lukefahr *et al.*, 1990). Jonson and Rask-Andersen (1993) clarified that weights of right and lift ovary in NZW rabbits ranged from 178 to 489 mg at 6 days post-coitum. Gosalvez *et al.* (1987) found that California multifarious does showed higher value of ovarian weight than precipitous (371.5 vs. 290.4 mg) reaching maximal weight (385.0 mg) from the 28th to 30th days of gestation and decreasing after kindling until a minimum value on the first day post-partum (243.6 mg). They added that, ovaries weight in rabbit does increased from 328.9 to 333.0 mg when the number of litters increased from 0-7 to 8-14 litters. Also, they reported that, the weights of the left and right ovaries were approximately close to each other (326.4 vs. 332.5 mg). The highest ovarian weight may be attributed to the increase in levels of FSH

and LH hormones which enhance development and growth of ovarian follicles and increase the number of growth or mature ova (Rommers *et al.,* 2004).Uterine capacity is defined as the maximal number of fetuses that the dam is able to support at birth when ovulation rate is not a limiting factor (Christenson *et al.,* 1987). Moce *et al.* (2004) noticed that the uterine capacity begins to affect litter size before implantation in rabbits; embryonic survival depends mainly on embryo viability and oviduct and uterine environment; available uterine space is a imitating component of fetal survival, which is related to an adequate vascular supply for nutrient exchange from the maternal to fetal blood streams and an adequate surface area for development of the placenta. Selection on uterine capacity could be a more efficient way of improving litter size in rabbits than direct selection on litter size, although more research is needed to confirm this possibility (Blasco *et al.,* 1994). Argente et al. (2003) found differences in doe traits between the high- and low uterine capacity in rabbit's lines.

After implantation, there is a critical moment for fetal survival in rabbits between the day 8 and 17 of gestation, when the hemochorial placenta of rabbit has finished its development and the nutrition of the fetus begins to be controlled by the placenta (Adams and Chang, 1960). It has been reported that 52% of the fetal losses takes place between implantation and day 18 of gestation for unilateral ovariectomized does (Moce *et al.,* 2004) and 29% from implantation to birth for intact does (Argente *et al.,* 2003).

Ovulation rate could be measured by counting the number of corpus luteum at days 15 post-coitum (Hafez and Hafez, 2000). Hulot and Mariana (1985) recorded the ovulation rate 8 hours after mating to be 13 and 11 for Cal and NZW female rabbits, respectively. Abd El-Kafy (2000) reported that in primiporous NZW rabbits, number of corpus luteum was 7.0 at 48 hrs. post-coitum, but less than that reported by Ismail *et al.* (1992), which was 10.5 in Bouscat does rabbits. The present work was carried out to investigate the effect of breed group of Gabali, V-line and M-line (as new synthetic line, 50% Gabali and 50% V-line) on reproductive activity at the 12 day post-coitum.

Materials and methods

The experimental work of this study was carried out within a project of "improvement of rabbit lines "with the cooperation of the Spanish agency of international cooperation through four successive seasons from 2007 to 2008 in the rabbitry farm of the Department of Animal Production, Faculty of Agriculture, Benha University, Egypt. In the base generation animals of V-line a synthetic maternal line originated in 1982 at the Department of Animal Science of Universidad Politecnica de Valencia, Spain. Litter size at weaning was considered as the criterion for selection in this line. The Moshtohor line (M-line) as an Egyptian synthetic line coming from a first cross between the Egyptian Sinai Gabali (50%) and the V-line (50%), followed by three consecutive generations of "inter se" mating (Iraqi *et al.,* 2010). Three does from each breed group were chosen randomly and sacrificed at 12 day of gestation (after palpation and pregnancy assurance). The weight of ovaries and uterus was recorded. Number of corpora lutea on the right and left ovary alongside the number and weight of survival embryos were recorded. The uterine horns were dissected surgically and embryos were examined for heart pulsation. The swelling in the horn were cut open and chorionic vesicles if present were transferred into a petri dish containing normal saline, carefully opened and embryos were released. Viable embryos were those showing spontaneous or induced heart beatings or those without any detectable symptoms of disintegration, e.g. their tone, color, proportion of the head and limbs in relation to the body and crown rump length were the same as for their counterparts with functioning hearts Embryos otherwise were considered dead. Swelling with partial or complete disintegrated embryonic membranes revealed typical cases of embryonic mortality. Ovarian weights, numbers of corpora lutea, implantation and viable embryos were recorded. Autopsy samples were taken from uterus of rabbits in different groups and fixed in 10% normal saline for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns by slide microtome. The obtained tissue sections were collected on glass slides, deparffinized and stained by hematoxylin and eosin stains (Banchroft et al., 1996) for histological examination through the electric light microscope. Statistical analysis: Data were analyzed using SAS Software (SAS, 2004) according to the following model:

$$Y_{ij} = \mu + B_i + e_{ij}$$

Where:

Y_{ij} = the j^{th} observation on the doe rabbit; μ = general mean, common element to all observations; B_i = the fixed effect of the i^{th} breed group and e_{ij}= random error associated with the individual observation. All data measured as percentages were subjected to arcsine transformation to approximate normal distribution before being analyzed. Tests of significance for the differences between means were carried out according to Duncan (1955).

Results and discussions

Reproductive activity

Both the number and weight of corpora lutea were determined. The data of ovulation rate estimated on the basis of counts of corpora lutea appear in Table 1. Rates for the three respective breeds when CL counts were done 1 days post coitum were 8.67, 8.1, and 9.2 in Gabali, V-line and M-line, respectively. Difference between mean was statistically significant ($p<0.05$) as presented in Table 2. Average numbers of implantations sites/doe were 7.36, 7.43, and 8.33 in Gabali, V-line and M-line, respectively. Difference between means was statistically significant ($p<0.01$) as presented in Table 2. Data on number of viable embryos/doe followed the same pattern reported for number of implantation (Table 1). Average number of viable embryos/doe was 5.02, 6.67, and 7.13 in Gabali, V-line and M-line respectively, showing significant differences ($p<0.05$) as presented in Table 2, of interest is the percentages of corpora lutea counted at 12 days post coitum associated with the formation of implantation sites. These percentages (implantation rate) were 84.89%, 91.72% and 90.54% in Gabali, V-line and M-line, respectively (Table 1), showing significant differences ($p<0.05$). Therefore, in this period the placenta would require an adequate surface area for its development and an adequate vascular supply for nutrient exchange from the maternal to fetal blood streams. After implantation, there is a critical moment for fetal survival in rabbits between the day 8 and 17 of gestation, when the hemochorial placenta of rabbit has finished its development and the nutrition of the fetus begins to be controlled by the placenta (Adams and Chang, 1960). Moce *et al.* (2004) noticed that the uterine capacity begins to affect litter size before implantation in rabbits; embryonic survival depends mainly on embryo viability and oviduct and uterine environment; available uterine space is a imitating component of fetal survival, which is related to an adequate vascular supply for nutrient exchange from the maternal to fetal blood streams and an adequate surface area for development of the placenta. These results are agreement with the findings of Garner *et al*, (1987), El-Terbany (1993), Ford *et al*. (2002). Valet *et al.* (2002), Argent *et al*, (2003) and Merchan *et al.* (2006) who found that, breed have significant effect on ovulation rates, number of implantation sites, number of viable embryos and implantation rate. Morphological differences for rabbit after 12 days post coitum: The means numbers of corpora lutea /doe are presented in Table 1. Results obtained show that average number of corpora lutea mounted 8.67, 8.1 and 9.2 in Gabali, V-line and M-line, respectively which show significant differences ($p<0.05$) as presented in Table 2 within the three studied breed groups. The average weights of corpora lutea mounted 12.9, 14.1 and 13.6 mg in Gabali,V-line and M-line, respectively which show significant differences within the three studied breed groups. However, the average weight of lutein tissue/doe mounted 111.84, 114.21and 125.12 mg in Gabali, V-line and M-line, respectively (Table 1). The weight of ovary means was 380, 440 and 405 mg in Gabali, V-line and M-line, respectively which show no significant differences due to breed group effect. Average of the early embryonic mortality was 20.69, 9.12 and 10.87% in Gabali, V-line and M-line, respectively. These results are in agreement with many studies El-Fouly *et al.* (1977), Gorbner *et al.* (1987), Radwan and El-Sayed (1990) and Blasco *et al.* (1994) who found that, breed have significant effect on number of corpora lutea /doe and weights of corpora lutea. On the other hand, results obtained disagreed with those of Hafez (1964) who reported that the average weight weights of corpora lutea pair in multifarious NZW does at 6 days post-coitus ranged from 266 to 892 mg with an average of 479 mg. There was no significant difference between the weight of ovaries in NZW and Chinchilla rabbits breeds.

Table 1. Least-square means of morphological traits in different breed group of rabbits after 12 days post coitum.

Observation (Imposed at 12 day post coitum)	No. of Obs.	Breed group[+]			Heterosis (%)
		Gabali	V-line	M-line	
Ovulation rates [(1)](OR)	9	8.67^{ab}±0.50	8.1^{b}±0.50	9.2^{a}±0.50	9.7
Number of implantation sites/doe(NIS)	9	7.36^{b}±0.63	7.43^{b}±0.63	8.33^{a}±0.63	12.6
Number of viable embryos/doe(NVE)	9	5.02^{b}±0.41	6.67^{b}±0.41	7.13^{a}±0.41	22.0
Implantation rate (IR) %	9	84.89	91.72	90.54	2.5
Number of corpora lutea /doe(NCL)	9	8.67^{ab}±0.50	8.1^{b}±0.50	9.2^{a}±0.50	9.7
Weight of corpora lutea (WCL), mg	9	12.9^{b}±0.001	14.1^{a}±0.001	13.6^{a}±0.001	0.74
Weight of lutein tissue/doe (WLT), mg	9	111.84	114.21	125.12	10.7
Weight of ovary (WO), mg	9	380	440	405	-1.2
Early embryonic mortality (EEM) %	9	20.69	9.12	10.87	-27.1

[+] Means with different superscript in the same row are significantly different at ($p<0.05$).

[++] Heterosis percentages were computed as: {(average of M-line – (average of the Gabali and V-line)) / (average of the Gabali and V-line)} x 100. [(1)]. Assessed by counting number of corpora lutea / doe.

Table 2. F-ratios of least squares analysis for breed group affecting morphological tarits[+] in doe rabbits after 12 days post coitum.

S.O.V	d.f.	F-ratios					
		OR	NIS	EAT	NCL	WCL	WO
Breed group	2	4.00*	1.73**	0.67*	4.00*	1.00*	1.75ns
Error d.f.	6						
Error MS		0.778	1.22	0.67	0.778	0.00005	0.001

n.s = non-significant, * = p<0.05 and ** = p<0.01.
[+]OR = *Ovulation rates* (1), NIS =*Number of implantation sites/doe*, EAT= *Number of embryos atresia*, NCL =*Number of corpora lutea /doe*, WCL =*Weight of corpora lutea (mg)*, WO =*Weight of ovary (mg).*

On the other hand, results in Table 1 showed also that doe rabbits of M-line had superiority in OR (9.7%), INS (12.6%), NVE (22.0%), IR (2.5%), NCL (9.7%), WCL (0.74%), WLT (10.7%), WO (-1.2%) and EEM (-27.1%) over the two foundations. This may be due to dominance and/or over dominance of genes action. These results are agreement with foundations of Argente *et al.* (2003) and Moce *et al.* (2004).

Histological studies:
The mucosal layer of uterus was lined by columnar epithelium with underlying connective tissue of the lamina propria which was rich by dilated blood capillaries in V-line and M-line than in Gabali breed. Maximum uterine proliferation Table 3 was observed in the endometrium of V-line Figure, 1 and followed by M-line Figures 2 and 5. The contrary picture was observed in Gabali Figures, 3 and 6. Concerning the thickness of myometrium, inner circular and outer longitudinal muscular layer was recorded with outer serosal layer. The muscular layer was thicker in M-line than in Gabali and V-line. This seems to be associated with the reproductive activity.

Table 3. Histological findings recorded in the uterus in different breed groups of rabbits at 12 day of gestation.

Breed group	Uterus lesions			
	Hyperplasia of the epithelial lining of the endometrium	Activation of glands in endometrium	Congestion of blood vessels	Hyperplasia of the myometrial layer
Gabali	++	+	+	++
V-line	+++	+++	+++	+++
M-line	+++	+++	+++	+++

+ = low, ++ = moderate and +++ = High.

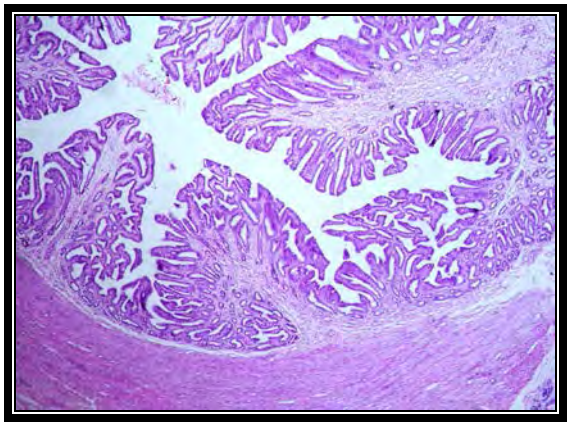

Fig. 1: Uterus of V-line rabbits showing hyperplasia of the epithelial Endometrium (H, E X40)

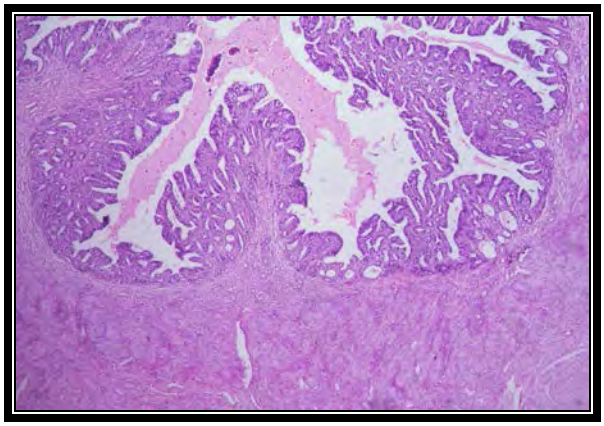

Fig. 2: Uterus of M-line rabbits showing hyperplasia of the epithelial Endometrium (H, E X40)

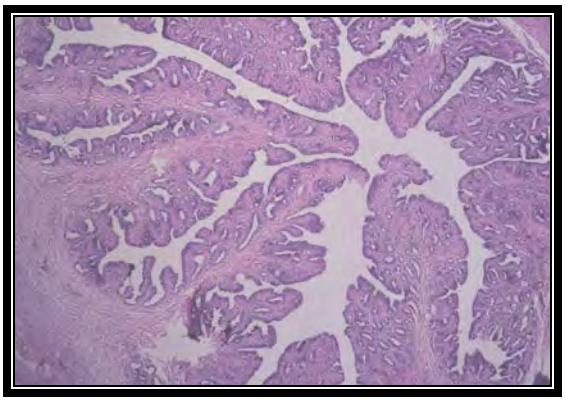

Fig. 3: Uterus of Gabali rabbits showing mild hyperplasia of the epithelial lining (H, E X40)

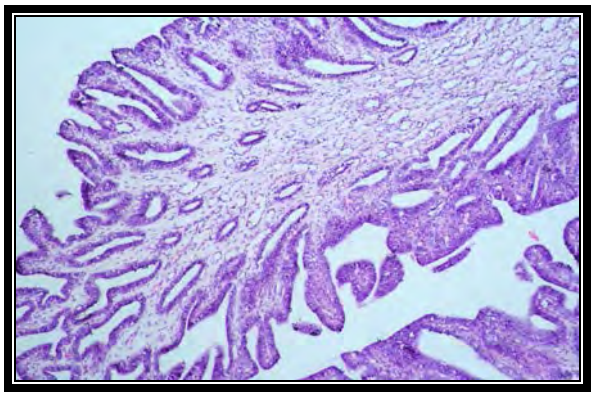

Fig . 4: Uterus of V-line rabbits showing activation of glands in endometrium. (H, E X40)

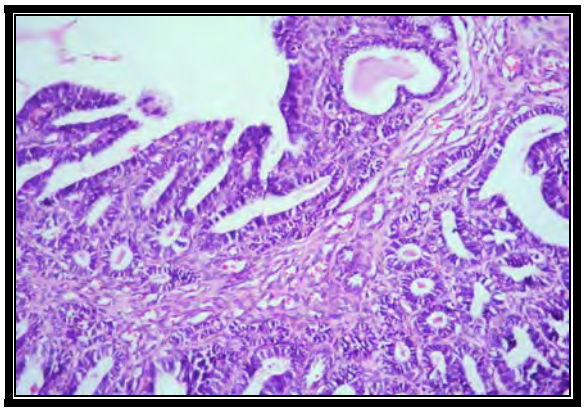

Fig. 5: Uterus of M-line rabbits showing activation gland and congestion of blood vessels. (H, E X40)

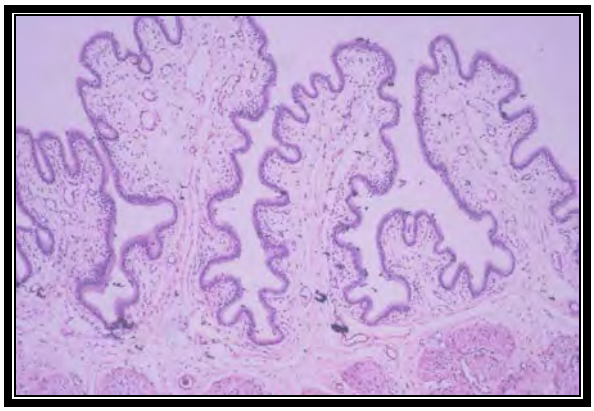

Fig. 6: Uterus of Gabali rabbits showing mild activation of glands. (H, EX40)

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The optimal synchronization time and place for *in vivo* embryo recovery by laparoscopy in doe rabbit

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Abstract

This work aimed to study the optimal synchronization time and place for *in vivo* embryo recovery by laparoscopy in rabbit does. A total number of 104 multiparous rabbit does from R line selected for growth rate were superovulated with a single subcutaneous injection of 50 IU eCG per female. Does were then artificially inseminated 60 h later and immediately injected muscularly with GnRh for ovulation, thereafter, embryos were recovered *in vivo* by ventral midline laparoscopy technique under anesthesia. The does were divided into four groups according to embryo recovery time and place as: 20 does were recovered 70 h post-insemination from uterus only (Group 1), 36 does were recovered 70 h post-insemination from oviducts and uterus (Group 2), 24 does were recovered 80 h post-insemination from uterus only (Group 3) and 24 does were recovered 80 h post-insemination from oviducts and uterus (Group 4). Ovulation rates were similar among the groups (average 10.4 ovulation sites). The recovery rate in does recovered in group 4 was significantly ($P<0.05$) higher than that in does recovered in the other groups (80.4% for group 4 *versus* 39.0, 48.3 and 18.7% for groups 1, 2 and 3, respectively). Rate of abnormality increased in embryos recovered from group 2 (29.9%) when compared with other groups (average 16.2%), however, this difference was not statistically significant. Embryo donor rates (donor with at least one normal embryo) in groups 3 and 4 (79.2% and 83.3% respectively) were significantly ($P<0.05$) higher than that in group 1 (45%). The number of normal embryos recovered in donor does was significantly ($P<0.05$) higher in groups 1 and 4 when compared with group 2 (8.7 and 9.2 versus 5.9 embryos per donor doe, respectively), and the lowest number of normal embryos was recovered from donors of group 3 (2.2 embryos per donor doe, $P<0.05$). A significant increase ($P<0.05$) was found in the number of embryos recovered at blastocyst stage in group 4 in comparison with group 1 (19.8 *versus* 3.3% blastocyst rate, respectively). Results indicated that delay recovery time to 80 h post-insemination and apply recovery from both oviducts and uterus leads to the highest recovery rates of normal embryos per donor doe, and this way could be considered as the optimal conditions for *in vivo* embryo recovery by laparoscopy techniques in rabbit does.

Keywords: Rabbits, Recovery time, Recovery place, Laparoscopy.

Introduction

The recovery of the embryo of rabbits is carried out usually after slaughtering the female. Few studies have been carried out concerning the in vivo embryo recovery of rabbits by laparotomies (Forcada and Lopez, 2000, Mehaisen *et al.*, 2009) or laparoscopies (Besenfelder *et al.* 1998; Kidder *et al.* 1999; Mehaisen *et al.*, 2005). Laparoscopic techniques avoid the formation of adhesions which are caused by laparotomic techniques and, consequently, laparoscopies are preferable to use when embryos are collected from highly valued animals (Torres and Sevellec, 1987).

In previous studies, it was shown that the number of embryos recovered from rabbit lines selected for growth rate was lower than that recovered from maternal lines selected for litter size (Viudes de Castro et al. 1995; Vicente *et al*. 2003). These studies and others (Mehaisen *et al*., 2005; Mehaisen *et al*., 2006) showed the convenience of the utilization of in vivo embryo-recovery by multiple laparoscopic techniques in such rabbit lines which present low productive traits, in order to increase the number of embryos recovered per donor doe.

The physiology of reproduction in the female rabbit is characterised by non-spontaneous ovulation (Besenfelder *et al.*, 2000). Mating induces a neuro-endocrinological reflex, which provokes an LH pulse that leads to ovulation. Ovulation takes place 10–12 h after the recorded LH peak (Harper, 1961). Twenty four hours post coitus (p.c.), all embryos can be found in the isthmic part of the oviduct (Bourdage and Halbert, 1988). López-Béjar (1995) reported that the first cell cycle is terminated 26 h.p.c. and the consecutive cleavages of embryos are expected to be completed within 26-32 h.p.c. (4-cell stage), 32-40 h.p.c. (8 cell stage), 40-47 h.p.c. (16 cell stage), 47-68 h.p.c. (morulae) and 68-76 h.p.c. (blastocyst). About 30% of embryos were found beyond the uterotubal junction 78 h.p.c., while more than 90% of the embryos have reached the uterine horn 84 h.p.c. (Bourdage and Halbert, 1988). Embryo implantation has been reported between 120 and 144 h.p.c. (López-Béjar, 1995). Depending on these information, most of laparoscopies used to recover normal embryos from rabbit does were done by

flushing the uterus 76-80 h post-coitus or insemination.

The objective of this study is to evaluate the optimal synchronization time and place for *in vivo* embryo recovery by laparoscopy in rabbit does selected for growth rate.

Materials and methods

Animals

A total number of 104 multiparous rabbit does were used from a synthetic breed (R line), selected for growth rate from weaning to slaughter (28-70 days of age) (Estany *et al.*, 1992). All does were kept individually under the same environmental conditions.

Artificial insemination

To synchronize the receptivity of females, a dose of 50 IU eCG (Gonaser, Hipra) were subcutaneously injected 60 hours before insemination. Does were artificially inseminated with 0.5 ml of semen pool from, at least, three fertile bucks of the same line. Ejaculates used were selected according to motility criteria, more than 70% of progressive motility, and diluted 1:3 with a Tris-citric-glucose extender described by Vicente et al. (2004). Immediately after insemination, ovulation was induced by an intramuscular injection of GnRh (2µg of Buserelin acetate, Hoechst, S.A.).

Embryo recovery

Embryos were recovered *in vivo* by ventral midline laparoscopy technique under anesthesia (Kidder *et al.*, 1999). First, anaesthesia was induced by an intramuscular injection of 16 mg xylazine (0.8 ml of Rompun; Bayer AG, Leverkusen, Germany), followed by an intravenous reinjection of ketamine chlorohydrate at the rate of 1.2 ml per kg body weight (Imalgene; Merial, S.A., Lyon, France) to maintain does under anaesthesia during laparoscopy. Anaesthetised donor does were placed in a head-down position with an angle of 45ºC. A verres needle was inserted in the lower right abdominal quadrant and attached to CO_2 automatic insufflators. After CO_2 abdominal distension, a trocar-cannula unit of 5 mm diameter was inserted into the abdominal cavity at 10 cm caudal to the sternum. Another accessory trocar-cannula unit was inserted 4-5 cm laterally to right of the former. After both were removed, they were respectively replaced by the laparoscope (Wolf paediatric 0º) and grasping forceps (28.5 cm length). For oviduct flushing, an epidural needle (1 mm of inner diameter, Vigor Epidural G17) was inserted near the ovary; a sterile polyethylene tube (inner diameter 0.3 mm) attached to a 5 ml sterile syringe was introduced 2-3 cm through the epidural needle into the oviduct. After fixation with the grasping forceps, ova and embryos were flushed from the oviducts to uterine horns with 5 ml of recovery medium [Dulbecco's phosphate-bufered saline (DPBS; Sigma-Aldrich Quimica S.A., Alcobendas, Madrid, Spain), supplemented with antibiotics (penicillin and streptomycin; Penivet 1)]. For flushing of uterine horns, they were firstly elevated with grasping forceps near to the oviduct-uterotubal junction and then flushed with 50 ml of recovery medium inserting a sterile epidural needle directly through abdominal wall into the uterine horn. Finally, the flushing media was aspirated from the vagina by a Foley Catheter (Minitube, Tiefenbach, Germany) connected with a vacuum pump inducing negative pressure of 20-40 mm Hg.

Experimental design

The does were divided into four groups according to embryo recovery time and place as: 20 does were recovered 70 h post-insemination from uterus only (Group 1), 36 does were recovered 70 h post-insemination from oviducts and uterus (Group 2), 24 does were recovered 80 h post-insemination from uterus only (Group 3) and 24 does were recovered 80 h post-insemination from oviducts and uterus (Group 4). In all groups, presumptive embryos were scored by morphological criteria. Only embryos in morulae or young blastocyst stage with a mass of homogeneous cells and without morphological abnormalities in mucin coat or zona pellucida were catalogued as normal embryos.

The ovulation rate (the number of corpora lutea on both ovaries), the number of oocytes, abnormal and normal embryos per doe, and the number of normal embryos recovered in morulae stage or blastocyst stage were recorded.

Statistical analysis

The effect of recovery time and place in the four groups defined previously on the ovulation rate, the recovery rate (oocytes + normal embryos + abnormal embryos/ovulation rate), abnormality rate (oocytes + abnormal embryos on total recovery per donor doe), the number of normal embryos recovered in ovulating does, and the percentage of embryos in blastocyst stage per donor doe were analyzed by one-way ANOVA (SPSS for Windows 11.0.1, SPSS Inc., Copyright 1989–2001, USA).

A chi-square test (SPSS for Windows 11.0.1, SPSS Inc.., Copyright 1989–2001, USA) was used to compare the effects of recovery time and place in the four groups on the embryo donor rate (donor does with at least one normal embryo respect to ovulating does).

Results

Ovulation rates ranged from 9.9 to 11.2 in donor does with no significant differences among groups (Table 1). Significant differences were observed in the recovery rate among the groups ($P<0.05$, Table 1); group 4 showed a higher recovery (80.4%) than

that in group 1 and 2 (39.0 and 48.3%, respectively), while the lowest recovery was found in group 3 (18.7%). Rate of abnormality was statistically similar among the groups. However, we observed that rate of abnormality tended to increase in embryos recovered from group 2 (29.9%) when compared with other groups (average 16.2%) (P<0.2, Table 1).

Table 1. Ovulation, recovery and abnormality rates as affected by laparoscopic recovery time and place in rabbit does.

Groups [1]	Ovulation rate LSM ± S.E (n)	Recovery rate [2] LSM ± S.E (n)	Abnormality rate [3] LSM ± S.E (n)
Group 1	10.5 ± 0.8 (20)	39.0 ± 9.2 (20) [b]	18.2 ± 12.2 (11) [xy]
Group 2	10.5 ± 0.7 (36)	48.3 ± 6.7 (36) [b]	29.9 ± 8.2 (26) [x]
Group 3	9.9 ± 0.1 (24)	18.7 ± 2.4 (24) [c]	11.1 ± 6.6 (21) [y]
Group 4	11.2 ± 0.6 (24)	80.4 ± 6.2 (24) [a]	19.3 ± 6.4 (22) [xy]

LSM ± S.E.: least square means ± standard error; (*n*): number of donor does; Values with different letters (a, b, c) in the same column are statistically different (P < 0.05); Values with different letters (x, y) in the same column are different (P < 0.2).
[1]Group 1: does were recovered 70 h post-insemination from uterus only; Group 2: does were recovered 70 h post-insemination from oviducts and uterus; Group 3: does were recovered 80 h post-insemination from uterus only; Group 4: does were recovered 80 h post-insemination from oviducts and uterus.
[2]Recovery rate: oocytes + normal embryos + abnormal embryos/ ovulation rate.
[3]Abnormality rate: percentage of oocytes and abnormal embryos per doe with at least one recovered oocyte or embryo.

Embryo donor rates in groups 3 and 4 were significantly higher than that in group 1 (79.2% and 83.3% versus 45%, respectively, P<0.05, Table 2). The number of normal embryos recovered in donor does was significantly higher in groups 1 and 4 when compared with group 2 (8.7 and 9.2 versus 5.9 embryos per donor doe, respectively, P<0.05, Table 2), and the lowest number of normal embryos was recovered from donors of group 3 (2.2 embryos per donor doe, P<0.05). A significant increase in the number of embryos recovered at blastocyst stage was found in group 4 when compared with group 1 (19.8 *versus* 3.3% blastocyst rate, respectively, P<0.05), but similar to that in groups 2 and 3 (7.9 and 4.3%, respectively) (Table 2).

Table 2. Embryo donor rate, number of normal embryos and blastocyst rate as affected by laparoscopic recovery time and place in rabbit does.

Groups [1]	Embryo donor rate [2] % (n)	Normal embryos [3] LSM ± S.E	Blastocyst rate [4] LSM ± S.E
Group 1	45.0 (9) [b]	8.7 ± 1.4 [a]	3.3 ± 3.3 [b]
Group 2	58.3 (21) [ab]	5.9 ± 0.8 [b]	7.9 ± 3.9 [ab]
Group 3	79.2 (19) [a]	2.2 ± 0.2 [c]	4.4 ± 3.1 [ab]
Group 4	83.3 (20) [a]	9.2 ± 0.9 [a]	19.8 ± 6.8 [a]

LSM ± S.E.: least square means ± standard error; (*n*): number of donor does; Values with different letters (a, b, c) in the same column are statistically different (P < 0.05).
[1]Group 1: does were recovered 70 h post-insemination from uterus only; Group 2: does were recovered 70 h post-insemination from oviducts and uterus; Group 3: does were recovered 80 h post-insemination from uterus only; Group 4: does were recovered 80 h post-insemination from oviducts and uterus.
[2]Embryo donor rate: percentage of donor does with at least one normal embryo respect to ovulating does.
[3]Normal embryos: number of normal embryos recovered in ovulating does with at least one normal embryos.
[4]Blastocyst rate: percentage of embryos in blastocyst stage recovered in ovulating does with at least one normal embryos.

Discussion

R-line rabbits have been established within a program of selection for growth rate in the department of Animal Science (UPV, Spain), and have shown low reproductive performance and prolificacy as well as low normal embryo recovery rates (Vicente *et al.*, 2003). The use of laparoscopic techniques has been considered as a useful methodology to ensure the greatest number of recovered embryos to finally preserve the genetic resources of lines similar to the R line (Mehaisen *et al*., 2004). However, our previous studies indicated that many alternative attempts in superovulation

treatments, vitrification protocols and synchronization time of recovery are necessary to improve embryo stocks of such lines (Mehaisen *et al*., 2005 and 2006). In this study, we applied the laparoscopy technique for in vivo recovery of donor does, and studied if time of recovery (early at 70 h post-insemination or late at 80 h post-insemination) and place of recovery (flushing from uterus only or from oviduct and uterus) have effect on embryo recovery and donor rates focusing on its stage of development before vitrification. In this study, we did not follow up the development rate of these recovered embryos after vitrification because we think that effects will be similar on embryos already recovered in normal status as indicated in previous work (Lavara *et al*., 2003).

In the present work, we used a unique subcutaneous dosage of 50 IU of eCG to improve the receptivity and ovulation rate in does with a minimum possible deleterious effects of the higher doses (Mehaisen *et al*., 2005). The average ovulation rate recorded in the R line does in this study (10.4) was similar to that obtained by Mehaisen *et al.* (2004) in does of the same line recovered in vivo by laparoscopy. When late laparoscopy was applied on does from both oviduct and uterus, a significant increase in recovery rate was observed (80.4%) in comparison with does recovered earlier (39% from uterus only or 48.3% from oviduct and uterus, Table 1). These results are in agreement with Bourdage and Halbert (1988) who reported that more than 90% of embryos reached the uterine horn 84 h.p.c. versus 30% of embryos were found beyond the uterotubal junction 78 h.p.c. However, a significant decrease in recovery was found in donor does when laparoscopy was applied at 80 h post-insemination but from uterus only (18.7%). This may be correlated with the lowest ovulation rate recorded for does from the same group (Table 1).

Rate of abnormality was statistically similar among the groups, ranging between 11.1 to 29.9% in R line (Table 1). In previous study, we recorded a higher abnormality rate in the same line reached to 34.1% in non-superovulated does and ranged from 22 to 48% in superovulated does (Mehaisen *et al*., 2006). Although high fertilizing failures in this rabbit line, we can suggest that late recovery by 80 h post-insemination reduces the abnormality rate than early recovery at 70 h post-insemination (15.2% versus 24.0%, respectively).

Another aspect of low reproductive performance for this line is the low capability of these does to produce normal embryos in compact morula or early blastocyst stages. Vicente *et al.* (2003) reported that 45% of donor does from line R versus 20% from line V failed to produce normal embryos without superovulatory treatments. When superovulation treatment was applied, Mehaisen *et al*. (2004) reported that only 53% of donor does able to produce at least one normal embryo with average of 5.7 normal embryos per donor doe. In this study, we observed similar results in embryo donor rates in groups 1 and 2 when does were subjected for early recovery (45% and 58.3%, respectively), but significant increase was obtained in embryo donor rates when does were recovered at later time in group 3 and 4 (79.2% and 83.3%, respectively, Table 2). However, we found that with applying recovery at late time, it is preferably flushing both oviduct and uterus to receive the maximum number of normal embryos per donor doe (9.2 normal embryos recovered from both oviduct and uterus versus 2.2 normal embryos recovered from uterus only at 80 h post-insemination, $P<0.05$, Table 2). Moreover, we observed that about 20% of normal embryos recovered in group 4 were in blastocyst stage versus lower rates in the other groups. These results coincide with the chronology of embryo development described by López-Béjar (1995).

Conclusions

Results indicated that delay recovery time to 80 h post-insemination and apply recovery from both oviducts and uterus leads to the highest recovery rates of normal embryos per donor doe, and this way could be considered as the optimal conditions for *in vivo* embryo recovery by laparoscopy techniques in rabbit does.

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Effect of probiotics and prebiotics on economic, microbial and immunological traits in local broilers' purebreds and crossbreds chickens

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Abstract

This experiment was carried out to study the effect of probiotics and lactose on growth performance, Salmonella colonization and immunity in Matrouh (MA) and Inshas (IN) local broilers and their crosses. Four hundred and eighty chicks produced from four genetic groups (MA x MA, IN x IN, MA x IN and IN x MA) were used. Ten groups of broilers chicks of each genetic group were categorized and offered different treatments of probiotics including *Lactobacillus acidophilus, Bacillus subtilis and Enterococcus faeceium* alone or accompanied by 2.5% Lactose in drinking water. Different parameters were evaluated including body weight, daily gain, feed intake, feed conversion, caecal Salmonella count, caecal pH and antibody titre against Salmonella. Results showed that *Enterococcus faeceium* had significant effects on body weight and daily gain of chicks. *Enterococcus faeceium* and *Bacillus subtilis* had significant effects on feed intake only at one week of age while *Bacillus subtilis* showed a significant difference on feed conversion only at 4 weeks of age. IN x MA crossbred proved to be the most effective in reducing Salmonella count at 4 weeks of age. All treatments caused reduction of caecal pH and *Lactobacillus acidophilus* with lactose 2.5% had the highest effect. MA x IN crossbred showed the strongest immunity reaction against Salmonella when compared with other breeds. *Enterococcus faeceium* together with lactose gave also the strongest immune reaction against Salmonella when compared with the other breeds.

Keywords: Food safety, chickens, Probiotics, prebiotics, Salmonella control, immunity, purebreds, crossbreds.

Introduction

Transmission of enteric pathogens to the public contacts of farm animals is a growing problem, particularly among children and old people (Smith *et al.*, 2004). One of the most frequent causative agents of food infections is Salmonella, which mostly can be found in animal herds (Fehlhaber, 2003). Salmonella are facultative intracellular Gram-negative bacteria that are found ubiquitously in nature and have the ability to infect wide range of hosts including humans, domesticated and wild mammals and birds. The principal clinical manifestations associated with Salmonella infection in humans are enteric fever (typhoid and paratyphoid) and a self-limiting gastroenteritis (salmonellosis) (Salez and Malo, 2004). Some Salmonella species are less pathogenic to birds (notably *Salmonella typhimurium* and *Salmonella enteritidis*) and can cause colonization of the gut, which leads to carcass contamination and subsequent human infection, without causing evident disease in the chicken (Bumstead, 2003). As control of this health hazard, antimicrobials were used as growth promoter and/or prophylactic agents against many pathogens that may enter the animal body through contaminated carcass meals, edible plastics, sewage, petrochemical residues and excrements (Gihan El-Moghazy, 2002). These antimicrobials include: Bacitracin, Chlortetracycline, Erythromycin, Lincomycin, Neomycin, Oxytetracycline, Pencillin, Streptomycin, Tylosin and Verginiamycin, which were added as growth promoters in poultry feed at a level of about 1400 g per ton of feed, which is lower than its minimum inhibitory concentration (subtheraputic level) and consequently encourages the selection of antibiotic resistant bacteria. Alternatives to growth-promoting and prophylactic uses of antimicrobials in agriculture include improved management practices, wider use of vaccines and introduction of probiotics, prebiotics and a combination of them (symbiotic) (McEwen and Fedorka-Cray, 2002). Probiotics, which means "for life" in Greek, has been defined as "a live microbial feed supplement" which beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). Lactose, as a commonly used prebiotic, markedly increases resistance to caecal colonization, organ invasion and horizontal transmission of Salmonella species in broilers when included in drinking water. The main role of this prebiotic is achieved through its utilization by the intestinal beneficial bacteria resulting in; reduced caecal pH, increased caecal lactic acid, acetic acid, propionic acid and buteric acid concentration and increased caecal oxidation-reduction potential which in return

considerably reduces Salmonella colonization in caeca of treated birds (El-Borollosy *et al.*, 2001). The aim of this work is to find safe growth promoters for chickens to be used as alternatives to antimicrobial growth promoters through estimation of the effect of three different probiotic strains (*Lactobacillus acidophilus, Enterococcus faecium* and *Bacillus subtilis*), Lactose and mixture of all on: growth performance, antibody titer against Salmonella in the serum of artificially inoculated broiler chicks and count of Salmonella living cells in their caeca.

Materials and methods

Chicks used

This experiment was carried out in the Poultry Farm of chickens, Faculty of Agriculture at Moshtohor, Benha University, Egypt, in April 2005.

Experimental work

Two local strains of Matrouh (MA) and Inshas (IN) were used. Pullets of each strain were randomly divided into two groups (100 hens / group); the first group was mated with 10 cocks from the same strain, while the second group was mated with 10 cocks from the other strain. Consequently, the pedigreed eggs from each individual breeding pen for the four mating groups (Table 1) were collected daily for ten days and incubated in one hatch then after.

Table 1. Number of chicks used in the experimental work and description of genetic group of sires and dams produced from them

Genetic group* of chicks	No. of chicks	Genetic group of sire	Genetic group of dam
MA x MA	120	MA	MA
IN x IN	120	IN	IN
MA x IN	120	MA	IN
IN x MA	120	IN	MA
Total	480		

* MA and IN= Matrouh and Inshas strains, respectively.

On hatching day, numbers of 120 chicks (12 chicks from each sire) were randomly chosen from each genetic group, then after wing banded to save its genetic groups and immediately transferred to the Moshtohors' Poultry Farm of chickens. Chicks from each genetic group were distributed randomly on ten treatments (12 chicks in each). Description of treatments supplied to the chicks is summarized in Table 2.

Table 2. Description of treatments used in the experimental work.

Treatment No.	Description of treatment
1	2.5% lactose added in drinking water
2	2.5% lactose in drinking water and *Lactobacillus acidophilus*
3	2.5% lactose in drinking water and *Enterococcus faecalis*
4	2.5% lactose in drinking water and *Bacillus subtilis*
5	2.5% lactose in drinking water and *Lactobacillus acidophilus, Enterococcus faecalis,* and *Bacillus subtilis*
6	Control negative group without any treatment
7	Control positive group treated with *Salmonella typhimurium* only
8	Treated with *Lactobacillus acidophilus*
9	Treated with *Enterococcus faecalis*
10	Treated with *Bacillus subtilis*

At hatch, chicks were challenged with 10^6 cfu (*Lactobacillus acidophilus, Enterococcus faecalis*, and *Bacillus subtilis*) by crop inoculation. At 3 days of age, all chicks were challenged with 106 cfu *Salmonella typhimurium* by crop inoculation, except the control negative group. Chicks were reared in floor brooder up to end of the experiment under continuous lighting program (fluorescent lamps, 10 watt/m^2). Starter, grower and finisher diets were adequately supplied to cover the requirements according to NRC (1994). The experimental diets (in mash form), the clean as well as residual feed were weighed. They were fed (without antibiotics, coccidiostats, or growth promoters) during rearing and growing periods on diet containing 23.01 %, 20 % crude protein, 3.6 %, 3.19 % crude fiber, respectively, as well as *ad libitum* drinking water. All birds were subjected to similar hygienic and environmental conditions and vaccinated against Newcastle and Gambaro diseases.

Procedure of experiment

Examination of Salmonella in materials

Ten samples from the source of water and feed offered to the chicks were collected to be examined for the presence of Salmonella. Samples from the litter present in the floor in which the chicks were delivered were examined also for the presence of Salmonella. In addition, two hundred chicks (five chicks from every treatment per genetic group) were

examined for the presence of Salmonella by cloacal swab.

Bacterial strains
Salmonella typhimurium was kindly obtained from Animal Health Research Institute, A.R.C., Giza, Egypt.

Probiotic strains
The used strain of *Lactobacillus acidophilus, Enterococcus faecalis, Bacillus subtilis* were isolated, purified, identified and stored from routine work in the Food safety Laboratory, Regional Center for Food and Feed, A. R. C., Giza, Egypt.

The preparation of infective dose of Salmonella
Salmonella typhimurium was propagated onto S.S agar medium and incubated at 37°C for 24 hours, and the growth was harvested, then washed three times and resuspended in phosphate buffer saline. The suspension was matched with Brown's Opacity tube number (1) in order to have a final concentration of 10^8 microorganisms per ml.

Detection of Salmonella was carried out according to NMKL (1994).

Biochemical and serological identification of Salmonella
Initial identification attempts were made using the criteria described by NMKL (1994) and API 20E (bioMerieux).
The strips were used according to the detailed procedure steps illustrated in the kit's manual. Serological identification of the suspected Salmonella strain was carried out according to NMKL (1994).

Determination of caecal colonization by *Salmonella typhimurium*
Caecal material was serially diluted in sterile saline solution and plated on brilliant green agar. The plates incubated for 18-24 hours at 37°C, and cfu were counted. Typical *Salmonella* colonies were confirmed by biochemical tests as mentioned before.

Determination of pH in the caecal contents
At thirty days of age and at the end of experiment, 5 chicks from each treatment/genetic group were slaughtered by cervical dislocation. Caecal contents were aseptically removed, and 0.2 g was suspended in 0.8 ml of sterile glass distilled water. One ml of distilled water was added to the suspension.

Estimation of Salmonella antibody titer in the serum of experimental chicks:
Collection of serum, procedure and interpretation of the results were performed according (Alton *et al.*, 1988).

Data and traits studied
Data of 480 chicks were recorded for traits of body weight (g) at 1st (BW1), 2nd (BW2), 3rd (BW3), 4th (BW4), 5th (BW5), 6th (BW6), 7th (BW7), 8th (BW8), 9th (BW9) and 10th (BW10) weeks of age. Daily weight gains during the periods from 1 to 4 (DG1-4), 4 to 8 (DG4-8), 8 to 10 (DG8-10) and 1 to 10 (DG1-10) weeks of age were computed. Feed intakes were recorded at the intervals of 1 (FI1), 2 (FI2), 3 (FI3), 4 (FI4), 5 (FI5), 6 (FI6), 7 (FI7), 8 (FI8), 9 (FI9) and 10 (FI10) weeks of age and expressed as g/bird/day. Feed conversion values (g feed/g gain) were computed at the intervals of 1 (FC1), 2 (FC2), 3 (FC3), 4 (FC4), 5 (FC5), 6 (FC6), 7 (FC7), 8 (FC8), 9 (FC9) and 10 (FC10) weeks of age.
Salmonella count and caecal pH traits were also studied, as well as antibody titer in serum was estimated according to procedure of (Alton et al., 1988). The antibody titer was expressed as ($-\log_2$). The criteria of response (performance parameters) are recorded & calculated in the present study according to Abdel-Azeem, (1997) which included: live body weight gain, feed intake and feed conversion.

Statistical analysis
Data of body weight, daily gain and feed conversion traits were analyzed using (Model 1), but Salmonella count and caecal pH traits were analyzed using (Model 2); and feed intake and antibody titer traits were analyzed using (Model 3) according to SAS program (SAS, 2004):

$Y_{ijkl} = \mu + G_i + T_j + X_k + (GT)_{ij} + e_{ijkl}$
(Model 1)
$Y_{ijk} = \mu + G_i + T_j + (GT)_{ij} + e_{ijk}$
(Model 2)
$Y_{ijk} = \mu + G_i + T_j + e_{ijk}$
(Model 3)
Where:
Y_{ijkl} and Y_{ijk} = the observation recorded on chick;
μ = the overall mean;
G_i = fixed effect of the i^{th} genetic group;
T_j = fixed effect of the j^{th} treatment;
X_k = fixed effect of k^{th} sex (levels= 1, 2 and 3 for males, females and dead chicks before sexing, respectively);
$(GT)_{ij}$ = Fixed effect of interaction between the i^{th} genetic group and j^{th} treatment; and
e_{ijkl} and e_{ijk}= the random deviation particular to the chick, assumed to be independently randomly distributed with zero mean and variance (σ_e^2).

Duncan's multiple range test (Duncan 1955) was used to detect the significant differences between means of genetic groups.

Results and discussion

Economical traits

Effects of different treatments on body weight were illustrated in (Table 3). Body weight of chicks treated with *Enterococcus faecalis* was the heaviest at 3, 4, 5, 6, 7, 8, 9 and 10 weeks of age when compared with control group (without any treatment) at the same ages which were 102.48, 224.08, 162.46, 319.5, 402.71, 490.47, 581.72, 704.45 and 847.53 grams, respectively, while means of body weight for control group were 150.49, 211.68, 244.81, 312.1, 383.31, 453.07, 582.9 and 699.82 grams, respectively. These results are in agreement with those reported by other investigators (Shivani-Katoch et al., 1996 and Kahraman et al., 1997). The body weight of chicks treated with *Bacillus subtilis* at 5, 6, 7, 8, 9 and 10 weeks appeared to follow the above mentioned treatment in its effect with values of 292.7, 367.43, 441.55, 520.28, 649.95 and 789.49 grams, respectively. These results are in agreement with those reported by many investigators (Jin *et al.*, 1996 and Samanya and Yamauchi, 2002).

The results showed that, addition of lactose in drinking water to chicks has negative effects on body weight when compared with the control group. On the contrary, (Maiorka *et al.*, 2001 and Douglas *et al.*, 2003) found that the addition of 2 or 4% lactose increased weight gain ($P<0.01$) from zero up to 21 days that may increase growth of commercial broiler chicks which may be due to breed variation.

Table 3. Least-square means and standard errors of body weight (g) traits[+] as affected by treatments in a crossbreeding experiment.

Treatment[*]	BW1	BW2	BW3	BW4	BW5	BW6	BW7	BW8	BW9	BW10
1	66.25^{bc}	98.64^{bc}	147.52^{bcd}	187.38^{de}	239.10^{cde}	301.38^{cde}	368.10^{cd}	448.37^{cde}	587.63^{cd}	717.41^{c}
	±1.50	±2.54	±4.03	±5.40	±11.27	±18.14	±21.56	±25.86	±37.2	±42.92
2	67.88^{bc}	98.26^{bc}	145.26^{bcd}	173.44^{f}	208.14^{f}	255.69^{f}	317.62^{e}	388.38^{f}	524.36^{e}	651.13^{d}
	±1.45	±2.45	±3.88	±5.20	±9.70	±14.93	±17.74	±21.28	±32.89	±37.85
3	65.19^{cd}	100.12^{bc}	145.42^{bcd}	179.43^{ef}	225.45^{def}	288.40^{de}	330.19^{e}	425.20^{def}	553.99^{de}	680.66^{cd}
	±1.44	±2.44	±3.88	±5.38	±11.20	±18.06	±21.46	±25.75	±37.22	±42.83
4	60.56^{d}	92.99^{c}	140.33^{cd}	169.33^{f}	215.12^{ef}	272.42^{ef}	321.04^{e}	407.19^{ef}	538.03^{cde}	654.08^{cd}
	±1.50	±2.50	±3.97	±5.32	±11.05	±17.89	±21.26	±25.50	±36.97	±42.55
5	63.92^{cd}	98.01^{bc}	147.78^{bcd}	170.85^{f}	221.95^{def}	287.45^{cde}	333.48^{de}	412.32^{ef}	537.19^{de}	654.67^{cd}
	±1.47	±2.47	±3.93	±5.26	±11.12	±17.96	±21.35	±25.61	±37.07	±42.66
6	73.45^{a}	111.09^{a}	150.49^{bc}	211.68^{ab}	244.81^{c}	312.10^{cd}	383.31^{c}	453.07^{cd}	582.9^{bc}	699.82^{c}
	±1.44	±2.38	±3.82	±5.11	±10.55	±17.32	±20.59	±24.70	±36.22	±41.69
7	68.22^{bc}	100.996^{bc}	138.61^{d}	198.79^{cd}	250.26^{cd}	323.52^{cd}	384.03^{c}	455.98^{cde}	581.2^{cd}	703.75^{cd}
	±1.44	±2.42	±3.85	±5.16	±10.68	±17.47	±20.76	±24.91	±36.48	±41.98
8	72.36^{a}	102.21^{b}	150.08^{bcd}	208.13^{bc}	248.14^{cd}	323.82^{c}	388.90^{c}	472.53^{c}	601.73^{bcd}	707.68^{c}
	±1.44	±2.38	±3.77	±5.05	±9.98	±17.37	±20.64	±24.76	±36.34	±42.00
9	70.54^{ab}	102.48^{b}	162.46^{a}	224.08^{a}	319.50^{a}	402.71^{a}	490.47^{a}	581.72^{a}	704.45^{a}	847.53^{a}
	±1.48	±2.45	±3.90	±5.28	±11.00	±17.83	±21.19	±25.42	±36.88	±42.45
10	67.54^{bc}	100.12^{bc}	157.79^{ab}	227.73^{a}	292.70^{b}	367.43^{b}	441.55^{b}	520.28^{b}	649.95^{b}	789.49^{b}
	±1.45	±2.41	±3.82	±5.17	±10.13	±17.65	±20.97	±25.16	±36.70	±42.23

+BW= Body weight at 1 week and up to 10 weeks, respectively.

[*] Treatments as described in Table 2.

[a-f] means with the same letters within each column of trait are non-significantly different ($P<0.05$).

Results in (Table 4) illustrated that daily gain of chicks treated with *Enterococcus faecalis* was higher than others during the intervals 4-8 and 1-10 weeks of age when compared with all treatments at the same ages. Means of daily gain for *Enterococcus faecalis* group were 12.33 and 12.28 grams, respectively. These results are in agreement with those reported by other investigators (Cho *et al.*, 1992 and Pisarski *et al.*, 1995). Means of daily gain for *Bacillus subtilis* group during the intervals 1-4 and 8-10 weeks of age were 7.63 and 17.70 grams, respectively. Daily gain of chicks treated with *Lactobacillus acidophilus* has no significant differences when compared with the control group (without any treatment) during all intervals of the experiment. The data cleared that, addition of lactose in drinking water to chicks has negative effects on daily gain when compared with control group. On the contrary, (Maiorka *et al.*, 2001 and Douglas *et al.*, 2003) found that addition of 2 or 4% lactose increased weight gain ($P<0.05$) from zero up to 21 days that may increase growth of commercial broiler chicks.

There were not significant differences among different treatments on feed intake except at one week of age (Table 5). The highest feed intake for group which treated with *Enterococcus faecalis* and *Bacillus subtilis* group then *Lactobacillus acidophilus*.

Highly significant differences among different treatments on feed intake except at the 2nd, 6th and 7th weeks of age (Table 5). The highest feed intake was found in group treated with 2.5% lactose at 2, 5, 6 and 7 weeks followed by those treated with

lactobacillus acidophilus and 2.5% lactose 3, 5 and 6 weeks of age. While, the lowest feed intake were found in control groups at all periods of estimation except at the 3rd and 4th weeks of age only. Highly significant effects were found on feed conversion due to treatments applied at all periods of estimation, except at 3 and 8 weeks of age only. The highest feed conversion was found in group treated with Enterococcus faecalis at 4, 5, 6, 7 and 10 weeks of age followed by those treated with control negative group at 6, 7 and 9 weeks of age.

Table 4. Least-squares means and standard errors of daily gain (g) traits[+] as affected by treatments in a crossbreeding experiment.

Treatment*	DG1-4	DG4-8	DG8-10	DG1-10
1	5.74^{cd}±0.22	9.32^{bc}±0.70	17.71^{a}±1.77	10.31^{c}±0.66
2	5.02^{e}±0.22	7.88^{d}±0.58	17.27^{a}±1.56	9.26^{d}±0.58
3	5.43^{de}±0.22	9.15^{bc}±0.70	16.72^{a}±1.77	9.73^{cd}±0.66
4	5.17^{de}±0.22	8.81^{bc}±0.69	16.13^{a}±1.75	9.40^{cd}±0.65
5	5.06^{e}±0.22	8.64^{c}±0.69	15.80^{a}±1.76	9.33^{cd}±0.66
6	6.59^{b}±0.21	8.87^{bc}±0.67	16.14^{a}±1.72	9.93^{c}±0.64
7	6.19^{bc}±0.21	9.09^{c}±0.67	16.16^{a}±1.73	10.00^{cd}±0.64
8	6.47^{b}±0.21	9.56^{bc}±0.67	15.59^{a}±1.73	10.06^{cd}±0.64
9	7.33^{a}±0.22	12.33^{a}±0.69	17.49^{a}±1.75	12.28^{a}±0.65
10	7.63^{a}±0.21	10.41^{b}±0.68	17.70^{a}±1.74	11.41^{b}±0.65

[+] DG = daily gains during 1-4, 4-8, 8-10 and 1-10 weeks of age.
[*] Treatments as described in Table 1.
[a-c] means with the same letters within each column of trait are non-significantly different (P<0.05).

Table 5. Least-squares means and standard errors of feed intake (g/bird/day) traits[+] as affected by treatments in a crossbreeding experiment.

Treatment*	FI1	FI2	FI3	FI4	FI5	FI6	FI7	FI8	FI9	FI10
1	3.1^{b} ± 0.28	10.83^{a} ±0.43	18.59^{a} ±0.82	27.68^{ab} ±5.40	29.5^{ab} ±1.25	38.32^{a} ±1.62	43.11^{a} ±2.29	47.26^{ab} ±1.98	57.60^{ab} ±3.29	66.39^{ab} ±3.85
2	3.17^{b} ± 0.28	10.84^{a} ±0.43	15.86^{ab} ±0.82	25.69 ±5.20	28.94^{ab} ±1.25	35.75^{a} ±1.62	43.04^{a} ±2.29	51.45^{a} ±1.98	62.46^{a} ±3.29	69.25^{a} ±3.85
3	2.73^{b} ± 0.28	10.80^{a} ±0.43	16.86^{ab} ±0.82	27.88^{ab} ±5.38	27.42^{b} ±1.25	37.28^{a} ±1.62	42.89^{a} ±2.29	47.69^{ab} ±1.98	56.48^{ab} ±3.29	65.73^{ab} ±3.85
4	2.86^{b} ± 0.28	10.93^{a} ±0.43	17.16^{ab} ±0.82	27.13^{ab} ±5.32	30.12^{ab} ±1.25	38.46^{a} ±1.62	44.10^{a} ±2.29	48.12^{ab} ±1.98	56.42^{ab} ±3.29	61.46^{ab} ±3.85
5	3.33^{ab} ± 0.28	11.37^{a} ±0.43	16.87^{ab} ±0.82	26.15^{b} ±5.26	30.14^{ab} ±1.25	39.76^{a} ±1.62	45.40^{a} ±2.29	48.40^{ab} ±1.98	56.34^{ab} ±3.29	64.32^{ab} ±3.85
6	2.95^{b} ± 0.28	10.01^{a} ±0.43	16.21^{ab} ±0.82	26.84^{ab} ±5.11	26.97^{b} ±1.25	35.14^{a} ±1.62	40.56^{a} ±2.29	44.39^{b} ±1.98	50.00^{b} ±3.29	55.49^{b} ±3.85
7	2.95^{b} ± 0.28	10.01^{a} ±0.43	16.21^{ab} ±0.82	26.84^{a} ±5.11	26.97^{a} ±1.25	35.14^{a} ±1.62	40.56^{a} ±2.29	44.39^{ab} ±1.98	50.00^{ab} ±3.29	55.49^{ab} ±3.85
8	3.59^{ab} ± 0.28	10.60^{a} ±0.43	16.80^{ab} ±0.82	26.03^{b} ±5.05	27.96^{b} ±1.25	37.07^{a} ±1.62	41.43^{a} ±2.29	46.87^{ab} ±1.98	52.88^{ab} ±3.29	61.62^{ab} ±3.85
9	4.16^{a} ± 0.28	11.22^{a} ±0.43	17.23^{ab} ±0.82	26.13^{b} ±5.28	28.33^{b} ±1.25	36.25^{a} ±1.62	43.92^{a} ±2.29	48.42^{ab} ±1.98	56.38^{ab} ±3.29	64.03^{ab} ±3.85
10	4.09^{a} ± 0.28	10.71^{a} ±0.43	15.62^{b} ±0.82	26.43^{b} ±5.17	27.82^{b} ±1.25	36.39^{a} ±1.62	44.18^{a} ±2.29	48.86^{ab} ±1.98	55.90^{ab} ±3.29	62.82^{ab} ±3.85

[+] FI = Feed Intake at 1st to 10th week of age.
[*] Treatments as described in Table 1.
[a-c] means with the same letters within each column of trait are non-significantly different (P<0.05).

Table 6. Least-squares means and standard errors of feed conversion (g feed / g gain) traits [+] as affected by treatments in a crossbreeding experiment.

Treatment*	FC1	FC2	FC3	FC4	FC5	FC6	FC7	FC8	FC9	FC10
1	0.11 [ab]	0.29 [b]	0.46 [a]	0.61 [bcd]	1.28 [b]	0.56 [abc]	0.56 [bcd]	0.54 [a]	0.33 [a]	0.50 [ab]
	±0.11	±0.05	±0.16	± 0.45	±1.76	±0.12	± 0.19	± 0.21	±0.13	± 0.08
2	0.12 [ab]	0.36 [ab]	0.38 [a]	0.99 [bc]	4.17 [a]	0.74 [a]	0.81 [bc]	0.68 [a]	0.52 [ab]	0.63 [a]
	±0.06	±0.03	±0.09	±0.26	± 0.98	±0.07	± 0.11	±0.11	±0.07	± 0.04
3	0.14 [ab]	0.36 [ab]	0.43 [a]	1.49 [ab]	1.93 [ab]	0.64 [ab]	1.31 [a]	0.46 [a]	0.49 [ab]	0.54 [ab]
	±0.05	±0.03	±0.08	±0.24	± 0.88	±0.06	± 0.10	±0.11	±0.07	± 0.04
4	0.15 [a]	0.39 [a]	0.35 [a]	1.86 [a]	1.11 [b]	0.61 [ab]	0.84 [bc]	0.51 [a]	0.43 [b]	0.57 [a]
	±0.06	±0.03	±0.09	±0.26	±0.98	±0.07	±0.12	±0.13	±0.08	±0.05
5	0.00 [b]	0.31 [b]	0.31 [a]	1.23 [bc]	0.87 [b]	0.50 [abc]	0.88 [b]	0.60 [a]	0.46 [ab]	0.52 [ab]
	±0.06	±0.03	±0.09	±0.23	±0.88	±0.06	± 0.10	±0.11	±0.07	±0.04
6	0.08 [ab]	0.26 [b]	0.48 [a]	0.53 [d]	1.70 [ab]	0.49 [bc]	0.55 [cd]	0.56 [a]	0.41 [b]	0.56 [ab]
	±0.06	±0.03	±0.09	±0.25	±0.98	±0.07	± 0.11	±0.12	±0.07	±0.05
7	0.08 [ab]	0.36 [ab]	0.45 [a]	0.64 [cd]	1.50 [b]	0.53 [abc]	0.76 [bcd]	0.84 [a]	0.64 [ab]	0.56 [ab]
	±0.06	±0.03	±0.09	±0.25	±0.95	±0.07	± 0.11	±0.12	±0.07	±0.05
8	0.10 [ab]	0.34 [ab]	0.49 [a]	0.52 [cd]	2.22 [ab]	0.51 [bc]	0.62 [cd]	0.54 [a]	0.43 [b]	0.57 [ab]
	±0.06	±0.03	±0.09	±0.24	±0.92	±0.06	± 0.11	±0.12	±0.07	±0.04
9	0.14 [ab]	0.33 [ab]	0.35 [a]	0.45 [cd]	0.67 [b]	0.39 [c]	0.52 [d]	0.59 [a]	0.49 [ab]	0.45 [b]
	±0.06	±0.03	±0.09	±0.25	±0.94	±0.07	± 0.11	±0.12	±0.07	±0.05
10	0.14 [ab]	0.32 [b]	0.34 [a]	0.50 [d]	2.09 [b]	0.51 [abc]	0.58 [cd]	0.64 [a]	0.49 [ab]	0.54 [ab]
	±0.06	±0.03	±0.09	±0.24	±1.00	±0.07	± 0.11	±0.12	±0.07	±0.05

[+] FC = Feed conversion at 1st to 10th week of age.
[*] Treatments as described in Table 2.
[a-c] means with the same letters within each column of trait are non-significantly different (P<0.05).

Microbiological and Immunological traits

Salmonella colonization and caecal pH

Tables (7 & 8) revealed that, genetic group was found to have highly significant effects (P<0.001) on Salmonella colonization at 4 weeks of age, while no significant effect of these groups on caecal pH was noticed. These results are in agreement with (Girard-Santosuosso *et al.*, 1998 and Kaiser and Lamont, 2001) who reported significant effect of genetic line (P<0.05) on Salmonella in caecal content. No significant differences between MA purebred and IN purebred on salmonella count at 4 weeks of age. Genetic group of IN x MA crossbred significantly decreased Salmonella colonization at 4 weeks of age than MA x IN crossbred, while no significant differences between MA x IN crossbred and both of MA and IN purebreds on Salmonella colonization at 28 days of age was noticed. However, IN x MA crossbred significantly decreased Salmonella colonization at 4 weeks of age than both of MA and IN purebreds. Also, all the used treatments significantly decreased caecal pH (P<0.001) at 4 weeks of age, except 2.5% lactose alone in drinking water, while 2.5% lactose and *Lactobacillus acidophilus* recorded the best effect for caecal pH reduction (Table 8). This result could be attributed to the effect of both *Lactobacillus acidophilus* and lactose which caused the increase of the lactic acid concentrations of their caecal contents, which were directly correlated to decrease caecal pH, values . These results are in agreement with (Hinton *et al.*, 1990; and Vandevoorde *et al.*, 1991) who stated that the addition of probiotic and prebiotic had significant effect on caecal pH, while Kahraman *et al.* (1997) showed that caecal pH did not differ in group which treated with probiotic from the control group.

Table 7. *Salmonella* count as affected by genetic group[+] and treatment at 4 weeks of age.

Treatment[*]	MA x MA	IN x IN	MA x IN	IN x MA
1	10^3	10^3	10^3	negative
2	negative	negative	negative	negative
3	negative	negative	negative	negative
4	negative	negative	negative	negative
5	negative	negative	negative	negative
6	negative	negative	negative	negative
7	10^4	10^4	10^4	10^4
8	negative	negative	negative	negative
9	negative	negative	negative	negative
10	negative	negative	negative	negative

[*] Treatments as described in Table 2. [+] Genetic groups as described in Table 1.

Table 8. Caecal pH as affected by genetic group[+] and treatment at 4 weeks of age:

Treatment[*]	MA x MA	IN x IN	MA x IN	IN x MA
1	7.16	7.17	7.07	6.89
2	6.03	6.07	6.07	5.99
3	6.41	6.73	6.54	6.68
4	6.80	6.70	6.55	6.70
5	6.52	6.73	6.72	6.73
6	7.51	7.60	7.41	7.56
7	7.24	7.49	7.67	7.34
8	6.85	6.58	6.23	6.59
9	6.31	6.67	6.95	6.81
10	6.48	6.67	6.74	7.03

[*] Treatments as described in Table 2.
[+] Genetic groups as described in Table 1.

Antibody titer:

Data from Tables (9, 10 and 11) concluded that, genetic group was found to have highly significant effects (P<0.01) on antibody titer at 4 weeks of age, (Table 10). These results are in agreement with (Girard-Santosuosso *et al*., 1998 and Kaiser and Lamont, 2001) who reported significant effect of genetic line (P<0.05) on immunity against Salmonella in caecal content. No significant differences between MA and IN purebreds on immunity against Salmonella at 4 weeks of age were noticed (Table 10). No significant differences between MA x IN and IN x MA crossbreds on immunity against Salmonella at 4 weeks of age, while MA x IN crossbred had significant differences with both MA and IN purebreds on immunity against Salmonella at 28 days of age. Little information has been reported for effects of Probiotic and Prebiotic on chicks' immunity.

Table 9. Antibody titer as affected by genetic group[+] and treatment effects.

Treatment[*]	MA x MA	IN x IN	MA x IN	IN x MA
1	1/640	1/640	1/640	1/2560
2	1/640	1/640	1/640	1/640
3	1/640	1/640	1/640	1/640
4	1/640	1/640	1/640	1/640
5	1/640	1/640	1/1280	1/640
6	0	0	0	0
7	1/640	1/640	1/640	1/640
8	1/640	1/640	1/640	1/640
9	1/640	1/1280	1/2560	1/640
10	1/1280	1/1280	1/1280	1/640

[*] Treatments as described in Table 2.
[+] Genetic groups as described in Table 1.

Treatment was found to have highly significant effects (P<0.001) on immunity against Salmonella at 4 weeks of age (Table 11). There were significant differences among different treatments on immunity against Salmonella at 4 weeks of age, the highest antibody titer (1288.20) for group which treated with *Enterococcus faecalis*. 2.5% lactose group appeared to follow the above mentioned treatment in its effect on immunity against Salmonella at 4 weeks of age.

Table 10. Least-squares means and standard errors for antibody titer trait as affected by genetic group of purebreds and crossbreds chicks.

Genetic group[*] of chicks	*Antibody titer*
Matrouh x Matrouh	657.99^{b}
Inshas x Inshas	641.13^{b}
Matrouh x Inshas	862.54^{a}
Inshas x Matrouh	763.57^{ab}

[a-c] Means with the same letters within each column of trait are not-significantly different (P<0.05).
[*] Genetic groups as described in Table 1.

Table 11. Least-squares means and standard errors for antibody titer trait as affected by treatment in purebreds and crossbreds chicks.

Treatment[*]	Antibody titer
1	$1091.70^{ab} \pm 79.49$
2	$624.01^{c} \pm 79.55$
3	$639.58^{c} \pm 79.49$
4	$647.43^{c} \pm 77.60$
5	$768.29^{c} \pm 77.66$
6	$0.00^{d} \pm 71.38$
7	$641.40^{c} \pm 75.89$
8	$638.71^{c} \pm 72.78$
9	$1288.20^{a} \pm 77.66$
10	$975.32^{b} \pm 75.89$

[*] Treatments as described in Table 2.
[a-c] means with the same letters within each column of trait are non-significantly different (P<0.05).

Bacillus subtilis appeared to follow the above mentioned treatment in its effect on immunity against Salmonella at 4 weeks of age (Table 11). However, *Lactobacillus acidophilus* group, *Enterococcus faecalis* group and *Bacillus subtilis* group which treated with lactose which treated with or without lactose had no significant effect on antibody titer at 4 weeks of age when compared with control positive group (treated with Salmonella). Also, the group which treated with *Enterococcus faecalis*, *Bacillus subtilis*, *Lactobacillus acidophilus* and 2.5% lactose had no significant effect on antibody titer at 4 weeks of age when compared with control positive group (treated with Salmonella).

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Identification of different animal protein sources in animal feed ingredients using PCR technique

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Abstract

Two sets of DNA primer sequences were evaluated to estimate their sensitivity in the detection of different animal proteins in feed ingredients. Species specific DNA primers set (a) was used to detect the presence of 0.05%, 0.1%, 1%, 1.5%, 3%, 4.5% and 100% of beef, pork and chicken proteins mixed with plant protein, while species specific DNA primers set (b) was used to estimate its ability to detect 0.05% of beef, pork, fish and chicken meat mixed together with plant-protein-based poultry feed. Another target of this study was to estimate the effect of microwave drying and autoclaving on the DNA extraction of beef sample. Results obtained from this study showed that, both primer sets (a) and (b) were able to detect 0.05% of beef, pork, poultry and fish (only from set b primer sequence) in the used substrates. Also, no negative effect of the used heat treatment methods was observed on the DNA extraction ability of the examined heat treated beef samples. None of the examined meat and bone meal samples were adulterated with pork derived protein. It was concluded that, PCR technique can be considered as a highly sensitive and reliable procedure which can be used for animal protein detection and identification.

Keywords: Animal DNA, adulteration, detection limit, DNA extraction, PCR.

Introduction

The risk of occurrence of bovine spongiform encephalopathy (BSE) results not only from infected or incubating animals but also from the use of concentrated feed or its incriminating ingredient, mammalian meat and bone meal, contaminated with the causal agent. The end products in the rendering process conversion of animal waste to meat and bone meals are a lipid fraction and a protein fraction, which may be the carrier of any infectious agent present in raw material that was not destroyed by the heating process (Bradley 1994). This is why feeding ruminant proteins to ruminants has been prohibited since 1988 as a ban of animal derived meals in the manufacturing of feedstuffs has been introduced by a European Community Regulation (European Union 2002) as a preventive measure to avoid the spread of Bovine Spongiform Encephalopathy (BSE). Cross-contamination of feedstuffs for ruminant with mammalian meat meal used in animal feed for other species is one of the main problems for feed manufacturing industries, which are very interested in adopting a control process program to monitor this processing cross-contamination. The detection of animal tissues in feedstuffs has therefore gained great interest in the last few years. For this purpose, a microscopic method, based on the analysis of animal bone fragments, has been developed. This method has been recognized as the 'official' method in the European strategy against the BSE. However, it is time consuming, requires specialised staff and only enables the detection of zoological classes (mammalian, avian and fish), while the species origin of bone fragments remains undetermined. The need for alternative analytical approaches has prompted numerous studies. Biomolecular techniques have been extensively investigated as they offer undoubtful advantages, such as having a high degree of specificity and being applicable even to heat processed products. Although DNA like proteins undergoes thermal denaturation, it has been observed that DNA can be still detected by short fragment amplification (Meyer and Candrian 1996). Polymerase Chain Reaction (PCR) has been applied for the detection of bovine tissue in animal feedstuffs (Krcmar and Rencova 2001 and Wang *et al.,* 2000). Lahiff *et al.* (2001) developed a PCR to recognize ovine, porcine and poultry DNA in feedstuffs. Myers *et al.* (2003) identified different species in feedstuffs using universal primers coupled with restriction endonucleases. More recently, Bottero *et al.* (2003) developed a method which involved the ability of primers to amplify wider target sequences. This PCR based assay demonstrated to be highly sensitive and useful in routine feedstuff analysis for the detection of all vertebrates.

The aim of the present study was to:

1- Estimate the ability of species specific DNA primers (a) to detect the presence of 0.05%, 0.1%, 1%, 1.5%, 3%, 4.5% and 100% of beef, pork and chicken proteins mixed with plant protein.
2- Estimate the ability of species specific DNA primers (b) to detect 0.05% of beef, pork, and fish and chicken meat mixed together with plant-protein-based poultry feed.

3- Estimate the effect of microwave drying and autoclaving on the DNA extraction of beef sample.

Materials and Methods

Estimation of the specificity of the used primer sequences

Primer sequences (a) for ruminant, pork and poultry (Table 1) and primer sequences (b) for ruminant, pork, poultry and fish (Table 2) were used to amplify control beef, pork, poultry and fish DNA (Biotool, Spain) to verify its specificity as follows:

PCR amplification was performed in a final volume of 50 ml containing 75 mM Tris–HCl (pH 8.8), 1 unit of Platinum Taq DNA Polymerase (Invitrogen, USA), 0.1 mg/ml BSA (Roche Diagnostics GmbH, Mannhein, Germany), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia, Uppsala, Sweden), 2 mM MgCl2, 25 pmol of each primer and 250 ng of DNA templates. Amplification was performed in a Thermal Cycler Biometra (Applied Biosystems, CA) with the following cycling conditions: 1- For primers set (a) After an initial heat denaturation step at 95 ^{o}C for 10 min, 35 cycles were programmed as follows: 95 ^{o}C for 30 s, 60 ^{o}C for 30 sec and 72 ^{o}C for 1 min. 2- For primers set (b): After an initial heat denaturation step at 94 ^{o}C for 10 min, 35 cycles were programmed as follows; 94 ^{o}C for 30 s, 60 ^{o}C for 1 min, 72 ^{o}C for 1 min and final extension at 72 ^{o}C for 5 min. Following amplification, 10 ul of 50% sucrose solution were added to the PCR mixtures resulting in a total volume of 60 ul from which 25 ul were pipette into wells in 1.8% melting agarose (Fisher Scientific, USA). The PCR reaction samples were separated by horizontal gel electrophoresis (Biorad, USA) and digital images were obtained using gel documentation system, USA (Guan and Levin, 2002).

Estimation of the sensitivity of the used primers (set a) to detect different concentrations of animal DNA

Beef, pork and chicken meat were collected from retailers (0.5 kg per each), dried at 60^{o}C for 16 hrs (AOAC 2006), ground with sterile pestle and mortar and all mixed with ground maize in a percentage of 0.05%, 0.1%, 1%, 1.5%, 3%, 4.5% and 100%. DNA extraction was performed using Dneasy Tissue kit (Qiagen, Hilden, Germany), from all samples. The DNA was quantified by spectrophotometry (Nanodrop, USA). PCR amplification was performed as described before.

Estimation of the ability of primers set (b) to detect 0.05% of animal DNA

The dried and ground beef, pork and chicken meet together with a sample of fish meal were mixed with plant-protein-based poultry feed in a percentage of 0.05%. DNA extraction and PCR analysis were performed as described before.

Estimation of the effect of microwave drying and autoclaving on DNA extraction of beef sample:

A sample of 5 grams of raw beef was put in sterile glass Petri dish and dried in the microwave (National, Dimension 4, Japan) at low power for 25 min after which it was ground with sterile mortar and pestle. Also 250 gm beef sample was autoclaved at 121^{o}C for 15 min and then dried at 60^{o}C for 16 hrs. After drying the autoclaved sample was grounded using sterile mortar and pestle. DNA extraction and PCR analysis were performed as described before using ruminant specific primer sequence from set b.

Table 1. Design of oligonucleotides for different animal species used in this study (Primers set a) according to Kiyoshi et al. (2002).

Species	Gene	Primer sequence	Amplicom Pb
Beef	ART2	5- CTC CCA GCA TCA GAG TCT TT -3 5- CTG TGG TGT TGG AGA AGA CT -3	181
Pork	PRE1	5- GCA TAT GGA GGT TCC CAG GC -3 5- ACT AGG AAC TAT AAG GTT TGC -3	179
Chicken	CR1	5- ATA GAA TCA TAG AAT GGC CTG- 3 5 TTT TCA CAC AGA GGG TGG TG- 3	201

Table 2. Design of oligonucleotides for different animal species used in this study (Primers set b) according to Dalmasso et al. (2004).

Species	Gene	Primer sequence	Amplicom Pb
Beef	16S rRNA	5- GAA AGG ACA AGA GAA ATA AGG -3 5- TAG GCC CTT TTC TAG GGC A -3	104
Pork	12S rRNA-tRNA	5- CTA CAT AAG AAT ATC CAC CAC A -3 5- ACA TTG TGG GAT CTT CTA GGT -3	290
Fish	12S rRNA	5- TAA GAG GGC CGG TAA AAC TC -3 5- GTG GGG TAT CTA ATC CCA G -3	224
Chicken	12S rRNA	5- TGA GAA CTA CGA GCA CAA AC 30 5 GGG CTA TTG AGC TCA CTG TT 30	183

Results and Discussion

Figures 1, 2 and 3 clarified that, the used primer sequences of set (a) could detect all the used concentrations of the dried and ground beef, pork and chicken meat mixed with the ground corn. Also specificity of the used primers was clear by obtaining the specific bands at 181, 179 and 201 bp of beef, pork and poultry DNA respectively.

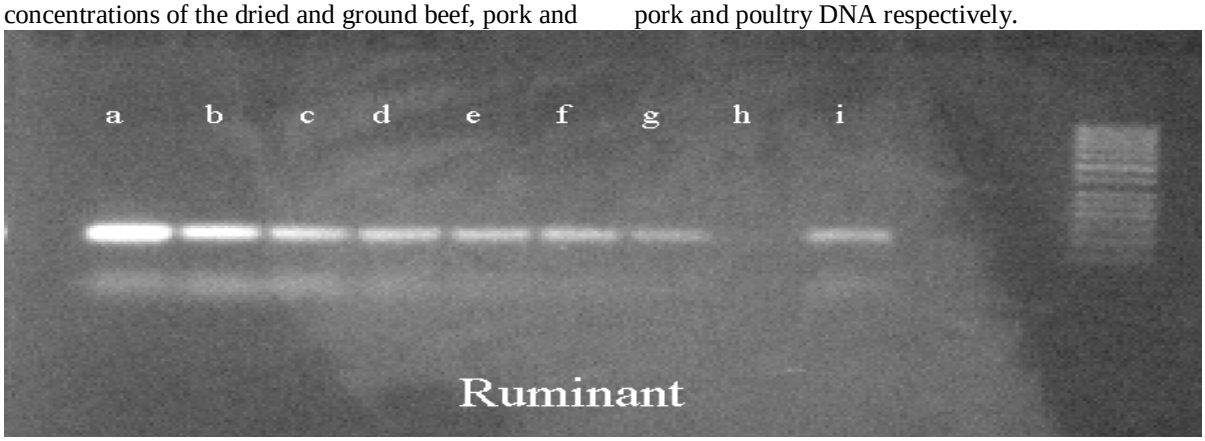

Figure 1. Gel of Beef DNA in various concentrations using set a primers (181 bp):
a)100% beef DNA, b) 4.5% beef DNA, c) 3% beef DNA, d) 1.5% beef DNA, e) 1% beef DNA, f) 0.1% beef DNA, g) 0.05% beef DNA, h) 0% beef DNA and i) standard beef DNA.

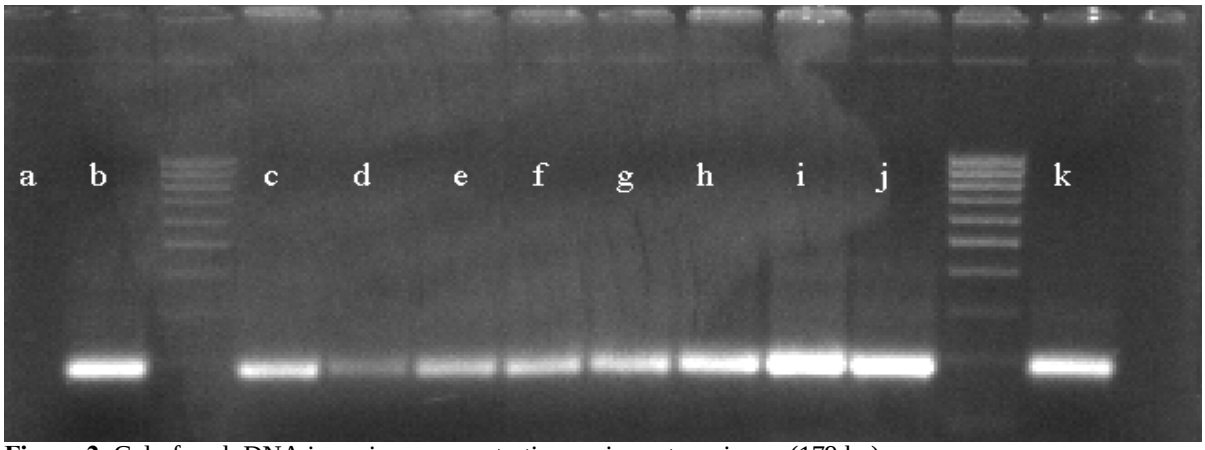

Figure 2. Gel of pork DNA in various concentrations using set a primers (179 bp):
j) 100% pork DNA, i) 4.5% pork DNA, h) 3% pork DNA, g) 1.5% pork DNA, f) 1% pork DNA, e) 0.1% pork DNA, d) 0.05% pork DNA, a) 0% pork DNA and b, c, k) standard pork DNA.

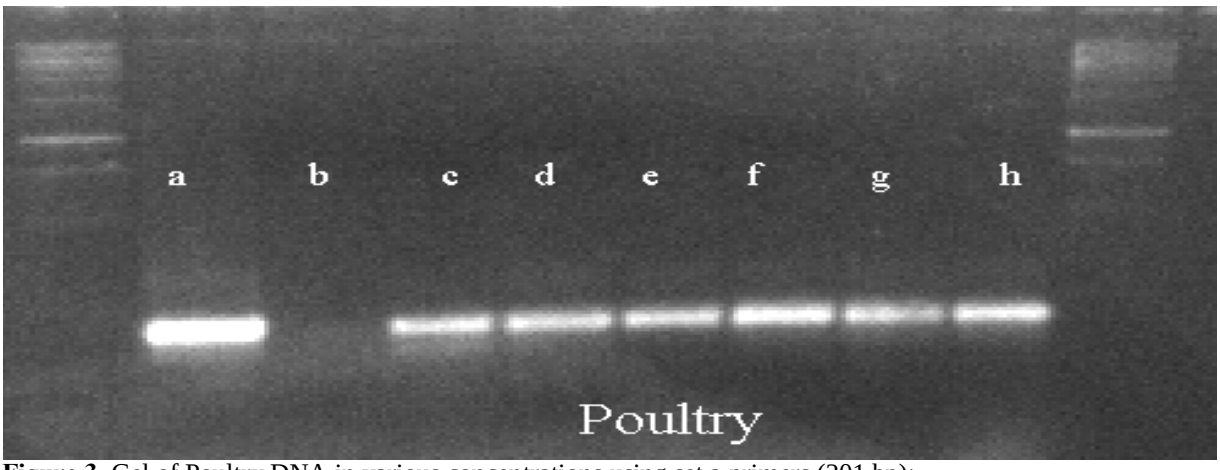

Figure 3. Gel of Poultry DNA in various concentrations using set a primers (201 bp):
a) 100% poultry DNA, h) 4.5% poultry DNA, g) 3% poultry DNA, f) 1.5% poultry DNA, e) 1% poultry DNA, d) 0.1% poultry DNA, c) 0.05% poultry DNA, and b) 0% poultry DNA .

Data from figure 4 showed the specificity of the used beef specific primer sequence from set (b) as it gave the specific band at 104 bp and also demonstrated its sensitivity as it could detect the DNA of 0.05% dried beef in poultry feed sample. Also data from this figure demonstrated that, neither autoclaving nor microwaving had a negative effect on the ability of DNA extraction from the examined beef sample.

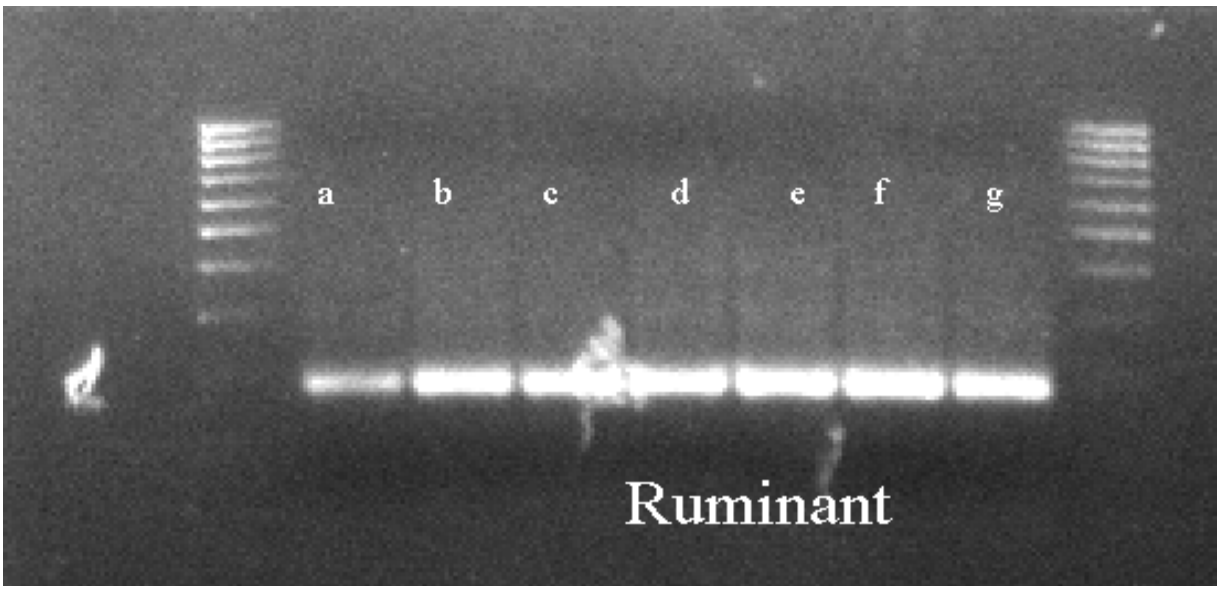

Figure 4. Gel of autoclaved, microwaved, 0.05% beef in poultry feed and standard beef DNA (104 bp):
a) Autoclaved beef DNA, b,c) microwaved beef DNA, d,e) 0.05% beef in poultry feed, f,g) standard beef DNA.

Figure 5 demonstrated the specificity and the sensitivity of pork specific primers of set (b) as it could form the specific band (290 bp) from the standard pork DNA and from the DNA extracted from poultry feed contained 0.05% dried and ground pork meat.

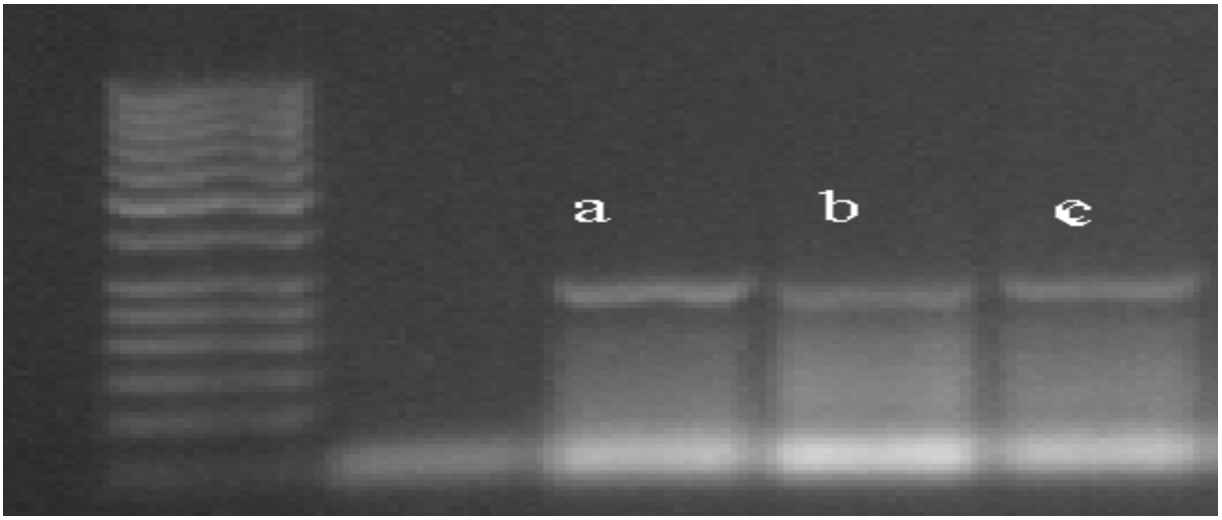

Figure 5. Gel of bands of pork DNA in 0.05% pork mixtures and standard pork DNA (290 bp):
a) Specific band with standard pork DNA b,c) specific bands of 0.05% pork DNA in poultry feed.

Figures (6&7) illustrated the specificity and sensitivity of the used poultry and fish primer sequences from set (b) as they could form the expected specific bands (183 and 224 bp, respectively) with their standard DNA and with their meat mixed with poultry feed at a percentage of 0.05%.

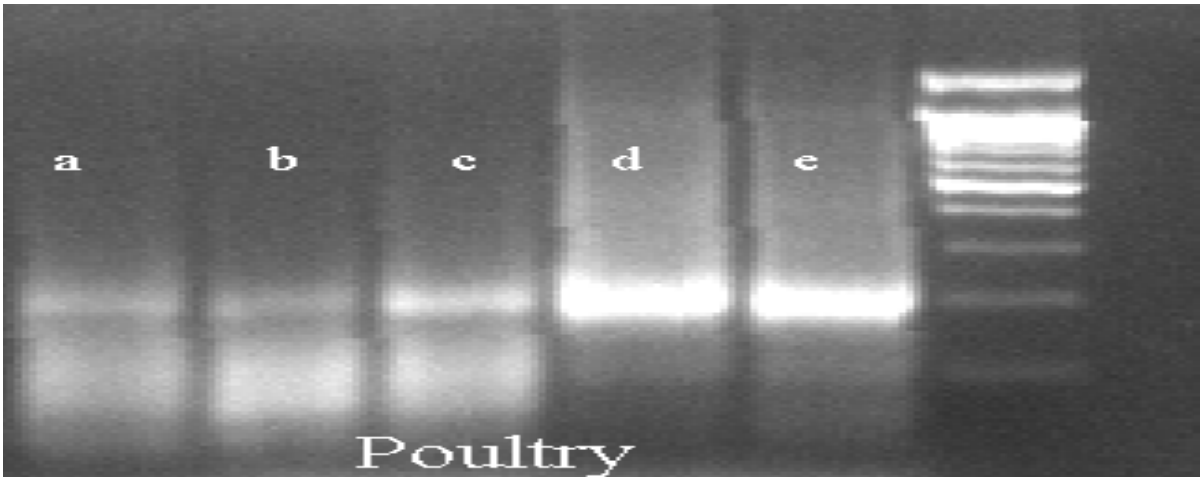

Figure 6. Gel of poultry DNA in 0.05% and in standard poultry DNA (183 bp):
a,b,c) 0.05% poultry DNA in poultry feed d,e) standard poultry DNA.

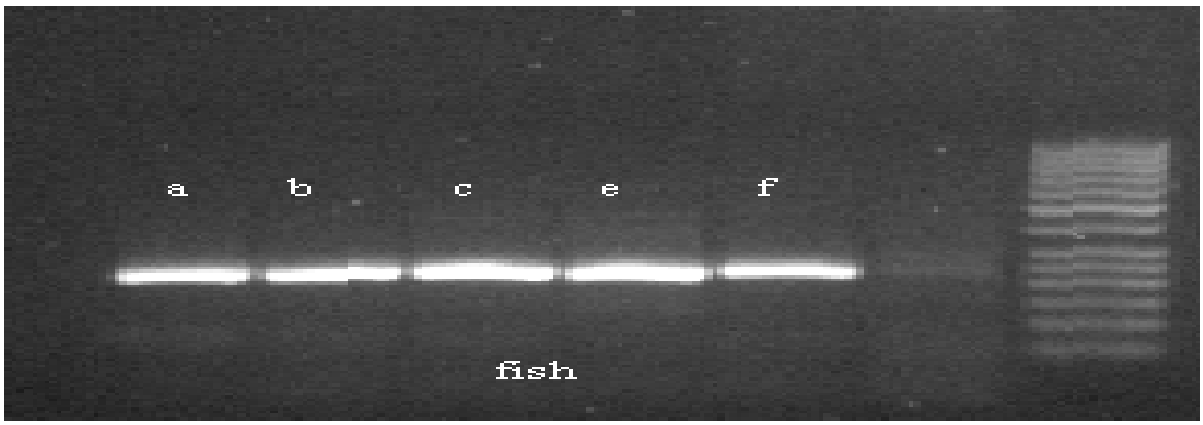

Figure 7. Gel of Fish DNA (224 bp):
a,b) Standard fish DNA, c,e,f) 0.05% of fish meal samples in poultry feed.

Figure 8 (a&b) demonstrated the specific bands at 104 bp obtained from analysis of MBM samples using beef specific DNA primers as a quality control tool to assure the presence of the extracted DNA in the analyzed samples, while negative results for the inclusion of pork derived proteins could be noticed by the absence of the pork specific bands at 290 bp.

At present, a critical point concerning the observance of the ban of animal meals in feedstuffs is represented by the reliability of the control tests. The low resolution efficiency of the microscopic method, which allows the detection of zoological classes but not of species, highlights the need for alternative analytical approaches.

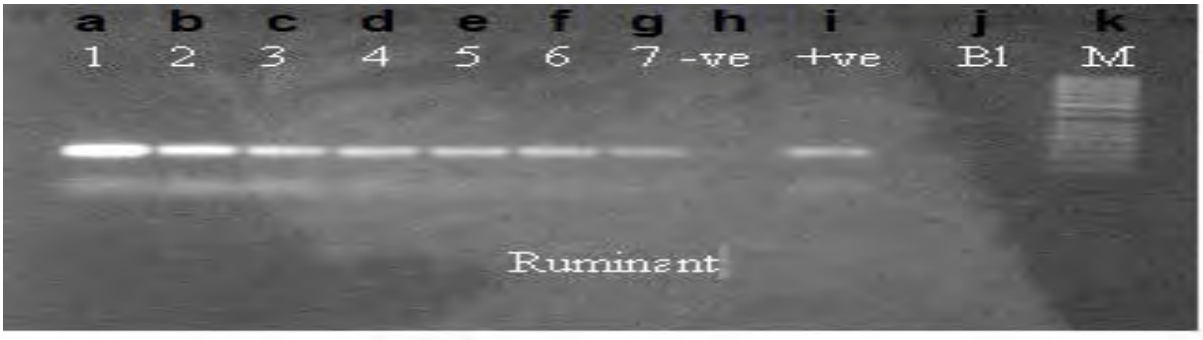

Figure 8 (a): Result of 5 samples negative for ruminant DNA.
Lane a, b, c, d, e, f and g samples positive for ruminant DNA Lane h control negative pork DNA and lane I control positive ruminant DNA Lane j blank and lane k ladder

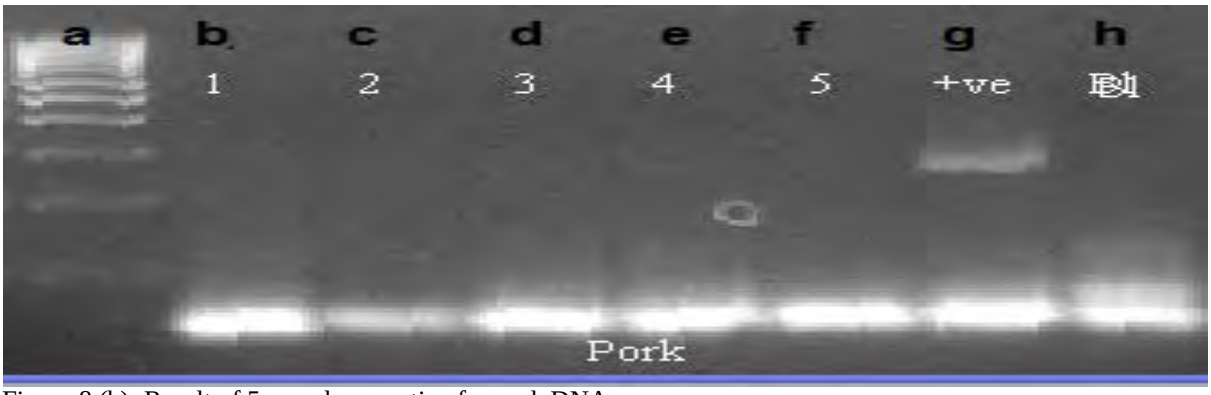

Figure 8 (b): Result of 5 samples negative for pork DNA.
Lane a Marker DNA, Lanes b, c, d, e and f samples negative for pork DNA Lane g control pork DNA and lane h blank

Technologies based on DNA analysis seem to fulfil this need. The present paper describes the application of PCR to detect ruminant, poultry, fish and pork materials in feedstuffs. The data obtained from this study agreed with that obtained by Kiyoshi *et al.* (2002) who used the same primer sequences in set (a) and ends with the same results ensuring its specificity and sensitivity. Also, Higgins *et al*. (1992) and Dalmasso *et al.* (2004) used the same primer sequence set (b) used in this study and came to the same conclusion (the specificity and the same level of sensitivity). The negative effect of the heat treatment on DNA of beef sample agreed with that obtained from Dalmasso *et al.* (2004) who erased the advantage of relying on DNA based method over protein based methods as the protein can be destructed by heat treatments while DNA could resist destruction and can be detected even in short chains.The PCR described in this paper proved to be very specific and sensitive, with a very low detection limit (0.05%) when DNA mixtures were tested. The same assay, applied on experimental mixtures of examined meat in vegetable, showed the same detection limit of the microscopic official method (European Union 1998). In conclusion, the PCR approaches proposed in this study can be considered as reliable and accurate methods for the control of food and/or feedstuffs. The test could be useful in the control of different products, and to verify the origin of the raw materials. Also adulteration of any food or feed ingredients with any extraneous protein sources could be detected by this method of analysis. Another conclusion is that, microwave drying and autoclaving has negative effect on the ability of DNA extraction from the examined substrate.

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Productivity, blood biochemical changes and immune response of broiler chicks vaccinated with different Avian Influenza vaccines at one or seven days of age

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Abstract

An experiment was conducted to investigate the effect of different Avian Influenza "AI" vaccines (H5N1, H5N2, combinant H5N2+Newcastle Disease "ND", and Egyptian H5N1) and vaccination programs (at 1 or 7 days-old) on broiler productivity, physiology and immunity. 1,350 day-old Hubbard broiler chicks were divided into 9 groups. Eight groups of chickens (3 replicates per group) were vaccinated with H5N1, H5N2, AI+ND and the Egyptian vaccine H5N1 at 1 or 7 days-old. The Egyptian vaccine was prepared from the isolated H5N1 AI virus from the Egyptian infected chickens in 2006. The chickens of group 9 were kept as negative control. All chicks had *ad libitum* access to water, corn-soy-based starter diet from 1 to 21 days-old, grower diet from 22 to 30 days-old and finisher diet from 31 to 42 days-old. Productive traits were measured weekly and calculated from 1 to 42 days-old. Five blood samples per replicate were collected at 4, 7, 14, 21, 28, 35, and 42 days-old. Results of real time PCR indicated that birds were not challenged by AI disease. During the experimental period, results indicated that neither different vaccines nor age at vaccination affect broiler productivity (final body weight (2,212 g), body weight gain (51.6 g/bird/d), feed intake (91.7 g/bird/d), feed conversion ratio (1.77 g feed : g gain) or mortality rate (6.6 %)), ND titer (ranged from 3 to 7 $\log_2$) and relative spleen (ranged from 147 to 165 mg/100 g BW) or bursa (ranged from 65 to 123 mg/100 g BW) weights. On the other hand, this study revealed that, H5N2 and H5N2+ND vaccines were more protective than H5N1 or the Egyptian AI vaccines as indicated by the geometric mean of HI titer against AI virus of experimentally broiler chicks (higher vs. less than 5 $\log_2$, respectively). However, no differences were detected among the vaccinated chicks at 1 or 7 days-old for HI titer against AI virus in most ages. Moreover, fluctuated changes within the normal levels were noted at 14, 28 and 42 days-old in plasma total protein, albumin, globulin, aspartate aminotransferase, creatinine and urea in response to AI vaccines at 1 or 7 days-old. It could be concluded that, both H5N2 and H5N2+ND vaccines were more preferable for Hubbard broiler flocks than H5N1 or the Egyptian H5N1vaccines.

Keywords: Broiler, Avian Influenza vaccines (H5N1, H5N2, combinant H5N2 with Newcastle disease), productivity, blood biochemical constituents, immune response.

Introduction

Avian Influenza (AI) virus is a type A Orthomyxovirus and produces a variety of disease syndromes in various poultry species. On the basis of serological reactions to surface glycoprotein (hemagglutination and neuraminidase), AI virus subtypes classified into 16 hemagglutinin (H1-H16) and nine neuraminidase (N1-N9) subtypes (Kawaoka *et al*., 1990; Rohm *et al*., 1996; Easterday et al., 1997). Infection with AI virus can be a devastating viral disease causing enormous losses in the poultry industry worldwide (Capua and Alexander, 2004). Conventional control strategies are based mainly on surveillance, stamping out of infected flocks, movement restriction, and enforcement of biosecurity measures (Swayne, 2009). The estimated loss of the Egyptian poultry industry after the first emergence of highly pathogenic AI H5N1 in February 2006 was 1 billion US$ and affected the income of 1.5 million people whose livelihoods depended on poultry (Meleigy, 2007). About 30 million birds were culled or depopulated in Egypt in the first wave of 2006.

Beside the biosecurity and monitoring infection particularly in the densely populated poultry areas, the vaccination represents an option for control. Vaccination as a supportive tool in AI virus control strategies was implemented to limit the spread of H5N1 and to reduce the losses (Lee and Suarez, 2005 and EFSA, 2008). Different types of vaccines are already in use that decrease shedding of the virus, morbidity, mortality, and transmissibility; increase resistance to infection; and reduce field virus replication (Van den Berg *et. al.*, 2008 and Swayne, 2009). Evaluation of different types of AI vaccines (A/Goose/Guangdong/1/1996 "H5N1" and A/Chicken/Mexico/232/94/CPA "H5N2") used in Egypt may provide effective vaccination strategy. Moreover, the combinant vaccine of AI+Newcastle Disease (ND) was recently recommended and commercialized for more protection against AI and ND viruses. In addition, the scientists of the Egyptian National Research Center, Giza, Egypt provided an Egyptian vaccine which prepared from the isolated H5N1 virus from the Egyptian infected chickens in 2006 (Bahgat *et al*., 2009).

Results reported by Hafez *et al*. (2010) of hemagglutination inhibition (HI) titer against AI virus in some H5N2-vaccinated and infected commercial farms using homologous H5N2 antigen conducted by the National Laboratory for Veterinary Quality Control on Poultry Production in vaccinated commercial broiler farms in Egypt from 2007 to 2008 recommended vaccination for AI virus at one-day-old. However, Lebdah and Shahin (2010) stated that the more preferable age for AI vaccination is 7 days-old. Moreover, Kim *et al.* (2010) suggested that day-old chicks obtained from immunized dames should not be vaccinated immediately. Therefore, the aim of this study was to obtain new insights into evaluation of AI vaccines and vaccination programs used in Egypt for broiler flocks.

Materials and methods

Experimental design

This study was conducted in the commercial poultry farm of Poultry Services Center, Faculty of Agriculture, Cairo University to examine 4 AI vaccines (H5N1, H5N2, combinant H5N2+ND and Egyptian H5N1) using 2 vaccination ages (at 1st day vs. at 7th day) in addition to a non-vaccinated control group. The Egyptian vaccine was prepared from the isolated H5N1 AI virus from the Egyptian infected chickens in 2006 (Bahgat *et al.*, 2009). Each group was replicated 3 times (50 chicks per each replicate) in separated floor pens.

Birds, diets and management

1350 one-day old Hubbard broiler chicks were obtained from the 10th of Ramadan City Hatchery (Cairo Poultry Company, Egypt). All chicks were wing-banded, weighed and housed on a deep litter in semi-closed house system. Chicks had *ad libitum* access to water and a non-medicated corn-soy-based starter diet from 1 to 21 day of age, grower diet from 22 to 30 days of age and finisher diet from 31 to 42 days of age. All diets were in mash form. All chicks were exposed to the same managerial and environmental conditions and medical treatments.

Vaccines and vaccination program

Vaccination programs and the methods of vaccination for all experimental birds regarding their groups were presented in Tables (1 and 2). The inactivated oil emulsion AI vaccines, either H5N1 (Reassortan, Subtype Re-1 Strain, Hardinweik Biotechnology, China; Batch no. 2009013 and titer $\geq 10^{8}$ EID_{50}), H5N2 (Volvac, Boehringer Ingelhein, Mexico; Batch no. 0707084K and titer $\geq 10^{8.5}$ EID_{50}), combinant H5N2 with ND (Boehringer, Mexico; Batch no. 100415A and titer $\geq 10^{7.6}$ EID_{50} for H5N2 and $\geq 10^{8.2}$ EID_{50} for ND) and Egyptian one were tested in this experiment by two programs of vaccination (Table 1). The Egyptian isolated AI vaccine (Eg. AI; challenge AI virus) is an inactivated oil emulsion H5N1 AI vaccine with titer of 10^{6} EID_{50}, kindly supplied by the Environmental Research Division, National Research Center. Briefly, washed red blood cells (10%), sterile saline, sterile distilled water and phosphate buffered saline were used as reagents. The inactivated H5N1 antigen was obtained from Veterinary laboratories Agency (New Haw, Addlestone, Surrey KT153 NB, UK), with preparation date was Dec09 and Lot No. of 3/05. Then, for preparing the Egyptian vaccine, thirty Specific Pathogenic Free (SPF) embryonated chicken eggs from 7 to 10 days of hatch (obtained from Kom-Oshim Company, El-Fayoum Governorate, Egypt) were used for titration of the isolated virus.

Table 1. Vaccination program for all experimental groups.

Chicken age (day)	Control	Program 1 (at 1st day)				Program 2 (at 7th day)			
		H5N1	H5N2	AI+ND	Eg. AI	H5N1	H5N2	AI+ND	Eg. AI
1	---	H5N1	H5N2	AI+ND	Eg. AI	---	---	---	---
4	ND+IB	ND+IB	ND+IB	ND+IB	ND+IB	ND+IB	ND+IB	ND+IB	ND+IB
7	---	---	---	---	---	H5N1	H5N2	AI+ND	Eg. AI
14	Avinew	Avinew	Avinew	---	Avinew	Avinew	Avinew	---	Avinew
14	Bursine+	Bursine+	Bursine+	Bursine+	Bursine+	Bursine+	Bursine+	Bursine+	Bursine+
30	Avinew	Avinew	Avinew	Avinew	Avinew	Avinew	Avinew	Avinew	Avinew

H5N1= Inactivated oil emulsion H5N1 AI vaccine (Batch No. 2009013, and titer $>10^{8}$ EID_{50}).

H5N2= Inactivated oil emulsion H5N2 AI vaccine (Batch No. 0707084K, and titer $>10^{8.5}$ EID_{50}).

AI+ND= Combinant oil emulsion H5N2 AI with ND vaccines (Batch No. 100415A and titer $>10^{7.6}$ EID_{50} for H5N2 and $>10^{8.2}$ EID_{50} for ND).

Eg. AI= Local isolated AI virus (challenge AI virus). Inactivated oil emulsion H5N1 AI vaccine at titer of 10^{6} EID_{50}.

ND+IB= Live ND virus B1 strain and live Infectious Bronchitis virus H120 strain (Batch No. 0906V241V and titer $>10^{6.5}$ EID_{50} for B1 strain and $>10^{3.5}$ EID_{50} for H120 strain).

Avinew= Avinew vaccine against ND (Merial, France; Batch No. L265547 and titer $>10^{4.5}$ EID_{50}).

Bursine+= Bursine Plus vaccine against Infectious Bursal Disease (IBD; Gumboro, Fort Dodge Animal Health, Iowa, USA; Batch No. 1053252A and titer $>10^{3.5}$ EID_{50}).

Table 2. Vaccination methods and doses

Vaccine type	Method of use and doses
AI (H5N1)	Injection subcutaneous, 0.5 cm^3
AI (H5N2)	Injection subcutaneous, 0.5 cm^3
AI (AI+ND)	Injection subcutaneous, 0.5 cm^3
AI (Eg. AI)	Injection subcutaneous, 0.5 cm^3
ND+IB (B1/H120)	Drinking water
IBD (Bursine+)	Drinking water
ND (Avinew)	Drinking water

Eg. = Egyptian; ND = Newcastle disease; IB; Infectious bronchitis disease; IBD = Infectious bursal disease 'Gumboro'; AI = Avian Influenza 'Avian flu'.

Studied traits

Productive performance

Individual body weight (BW) and feed intake (FI) were recorded weekly to the nearest 10 g. Then BW, body weight gain (BWG), growth rate (GR), FI and feed conversion ratio (FCR) were calculated from 1 to 42 days of age per replicate for each treatment. Mortality was recorded daily and mortality rate (%) was calculated per replicate for each group from 1 to 42 days of age.

Immunological traits

Peripheral blood samples (3 ml) were collected from five chicks, at random, from each replicate, via heart puncture at 4 and 7 days of age, then, from the brachial vein weekly up to 42 days of age. Each 3 ml were separated into two sterile Wassermann tubes (1 ml in an empty tube for serum and 2 ml in another tube containing heparin for plasma). Sera were obtained after blood centrifugation at 6000 rpm for 10 minutes from the 1 ml of the obtained blood to determine the HI antibody titers against AI and ND. This was done at the Cairo Poultry Company central laboratory (Giza, Egypt). This was done according to the World Organization of Animal Health manual (OIE, 2005) at 4, 7, 14, 21, 28, 35 and 42 days of age. Hemagglutination units of homologous antigen were supplied by the company producing the vaccine that was used. The AI and ND antigens and antiserum (positive and negative) used in the HI-tests were obtained from the supplier of the AI and ND vaccines, respectively.

Real-time PCR, for pooled swab samples, for each replicate was done at 35 days-old in Cairo Poultry Company Central Laboratory (Giza, Egypt) to determine the infectious status of AIV (positive or negative?) according to Spackman *et al.* (2002). Swabs were collected from the trachea of 5 chicks per replicate and pooled. In brief, ribonucleic acid was extracted and purified from tracheal swabs from each replicate separately by using the RNeasy® Mini Kit (Ref. 74104 QIAGEN®, AMBION, Inc., Austin, Texas, USA). Ribonucleic acid extracts were amplified using a 1-step real-time PCR kit (TaqMan®, Qiagen, Valencia, CA, USA). Detection of H5 and N1 gene segments was done by AIV H5N1 Real Time RT-PCR Kit (Roche) according to the instructions of the manufacturer using a LightCycler 2.0 machine (Roche molecular systems, Inc., Branchburg, New Jersey, USA) as described by Aly *et al.* (2008).

Blood biochemical constituents

At 14, 28 and 42 days of age plasma samples were obtained from the 2 ml of the obtained heparinized blood by centrifugation at 6000 rpm for 10 minutes and was kept frozen at -20°C until it was used for biochemical measurements of plasma constituents. Plasma total protein (Gornall *et al.*, 1949 and Josephson and Gyllensward, 1957), albumin (Doumas *et al.*, 1971 and 1997) and globulin (Doumas, 1975) were determined colorimetrically as indicators of immunoglobulin status in the blood. Also, aspartate transaminase (AST=GOT "Glutamic-Oxaloacetic Transaminase") and alanine transaminase (ALT=GPT "Glutamic-Pyruvic Transaminase") were measured colorimetrically by using commercial Kits purchased from Biomerieux laboratory reagents and products, France according to (Reitman and Frankel, 1957) as indicators of liver function. Levels of urea (Folin and Denis, 1912; Folin, 1934) and creatinine were measured kinetically according to Bartels *et al.* (1972) as indicators of kidney function.

All these plasma constituents were measured at 14, 28 and 42 days of age in the Biochemistry Laboratory, Faculty of Agriculture Research Park (FARP), Faculty of Agriculture, Cairo University.

Statistical analysis

The experiment was designed as a factorial randomized design of 9 groups (4x2+1). There were 4 AI vaccines (H5N1 vs. H5N2 vs. combinant H5N2+ND vs. Egyptian H5N1) by 2 vaccination ages (at 1st day vs. at 7th day) plus an un-vaccinated group 'negative control'. Each group was replicated 3 times (50 chicks per replicate). Overall mean and standard error (SE) for all traits were calculated by using Means procedure (SAS, 2004). One-way ANOVA with 9 groups (4 vaccines x 2 vaccination ages + a negative control group) or 3 vaccination groups (negative control group vs. vaccination at day-old vs. vaccination at 7 days of age) was performed for all data by using GLM procedure (SAS, 2004). Non normal distributed data were transformed to log form before the analysis. Detected differences among the experimental groups were tested using Tukey's honest significant differences test after ANOVA (SAS, 2004). Values were

considered statistically different at $P < 0.05$. Results were reported as least square means with standard error of the mean (SEM). The statistical model used was as follows: $Y_{ij} = \mu + T_i + E_{ij}$, whereas: Y_{ij}= the observation; j^{th} individual under i^{th} treatment; μ= the overall mean; T_i= the effect of the i^{th} treatment; E_{ij}= the uncontrolled deviation attributed to the individuals.

Results and discussion

Productive performance

Productive performance traits of broiler chicks from 1 to 42 days of age in response to AI vaccination types and programs were represented in tables (3.a and 3.b). In general, neither AI vaccination types nor ages affected final body weight (BW; 2,212 g), BW gain (51.6 g/bird/d), feed intake (91.7 g/bird/d), feed conversion ratio (1.778 g feed:g gain) or mortality rate (6.6 %). Published data about broiler performance in the Hubbard management broiler guide at 42 d of age were 2379 g for BW and 1.71 for FCR agree with our results. Information available in the literature about the effect of AI vaccines or vaccination ages on broiler performance is scarce.

Nasr (2008) studied the effect of AI vaccination dose (0.50 vs. 0.25 ml) and age (at 1 or 7 days-old) of AI-H5N2 vaccination on BW and immune response of maternally-immune broiler chicks. He reported no marked effect of vaccination age on BW which is in agreement with our results. However, he noticed that AI-H5N2 vaccinated chicks showed higher BW than non-vaccinated ones. In another trial, he also reported that BW of commercial broiler chickens vaccinated with full dose (0.50 ml) H5N1 at 1 or 7 days of age was not affected.

Table 3.a. Effect of Avian Influenza (AI) vaccination on productive traits of broiler chickens from 1 to 42 d of age.

Item	Final body weight, g	Body weight gain, g/bird/d	Feed intake, g/bird/d	Feed conversion ratio, g:g	Mortality rate, %
Control	2285	53.37	93.07	1.743	7.67
Vaccination at one-day-old					
AI-H5N1	2215	51.70	89.87	1.739	5.00
AI-H5N2	2215	51.70	93.50	1.808	5.33
AI + Newcastle Disease	2206	51.50	91.30	1.771	6.33
Egyptian AI	2130	49.70	94.43	1.901	6.67
Vaccination at 7 d of age					
AI-H5N1	2215	51.73	92.17	1.784	7.00
AI-H5N2	2162	50.50	88.90	1.765	7.00
AI + Newcastle Disease	2237	52.23	90.57	1.734	7.33
Egyptian AI	2241	52.37	91.80	1.758	6.67
SEM[1]	44.71	1.067	3.461	0.0705	1.711
Probability[2]	0.4676	0.4792	0.9694	0.8137	0.9692

[1] Standard error of the mean (3 replicates of 50 chicks per replicate for each group).
[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).
No significant differences were observed among groups.

Table 3.b. Effect of Avian Influenza (AI) vaccination program on productive traits of broiler chickens from 1 to 42 d of age.

Item	Final body weight, g	Body weight gain, g/bird/d	Feed intake, g/bird/d	Feed conversion ratio, g:g	Mortality rate, %
Overall mean	2212	51.64	91.73	1.778	6.56
SE[1]	14.91	0.355	1.015	0.0218	0.502
Control	2285	53.37	93.07	1.743	7.67
Vaccination at one-day-old	2191	51.15	92.28	1.805	5.83
Vaccination at 7 d of age	2214	51.71	90.86	1.760	7.00
SEM (n=3)[2, 3]	43.30	1.030	3.129	0.0663	1.511
SEM (n=12)[2, 3]	21.65	0.515	1.565	0.0331	0.755
Probability[3]	0.1761	0.1767	0.7385	0.5507	0.4196

[1] Standard error (27 replicates of 50 chicks per each replicate).
[2] Standard error of the mean (number of replicates with 50 chicks per each replicate).
[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).
No significant differences were observed among groups.

Immunological traits

Results in table (4.a) showed the effect of AI vaccination on antibody titers against AIV of broiler chickens from 1 to 42 d of age. At 4 and 7 days of age, no differences were detected among treated groups in comparison with the control group. It is apparent that the maternal antibodies were high since the breeders were vaccinated with H5N1 vaccine. However, from 14 days of age to the end of the experiment, birds vaccinated with H5N2 or H5N2 + ND at 1 or 7 days of age showed higher antibody titers against AIV than those vaccinated with H5N1 and the Egyptian-H5N1 vaccines or the non-vaccinated chicks. The geometric mean HI titers against AIV of broiler chicks vaccinated with H5N2 or AI-H5N2+ND vaccines, during these ages, were higher than 5 $\log_2$. In contrast, chicks vaccinated with H5N1 and the Egyptian-H5N1 AI vaccines and those of the control group had antibody titers against AIV less than 5 $\log_2$ during the same period.

These results are in agreement with results obtained by Bahgat *et al.* (2009). They reported that sera from chicks vaccinated with killed H5N2 vaccine had the highest ELISA IgG reactivity in comparison with chicks vaccinated with killed H5N1 Egyptian isolate or the untreated chicks. Moreover, Brugh and Stone (1986) and Swayne (2009) reported that the HI titers will probably be indicative of the level of protection and immunity to avian influenza. In addition, Tian *et al.* (2005) and Kumar *et al.* (2007) supposed that HI antibody titers of 4 $\log_2$, or higher, of vaccinated chickens were protected from virus challenge. Abdelwhab *et al.* (2012) observed that the maternal antibodies could interfere with the immune response when a homologous vaccine strain was used. They also reported that, vaccine-derived maternal AIV H5N1 specific immunity in one-day old chicks was investigated as a factor of vaccine failure in long-term blanket vaccination campaigns in broiler chickens. These suggestions might explain, at least in part, the low titer levels obtained in this trial from the chicks vaccinated with AI-H5N1 and the Egyptian-H5N1 vaccines in compare to those vaccinated with H5N2 or AI-H5N2+ND vaccines.

Table 4.a. Effect of Avian Influenza (AI) vaccines on antibody titer ($\log_2$) against AI virus of broiler chickens from 1 to 42 d of age.

Item	At 4 d	At 7 d	At 14 d	At 21 d	At 28 d	At 35 d	At 42 d
Control	5.53	5.13	4.70^{b}	3.50^{b}	2.67^{b}	3.00^{b}	1.83^{c}
Vaccination at 1 d of age							
AI-H5N1	5.08	4.27	2.12^{c}	1.87^{bc}	1.90^{bc}	2.67^{b}	2.80^{c}
AI-H5N2	4.70	4.43	7.92^{a}	8.05^{a}	6.67^{a}	5.72^{a}	5.87^{ab}
AI+Newcastle Disease	5.23	4.23	8.40^{a}	8.02^{a}	7.15^{a}	6.08^{a}	6.87^{a}
Egyptian AI	5.40	5.17	1.45^{c}	0.57^{c}	0.70^{c}	0.27^{c}	0.17^{d}
Vaccination at 7 d of age							
AI-H5N1	5.06	4.47	1.40^{c}	1.87^{bc}	2.07^{bc}	2.13^{bc}	2.07^{c}
AI-H5N2	4.80	4.60	7.25^{a}	7.53^{a}	6.70^{a}	6.43^{a}	5.93^{ab}
AI+Newcastle Disease	5.33	4.57	7.58^{a}	7.32^{a}	7.17^{a}	5.33^{a}	5.47^{b}
Egyptian AI	5.30	4.83	3.00^{bc}	0.55^{c}	0.56^{c}	0.33^{c}	0.23^{d}
SEM (n=3)[1]	0.311	0.340	0.369	0.398	0.390	0.406	0.262
Probability[2]	0.6221	0.4580	<.0001	<.0001	<.0001	<.0001	<.0001

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.

[1] Standard error of the mean (number of replicates with 5 samples per each replicate).

[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).

Chicks in the current trial were produced from breeders repeatedly immunized with a commercial inactivated vaccine based on the H5N1 strain. In addition, Ellis *et al.* (2004) stated that the use of killed H5N2 vaccine in the face of highly pathogenic AI H5N1virus challenge was able to protect chickens from the disease and can reduce virus transmission. Also, these findings were in agreement with Tian *et al.* (2005). In contrast, Nasr (2008) concluded that the imported AI H5N1 vaccine is better than H5N2 for breeder, layer and broiler poultry flocks in Egypt.

No differences were detected among the vaccinated chicks at 1 or 7 days of age in HI titers against AIV in comparison with non-vaccinated chicks at all ages during the experimental period (Table 4.b). In contrast, Lebdah and Shahin (2010) noted that the more preferable age for AI vaccination is 7 days of age. Moreover, Kim *et al.* (2010) suggested that day-old chicks produced from immunized dames should not be vaccinated immediately. On the other hand, the results reported by Hafez *et al.* (2010) of HI titers against AIV in some H5N2-vaccinated and infected commercial broiler farms using homologous H5N2 antigen conducted by the National Laboratory for Veterinary Quality Control on Poultry Production in Egypt from 2007 to 2008 recommended vaccination for AIV at 1 day of age. These results showed that HI titers for commercial broiler chicks vaccinated at 1

day-old ranged from 5.2 to 9 log_2 at 32 days of age and from 2.0 to 7.2 log_2 at 45 days of age. However, they reported that the HI titers for the chicks vaccinated at 7 days of age ranged from 2.0 to 3.0 log_2 at 44 days-old. Hafez *et al.* (2010) noted that most of the reported positive cases (65%) in 2007 and 2008 under field conditions have had high AI HI titers using the commercial homologous HI antigen (4 to 9.6 log_2). Moreover, they also reported that the field virus was detected by real-time reverse transcription PCR and clinical signs as well as lesions were observed in some vaccinated flocks.

In addition, Kilany *et al.* (2011) reported that since March 2006, Egypt embarked on inactivated H5 vaccines to combat the severe outbreaks of high pathogenic AI H5N1endemic virus in commercial poultry. Different H5 vaccines supplied by several companies are applied extensively in the field with highly variable vaccination regimes (Hafez *et al.*, 2010).

The insufficient efficacy of the current H5 vaccines to protect chickens against the newly emerging 2.2.1 variant HP AI H5N1 strains in Egypt has been recently proved (Kim *et al.*, 2010). These groups of antigenically distinct variant viruses were first and mainly detected from vaccinated commercial chicken farms since late 2007, 18 months after implementation of the nation wide blanket vaccination policy (Balish *et al.*, 2010 and Abdelwhab *et al.*, 2010). Due to the immune pressure exerted by the vaccine and continuous replication of the virus in different poultry species and mammals, major antigenic alterations were observed in the immunogenic epitopes of the H5 molecule which could be the most important cause for the so called vaccinal-outbreaks (Arafa *et al.*, 2010 and Hafez *et al.*, 2010).

Results in Tables 5.a and 5.b indicated that neither different AI vaccines nor age at vaccination affect the antibody titers against Newcastle Disease (ND) virus for all ages except at 35 days of age. At this age, chicks vaccinated with different AI vaccines at 7 days of age showed numerically higher antibody titer values against ND virus than non-vaccinated chicks. Chicks vaccinated at 1 day of age were intermediated. Titer values ranged from 5 to 6 log_2 which led to approximately the same physiological protection level.

Results in Tables 6.a and 6.b indicated that neither different vaccines nor vaccination programs have significant effect on relative immune organs (bursa and spleen) weights at 28, 35 or 42 days of age. In general, relative spleen weight ranged from 147 to 165 mg/100 g BW and relative bursa weight ranged from 65 to 123 mg/100 g BW.

Huff *et al.* (1988) reported that the relative spleen weight was 40 mg/100g BW for Hubbard chickens in a control group at 21 days of age. Moreover, Edrington *et al.* (1997) showed that relative spleen weight was 130 mg/100g BW in broiler chicken (Peterson x Hubbard) at the same age.

However, no information is available in the literature about the effect of AI vaccines or vaccination ages on the relative spleen or bursa weights of broiler chickens. Results of real time PCR indicated no AI virus infection was detected in all experimental groups. These findings prove that all groups were not challenged by AI disease. This might explain non detectable changes in productivity, especially mortality rate, or in the immune response.

Table 4.b. Effect of different Avian Influenza (AI) vaccination age on antibody titers (log_2) against AI virus of broiler chickens from 1 to 42 d of age.

Item	At 4 d	At 7 d	At 14 d	At 21 d	At 28 d	At 35 d	At 42 d
Overall mean	5.16	4.63	4.87	4.36	3.95	3.55	3.47
SE[1]	0.100	0.114	0.555	0.623	0.546	0.461	0.485
Control	5.53	5.13	4.70	3.50	2.67	3.00	1.83
Vaccination at 1 day-old	5.10	4.53	4.97	4.63	4.10	3.69	3.93
Vaccination at 7 day-old	5.13	4.62	4.81	4.32	4.12	3.56	3.43
SEM (n=3)[2]	0.302	0.337	1.732	1.933	1.682	1.435	1.466
SEM (n=12)[2]	0.151	0.169	0.866	0.967	0.841	0.718	0.733
Probability[3]	0.4369	0.2882	0.9861	0.8722	0.7229	0.9132	0.4531

[1] Standard error (27 replicates of 5 samples per each replicate).
[2] Standard error of the mean (number of replicates with 5 samples per each replicate).
[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).
No significant differences were observed among groups within column.

Table 5.a. Effect of Avian Influenza (AI) vaccines on antibody titers (log_2) against Newcastle Disease (ND) virus of broiler chickens from 1 to 42 d of age.

Item	At 4 d	At 7 d	At 14 d	At 21 d	At 28 d	At 35 d	At 42 d
Control	6.77	5.73	3.13	4.20	3.81	4.98	6.40
Vaccination at 1 d of age							
AI-H5N1	6.78	5.43	2.83	4.33	4.47	6.16	7.00
AI-H5N2	6.87	5.30	3.04	3.68	5.27	6.33	6.13
AI+ND	6.87	5.80	3.62	4.32	4.78	6.31	6.40
Egyptian AI	6.73	5.83	3.02	3.42	4.20	5.83	7.00
Vaccination at 7 d of age							
AI-H5N1	6.67	5.33	3.00	3.53	3.31	6.51	6.87
AI-H5N2	6.20	5.27	3.02	3.38	3.82	6.47	6.73
AI+ND	6.20	5.47	2.63	3.47	6.25	6.40	6.77
Egyptian AI	6.93	5.57	3.40	4.46	4.05	6.15	6.35
SEM (n=3)[1]	0.194	0.224	0.188	0.319	0.701	0.488	0.362
Probability[2]	0.0979	0.5008	0.0623	0.1088	0.1883	0.4965	0.6473

[1] Standard error of the mean (number of replicates with 5 samples per each replicate).
[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).
No significant differences were observed among groups within column.

Table 5.b. Effect of different Avian Influenza (AI) vaccination age on antibody titers (log_2) against Newcastle Disease (ND) virus of broiler chickens from 1 to 42 d of age.

Item	At 4 d	At 7 d	At 14 d	At 21 d	At 28 d	At 35 d	At 42 d
Overall mean	6.67	5.53	3.08	3.87	4.44	6.13	6.63
SE[1]	0.074	0.074	0.075	0.121	0.255	0.162	0.116
Control	6.77	5.73	3.13	4.20	3.81	4.98[b]	6.40
Vaccination at 1 day-old	6.81	5.59	3.13	3.94	4.68	6.16[ab]	6.63
Vaccination at 7 day-old	6.50	5.41	3.01	3.71	4.36	6.38[a]	6.68
SEM (n=3)[2]	0.212	0.221	0.231	0.366	0.778	0.434	0.359
SEM (n=12)[2]	0.106	0.110	0.115	0.183	0.389	0.217	0.180
Probability[3]	0.1234	0.3223	0.7627	0.4362	0.5881	0.0271	0.7863

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.
[1] Standard error (27 replicates of 5 samples per each replicate).
[2] Standard error of the mean (number of replicates with 5 samples per each replicate).
[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).

Table 6.a. Effect of different Avian Influenza (AI) vaccination on relative spleen and bursa weights (mg/100 g Body Weight) of broiler chickens at 28, 35 and 42 d of age.

Item	Spleen weight, mg/100 g BW			Bursa weight, mg/100 g BW		
	At 28 d	At 35 d	At 42 d	At 28 d	At 35 d	At 42 d
Control	136	162	139	105	96	75
Vaccination at one-day-old						
AI-H5N1	174	154	125	104	92	65
AI-H5N2	140	179	154	116	90	62
AI + Newcastle Disease	132	148	192	119	75	59
Egyptian AI	139	151	174	159	73	65
Vaccination at 7 d of age						
AI-H5N1	167	167	181	120	81	71
AI-H5N2	145	197	163	118	77	73
AI + Newcastle Disease	158	163	133	127	93	61
Egyptian AI	133	168	153	142	84	51
SEM[1]	10.9	12.8	21.3	13.1	8.5	6.2
Probability[2]	0.0536	0.1910	0.3466	0.0867	0.4470	0.1640

[1] Standard error of the mean (number of replicates with 5 samples per each replicate).
[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).
No significant differences were observed among groups within column.

Table 6.b. Effect of Avian Influenza (AI) vaccination program on relative spleen and bursa weights (mg/100 g Body Weight) of broiler chickens at 28, 35 and 42 d of age.

Item	Spleen weight, mg/100 g BW			Bursa weight, mg/100 g BW		
	At 28 d	At 35 d	At 42 d	At 28 d	At 35 d	At 42 d
Overall mean	147	165	157	123	85	65
SE[1]	3.7	4.3	7.1	4.5	2.8	2.1
Control	136	162	139	105	96	75
Vaccination at one-day-old	147	158	161	125	83	63
Vaccination at 7 d of age	151	174	158	127	84	64
SEM (n=3)[2]	11.3	12.9	21.5	13.4	8.5	6.3
SEM (n=12)[2]	5.6	6.5	10.7	6.7	4.2	3.1
Probability[3]	0.4926	0.2037	0.6444	0.3547	0.3312	0.2061

[1] Standard error (27 replicates of 5 samples per each replicate).
[2] Standard error of the mean (number of replicates with 5 samples per each replicate).
[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).
No significant differences were observed among groups within column.

Blood parameters

Results in tables (7.a and 7.b) shown a fluctuated effect of AI vaccination on plasma protein, albumin and globulin contents in broiler chickens but, all were in normal levels. Results obtained at 14 day of age indicated that birds vaccinated with the local (Egyptian H5N1) vaccine at 1 day-old (4.81 g/dl) had lower plasma protein content than those vaccinated with the same vaccine at 7 days-old (5.48 g/dl).

Table 7.a. Effect of different Avian Influenza (AI) vaccination on plasma total protein, albumin and globulin (g/dl) of broiler chickens at 14, 28 and 42 d of age.

Item	Protein, g/dl			Albumin, g/dl			Globulin, g/dl		
	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d
Control	5.43^{a}	5.87	5.46^{ab}	3.44	3.16	4.01^{ab}	2.00	2.71	1.45^{ab}
Vaccination at one-day-old									
AI-H5N1	5.39^{ab}	6.03	5.72^{a}	3.52	3.15	3.82^{b}	1.87	2.88	1.90^{a}
AI-H5N2	5.40^{ab}	5.95	5.44^{ab}	3.31	3.22	3.85^{b}	2.10	2.73	1.60^{a}
AI + Newcastle Disease	5.38^{ab}	6.00	5.55^{ab}	3.32	3.36	3.92^{ab}	2.06	2.64	1.64^{a}
Egyptian AI	4.81^{b}	5.89	5.53^{ab}	3.34	3.37	3.94^{ab}	1.47	2.52	1.59^{a}
Vaccination at 7 d of age									
AI-H5N1	5.15^{ab}	5.72	5.32^{b}	3.41	3.51	4.25^{a}	1.74	2.21	1.07^{b}
AI-H5N2	5.13^{ab}	5.65	5.32^{b}	3.22	3.32	3.78^{b}	1.91	2.33	1.54^{ab}
AI + Newcastle Disease	5.26^{ab}	5.77	5.60^{ab}	3.26	3.26	3.87^{ab}	2.00	2.51	1.73^{a}
Egyptian AI	5.48^{a}	5.83	5.69^{ab}	3.45	3.39	3.97^{ab}	2.03	2.44	1.73^{a}
SEM[1]	0.133	0.183	0.083	0.098	0.088	0.085	0.171	0.178	0.113
Probability[2]	0.0219	0.8623	0.0096	0.4566	0.0983	0.0162	0.2480	0.2212	0.0008

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.
[1] Standard error of the mean (number of replicates with 5 samples per each replicate).
[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).

Table 7.b. Effect of Avian Influenza (AI) vaccination program plasma total protein, albumin and globulin (g/dl) of broiler chickens at 14, 28 and 42 d of age.

Item	Protein, g/dl			Albumin, g/dl			Globulin, g/dl		
	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d
Overall mean	5.27	5.86	5.52	3.36	3.31	3.93	1.91	2.55	1.58
SE[1]	0.049	0.059	0.032	0.033	0.031	0.032	0.059	0.061	0.046
Control	5.43	5.87	5.46	3.44	3.16	4.01	2.00	2.71[a]	1.45
Vaccination at one-day-old	5.25	5.97	5.56	3.37	3.28	3.88	1.87	2.69[a]	1.68
Vaccination at 7 d of age	5.25	5.74	5.48	3.34	3.37	3.97	1.92	2.37[b]	1.52
SEM (n=3)[2]	0.149	0.174	0.095	0.099	0.182	0.095	0.178	0.174	0.135
SEM (n=12)[2]	0.074	0.087	0.048	0.050	0.091	0.048	0.089	0.087	0.068
Probability[3]	0.5133	0.2023	0.4246	0.6235	0.0881	0.2955	0.8174	0.0279	0.1351

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.

[1] Standard error (27 replicates of 5 samples per each replicate).

[2] Standard error of the mean (number of replicates with 5 samples per each replicate).

[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).

However, this effect was disappeared at 28 and 42 days of age. Moreover, no differences were detected among treatments for plasma protein at 14 and 28 days of age, albumin or globulin contents at 28 days of age. On the other hand, there is a clear effect at 42 days of age indicated that birds vaccinated with H5N1 vaccine at 1 day-old had higher protein and globulin and lower albumin contents than those vaccinated at 7 days-old. In addition, birds vaccinated with AI H5N2 at 7 days-old shown the lowest protein and albumin contents in plasma at 42 days of age.

Regarding the effect of age at vaccination, no differences were observed for all plasma protein, albumin and globulin contents at any age except for globulin content of plasma at 28 days of age that was lower for birds vaccinated at 7 days-old in compare to birds vaccinated at 1 day-old or non-vaccinated chicks.

Avian Influenza vaccination with different vaccines at 1 or 7 days-old did not affect liver function as indicated by GOT=AST and GPT=ALT contents in plasma except at 28 days of age for chicks vaccinated with the Egyptian vaccine at 1 day-old (Table 8.a). These chicks showed the higher GOT content (34.8 mg/dl) in plasma comparing to chicks vaccinated with the combinant AI H5N2 with ND vaccine at 7 days-old. However, all values are in normal levels and no differences were observed in response to the age of vaccination (Table 8.b).

Avian Influenza vaccination with different vaccines at 1 or 7 days-old did not affect kidney function as indicated by creatinine and urea contents in plasma except at 42 days of age for chicks vaccinated with the Egyptian vaccine at 1 day-old (Table 9.a). These chicks showed the higher creatinine content (2.83 mg/dl) in plasma comparing to chicks vaccinated with the AI H5N1 vaccine at the same age (2.03 mg/dl). However, all values are in normal levels. No differences were observed in response to the age of vaccination except at 14 days of age for urea content in plasma that was lower for chicks vaccinated at 1day-old than those vaccinated at 7 days-old with un-vaccinated chicks intermediate (Table 9.b). These findings indicated that the Egyptian vaccine increased the GOT level in plasma at 28 day of age and the creatinine level in plasma at 42 days of age which might disturb liver and kidney functions at these ages. However, in general all contents were within the normal levels. The information regarding blood biochemical changes in response to AI vaccination in the literature is scarce. Therefore, limited discussion is available for these traits. Egbal *et al.* (2007) studied the sero-biochemical changes of infected chicks with AI virus. Their results confirmed the biochemical evidence of liver and kidneys damage of chickens infected with highly pathogenic strains of AI virus. These results were in fairly good accord with the statement of Swayne and Halvorson (2003) that highly pathogenic AI produced a variety of edematous, hemorrhagic and necrotic lesions in visceral organs.

Elevation of serum GOT and GPT suggested pathologic involvement of liver and this was supported by the macroscopic lesions observed in infected chickens. Since the liver is considered to be the major source of plasma proteins, damage to this organ leads to decrease of total protein. These changes were correlated with the changes in serum enzymes originating in the liver. On the other hand, the reduction in albumin level in their study was due to damage in the liver and kidneys by the highly pathogenic AI virus.

Table 8.a. Effect of different Avian Influenza (AI) vaccination on liver function (plasma GOT=AST "Glutamyl oxaloacetic transaminase=Aspartate aminotransferase" and GPT=ALT "Glutamyl pyruvic transaminase=Alanine aminotransferase"; mg/dl) of broiler chickens at 14, 28 and 42 d of age.

Item	GOT, mg/dl			GPT, mg/dl		
	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d
Control	32.7	32.7[ab]	33.3	39.5	43.6	44.5
Vaccination at one-day-old						
AI-H5N1	31.5	32.1[ab]	32.4	41.6	44.2	43.6
AI-H5N2	31.8	33.4[ab]	31.8	44.2	43.6	42.7
AI + Newcastle Disease	30.9	33.6[ab]	32.4	43.3	45.1	42.7
Egyptian AI	31.5	34.8[a]	33.0	42.4	42.4	43.3
Vaccination at 7 d of age						
AI-H5N1	31.5	33.9[ab]	31.5	43.6	43.0	44.5
AI-H5N2	30.7	33.6[ab]	33.4	44.5	43.9	43.6
AI + Newcastle Disease	32.4	31.8[b]	31.8	42.7	45.1	44.2
Egyptian AI	31.5	32.1[ab]	31.8	42.5	45.1	43.9
SEM[1]	0.74	0.63	0.68	1.64	0.98	0.82
Probability[2]	0.6390	0.0289	0.4131	0.5632	0.4890	0.6999

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.
[1] Standard error of the mean (number of replicates with 5 samples per each replicate).
[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).

Table 8.b. Effect of Avian Influenza (AI) vaccination program on liver function (plasma GOT=AST "Glutamyl oxaloacetic transaminase=Aspartate aminotransferase" and GPT=ALT "Glutamyl pyruvic transaminase=Alanine aminotransferase"; mg/dl) of broiler chickens at 14, 28 and 42 d of age.

Item	GOT, mg/dl			GPT, mg/dl		
	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d
Overall mean	31.6	33.1	32.4	42.7	44.0	43.7
SE[1]	0.24	0.23	0.23	0.54	0.33	0.26
Control	32.7	32.7	33.3	39.5	43.6	44.5
Vaccination at one-day-old	31.5	33.5	32.4	42.9	43.8	43.1
Vaccination at 7 d of age	31.5	32.9	32.1	43.3	44.3	44.1
SEM (n=3)[2]	0.72	0.69	0.68	1.58	0.99	0.77
SEM (n=12)[2]	0.36	0.35	0.34	0.79	0.50	0.39
Probability[3]	0.2867	0.3998	0.3219	0.1004	0.7498	0.1256

[1] Standard error (27 replicates of 5 samples per each replicate).
[2] Standard error of the mean (number of replicates with 5 samples per each replicate).
[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).
No significant differences were observed among groups within column.

Table 9.a. Effect of different Avian Influenza (A.I) vaccination on kidney function (plasma creatinine and urea; mg/dl) of broiler chickens at 14, 28 and 42 d of age.

Item	Creatinine, mg/dl			Urea, mg/dl		
	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d
Control	2.37	2.40	2.47[ab]	11.97	11.39	12.01
Vaccination at one-day-old						
AI-H5N1	2.58	2.56	2.03[b]	11.82	11.57	11.80
AI-H5N2	3.08	1.97	3.28[ab]	11.61	11.32	11.75
AI + Newcastle Disease	2.50	2.69	2.58[ab]	11.75	11.43	11.58
Egyptian AI	2.46	2.56	2.83[a]	11.99	11.03	12.49

Table 9a. cont.

Vaccination at 7 d of age						
AI-H5N1	2.37	2.13	2.53[ab]	12.39	11.34	12.14
AI-H5N2	2.43	2.62	2.34[ab]	12.37	11.03	12.19
AI + Newcastle Disease	2.37	2.12	2.43[ab]	12.51	10.75	12.24
Egyptian AI	2.71	2.53	2.62[ab]	12.32	10.91	12.20
SEM[1]	0.203	0.239	0.134	0.280	0.230	0.254
Probability[2]	0.2742	0.3384	0.0111	0.2407	0.2117	0.2826

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.
[1] Standard error of the mean (number of replicates with 5 samples per each replicate).
[2] One-way analysis of variance with 9 groups (control + 2 vaccination ages x 4 types of vaccines).

Table 9.b. Effect of Avian Influenza (AI) vaccination program on kidney function (plasma creatinine and urea; mg/dl) of broiler chickens at 14, 28 and 42 d of age.

Item	Creatinine, mg/dl			Urea, mg/dl		
	At 14 d	At 28 d	At 42 d	At 14 d	At 28 d	At 42 d
Overall mean	2.54	2.40	2.46	12.08	11.19	12.04
SE[1]	0.069	0.081	0.051	0.096	0.079	0.087
Control	2.37	2.40	2.47	11.97[ab]	11.39	12.01
Vaccination at one-day-old	2.65	2.44	2.43	11.79[b]	11.34	11.91
Vaccination at 7 d of age	2.47	2.35	2.48	12.40[a]	11.01	12.19
SEM (n=3)[2]	0.207	0.246	0.154	0.266	0.231	0.258
SEM (n=12)[2]	0.104	0.123	0.077	0.133	0.116	0.129
Probability[3]	0.3212	0.8594	0.9077	0.0078	0.0965	0.2932

Means followed by a common letter in the same column are not significantly different at 0.05 level of probability.
[1] Standard error (27 replicates of 5 samples per each replicate).
[2] Standard error of the mean (number of replicates with 5 samples per each replicate).
[3] One-way analysis of variance with 3 treatments (control + 2 vaccination ages).

This was supported by the findings of Ley *et al.* (1983) who mentioned that the decreased serum albumin concentration had been correlated with liver necrosis and increased filtration and excretion by the kidneys. Another possibility of decreased total protein is due to reduction in food intake due to anorexia. In addition, they also reported a significant increase in creatinine level of infected broiler chickens and a decrease in urea concentration of two infected layer farms and attributed that to the impair occurred in kidney functions. The other hypothesis of the decreased urea concentration in sera of infected chickens may be attributed to decreases in protein (feed) intake.

Conclusion

During the experimental period, results indicated that neither different vaccines nor vaccination ages against AI virus affected performance, blood biochemical constituents at most ages in broiler chickens. This study also revealed that, H5N2 and H5N2+ND vaccines were more preferable than H5N1 or the Egyptian-H5N1 vaccines for broiler chichens. This was indicated by the geometric mean HI titers against AI virus of broiler chicks (more vs. less than 5 $\log_2$, respectively). However, no significant differences were detected among the vaccinated chicks at 1 or 7 days of age in their HI titers against AI virus at all ages studied. Thus it is recommended to use either the H5N2 or H5N2+ND vaccines than H5N1 or the Egyptian-H5N1 vaccines for Hubbard broiler flocks derived from breeders repeatedly immunized with a commercial inactivated H5N1 vaccine.

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Improving growth, carcass and meat composition traits by applying bio-techniques in crossbreeding project between Ardi Saudi and Damascus goats

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Abstract

A crossbreeding program between Ardi Saudi goats (A) with Syrian goats (Damascus, D) was started in 2006 in two experiments (Jouf and Qassim) applying bio-techniques of estrous synchronization and artificial insemination. Breeding does of Ardi goats were randomly divided into two groups and each group was subdivided into two subgroups to be inseminated artificially from semen of elite bucks of the same breed and of Damascus breed. Crossbred does of ½D½A were backcrossed with Damascus bucks to get the genetic group of ¾D¼A and such breeding plan permitted to produce four genetic groups of AA, DD, ½D½A and ¾D¼A in each experiment separately. Data collected were: 1) live body weights and gains at birth and biweekly thereafter up 24 weeks of age, 2) carcass traits, and 3) meat compositions involving dry matter, crude protein, ether extract, and ash. The animal models were used to estimate heritabilities and common litter environmental effects, while a generalized least square procedure was used to estimate direct additive genetic effects $(G^I = G_A^I - G_D^I)$, direct heterosis (HI), maternal heterosis (HM) and direct recombination effects (RI). Heritabilities were moderately heritable and ranging from 0.12 to 0.36 for body weights, 0.11 to 0.25 for body gains, and 0.10 to 0.34 for carcass traits. All estimates of direct additive effects for body weights at 0, 4, 8, 12, 16, 20 and 24 weeks of age were significantly in favour of Damascus goats by 0.260, 1.504, 1.337, 1.505, 1.324, 1.873 and 2.080 kg in Jouf experiment and by 0.548, 1.876, 2.168, 2.975, 2.739, 3.594 and 4.046 kg in Qassim experiment, respectively. The respective estimates for body gains at interval of 0-4, 4-8, 8-12, 12-16, 16-20, and 20-24 weeks of age were also significantly in favour of Damascus goats by 47, 31, 7, 30, 33, and 28 g in Jouf experiment and by 47, 35, 22, 42, 18, and 32 g in Qassim experiment. The estimates for most carcass traits were moderate and significantly in favour of Damascus kids by 6.2 kg for pre-slaughter weight, and 4.2 kg for hot carcass weight, while the estimates for meat compositions traits were somewhat low and in favour of Damascus kids relative to Ardi kids by 4.4% in dry matter of the lean and 3.4% in ether extract. The heterotic increments were mostly significant and ranging from 3.0 to 11.4 % for body weights and daily body gains and 2.4 to 28.1 % for carcass traits relative to the parental purebreds, while the estimates for meat composition traits were mostly non-significant although crossbred have shown reductions in moisture, ether extract and ash in meat of the carcass. The maternal heterotic increments in body weights and daily body gains were mostly significant and the estimates ranged from 1.0 to 27.0 %. Direct recombination losses were of little importance for the majority of the traits and they were generally lower than the estimates of direct heterosis.

Keywords: Ardi Saudi Goats, Damascus goats, crossbreeding, bio-techniques, growth, carcass, meat compositions, additive and heterotoc effects.

Introduction

In Saudi Arabia, Ardi, Hibsi and Zumri are the most common breeds. Unfortunately, the genetic knowledge for these breeds is limited. Ardi goat is the best local breeds in Saudi Arabia, heaviest live weight 34.4 kg; faster body gain or growth rate of 149 g/day and milk yield of 160.3 kg/lactation (Dosari *et al.* 1996). Ardi goats could record higher production than the other local breeds under the same conditions and therefore more attention should be paid to this breed. Comparable to these native breeds, other exotic goat breeds like Damascus (Damascus) goats have shown more productivity. In this concept, Damascus breeds are normally dual purpose (meat and milk), and better adapted to the harsh climate. Performance of Damascus goats could record an average daily gain from birth to weaning at 80 days to be 174 and 157 g/day for male and females kids, respectively (ACSAD, 1996). In order to improve the productivity of goats in Saudi Arabia, there is a great deal to increase the productivity of native goat breeds through crossbreeding programs. In the last two decades, new assisted reproductive technologies in goats had been applied successfully (Lazaris *et al*, 2002; Wheeler *et al*, 2003; Baldassarre and Karatzas, 2004). In this concept, artificial insemination and estrus synchronization could be used as new powerful and successively tools in planned breeding programmes to increase the rates of genetic improvement in goats (Thibier, 1996; Leboeuf *et al.*, 2000, 2003). The main objectives of the present study were: (1) To characterize genetically the native

Ardi breed of goats, (2) Improving the productivity of such breed for meat production through crossbreeding, and (3) Applying updated technologies of estrous synchronization and artificial insemination to shorten the generation interval in such genetic improvement program.

Materials and methods

Project Breeding Plan

A crossbreeding program between Ardi Saudi goats (A) with Damascus Syrian goats (D) was started in 2006 in Saudi Arabia in two experiments of Jouf station and Qassim University. Breeding does of Ardi goats were randomly divided into two groups, 120 doe each in each experiment. Each group of Ardi does was subdivided into two subgroups to be inseminated artificially from semen of elite bucks of the same breed and of Damascus breed. In both experiments, does of Damascus breed were randomly inseminated from bucks of the same breed to produce purebred kids. Also, crossbred does of ½D½A were backcrossed with Damascus bucks to get the genetic group of ¾D¼A. Accordingly, the breeding plan permitted to produce four genetic groups of AA, DD, ½D½A and ¾D¼A in each experiment separately. The semen of bucks was randomly assigned to inseminate the does. For different genetic groups, a total number of 1400 kids fathered by 115 sires and mothered by 516 dams were obtained.

Management and feeding

All does were housed in semi-shaded/open front barn and ear-tagged. Goats were fed on a commercial concentrate and alfalfa hay. The amount of concentrate and hay were calculated according to the nutritional requirements for goats which dependent on animal ages and production status. Water, straw, salt and minerals supplemented in blocks were freely available to all animals. Animals were fed *ad libitum* individually.

Semen collection, evaluation and preparation for artificial insemination

A total number of 1800 ejaculates collected by artificial vagina from 298 bucks were evaluated for semen characteristics. Immediately after collection, the semen tubes were placed in a water bath at 37 °C and samples were evaluated for consistency and examined microscopically for individual motility, sperm concentration and percent of normal spermatozoa. All these steps were done within 10 min of collection using the standard techniques. Semen with good quality were extended with extenders using dilution rate of 1:15 to 1:20 (semen:diluent) according to sperm concentration. The diluted semen samples were gradually cooled and stored in a refrigerator at 5 °C to be used in artificial insemination (Azawi *et al.*, 1993).

Estrous synchronization and artificial insemination (AI)

Estrous synchronization was applied using intravaginal progesterone impregnated sponges containing 30 or 45 mg fluorogesteone acetate (FGA) where the sponges were administered to does and maintained in situ for 14 days. Forty eight to seventy two hours before sponge withdrawal 200-1000 IU/eCG was injected intramuscular. Artificial insemination (AI) was done 48 and/or 60 hours after sponge removal, using chilled diluted semen (0.5 ml containing at least 150-200x10^6 motile spermatozoa). Three types of extenders of milk, Na Citrate and Tris were used as based extenders for semen dilution. AI was carried out using insemination pipette and vaginal speculum. The hind legs of the doe was lifted and placed at an angle of 45° to the horizontal railing. The vaginal speculum was introduced into the vaginal passage and the cervix was located with the help of light and by gentle sideways or downward manipulation of the speculum. Semen was deposited up to a depth of 5-10 mm into the cervix (Cervical insemination). Pregnancy diagnosis was applied 30-45 days post insemination with the aid of ultrasound scanner.

Growth, slaughter and meat composition traits:

Live body weights were recorded at birth and biweekly thereafter at up to 24 weeks of age, while daily weight gains were computed at four weeks intervals. At 6 months of age, kids of Qassim experiment of the entire genetic groups were randomly taken for slaughtering test. Kids were slaughtered and dissected and hot carcasses were weighed and dressing percentages were calculated. The head, fur, giblets (representing heart + liver + kidneys + spleen) and viscera of the carcasses were also weighed. A sample of the lean were taken from each animal and chemically analysed. Dry matter (using an air-evacuated oven for 16 h), crude protein (N × 6.25), ether extract (EE) and ash in the lean were determined according to the AOAC (1990).

Models of statistical analysis

For genetic analysis, data of each experiment were analysed separately. Variances calculated by SAS program applying REML procedure (SAS, 1999) were used as starting values in the analyses of single-trait animal model.

Data of individual body weights and gains were analysed applying such single-trait animal model (Boldman *et al*, 1995):

$$\mathbf{y = Xb + Z_a u_a + Z_c u_c + e} \qquad \textbf{(Model 1)}$$

Where **y** = vector of observed growth trait for kids, **b**= vector of fixed effects (genetic group, sex, year-season, litter size in which the kid was born); u_a= vector of random effect of the kid; u_c= vector of random effects of non-additive common litter effects; X, Z_a and Z_p are the incidence matrices

relating records to the fixed effects, additive genetic effects, and permanent environment, respectively; and **e**= vector of random error.

Data of carcass traits and meat compositions were analysed applying the following single-trait animal model (Boldman *et al*, 1995):

$y= Xb + Z_a u_a + Z_c u_c + e$ (Model 2)

Where y = vector of observed growth trait for kids, b= vector of fixed effects (genetic group, year-season, age of kid); u_a= vector of random effect of the kid; u_c= vector of random effects of non-additive common litter effects; X, Z_a and Z_c are the incidence matrices relating records to the fixed effects, additive genetic effects, and common litter effects, respectively; and **e**= vector of random error.

The inverse of the numerator relationship matrix **(A^{-1})** was considered; Var(a)= $A\sigma^2_a$, Var(p)= $I\sigma^2_p$, Var(c)= $I\sigma^2_c$ and Var(e)= $I\sigma^2_e$. Inbreeding coefficients for progeny, sires and dams were calculated using program of Boldman *et al*. (1995). Heritabilities for different traits were computed by DFREML of the animal model using the following equations:

$$h^2_A = \frac{\sigma^2_A}{\sigma^2_A + \sigma^2_c + \sigma^2_e}$$ where σ^2_A, σ^2_c, and σ^2_e are variances due to the effects of direct additive effect, common litter environment, and random error, respectively.

Genetic model and estimation of crossbreeding effects

The procedure of generalized least squares (GLS) using CBE program of Wolf (1996) was used to estimate crossbreeding effects. Model of Dickerson was used in the program as summarized by Dickerson (1992) and Wolf *et al* (1995). The linear model used was:

$y = Xb + e$, $Var(y) = V$

Where **y** = vector of genetic groups means, **X** = incidence matrix of the coefficients for crossbreeding effects, **b** = vector of crossbreeding genetic parameters, **e** = vector of residual effects, and **V** = full covariance matrix of **y**. The coefficients relating genetic crossbreeding parameters to the means of the genetic groups are shown in Table 1 (Wolf *et al*., 1995). Because the reciprocal cross, Ardi x Damascus, was not carried out, the maternal additive effects showed a high co-linearity with the direct additive effects where the corresponding errors highly correlated. For this reason, the maternal additive effects have been excluded from the model and the estimates of the direct additive effects must be interpreted as a balance between direct and maternal additive effects. Differences between breeds in terms of direct additive genetic effects $(G^I = G^I_A - G^I_D)$, direct heterosis (H^I) and maternal heterosis (H^M) were estimated using the CBE program of Wolf (1996). Thus, we have three parameters to be estimated (a vector called b-vector):

$$b = \left[(G^I_A - G^I_D) \quad H^I \quad H^M\right]$$

The estimates of **b** were calculated by the method of generalized least squares (GLS) using the following equation: $\hat{b} = (X'V^{-1}X)^{-1}X'V^{-1}y$

Table 1. Genetic groups of kids with their sires and dams and coefficients of the matrix relating genetic group means of kids with crossbreeding parameters.

Genetic group				Mean	Coefficients of the matrix				
Kid	Sire	Dam	Grand-dam		D_A	D_D	H^I	H^M	R^I
AA	A	A		1	1	0	0	0	0
DD	D	D		1	0	1	0	0	0
½D½A	D	A		1	.5	.5	1	0	0
¾D¼A	D	½D½A	A	1	.5	.5	.5	1	0.25

D_A and D_D = Direct additive genetic effects for the Ardi breed and the Damascus breed, respectively; H^I = Direct heterosis; H^M = Maternal heterosis; R^I= Direct recombination genetic loss.

Results and discussion

Actual means and variations

Results presented in Tables 2 and 3 describe kid growth performance in this project. However, wide phenotypic variations in all traits were observed. The average individual weights in Jouf experiment were nearly similar to those in Qassim experiment. These lower body weights present a unique opportunity for changing performance while exploiting additive genetic variance and variability within population with the use of familiar methods such as selection based on individual or family merit. Comparing body weights obtained in the present study with those of the large European and Boer breeds that exceed 90 kg in body weight (Warmington and Kirton, 1990) led to the conclusion that European breeds have considerable potential in crossbreeding and formation of composite population for the commercial production of meat from goats (Shrestha, 2005; Shrestha and Fahmy, 2007b).

Results given in Table 3 describe the performance of carcass and meat composition traits. Carcass and meat composition traits were changed markedly with the animal's age or the weight at slaughter.

Table 2. Actual means, standard deviations (SD) and ranges for growth traits of kids in Jouf and Qassim experiments

Trait	Jouf experiment					Qassim experiment				
	No. of Records	Mean	SD	Min.	Max.	No. of Records	Mean	SD	Min.	Max.
Body weight (kg):										
0-week	974	3.89	0.90	0.85	6.75	423	3.37	0.72	1.80	5.80
4-week	786	8.65	2.15	3.25	17.94	356	7.23	1.98	3.00	15.85
8-week	681	12.04	3.29	5.00	24.65	326	10.67	3.01	5.20	22.90
12-week	641	15.21	4.15	5.55	27.75	299	13.86	3.77	6.00	27.30
16-week	620	18.17	4.66	5.50	34.50	275	16.62	4.14	7.40	30.90
20-week	634	20.62	5.02	8.40	42.00	263	19.17	4.78	9.50	33.41
24-week	603	23.35	5.33	8.60	45.75	249	22.11	5.32	11.00	36.40
Daily gain in weight (kg):										
0-4 weeks	786	0.17	0.07	0.00	0.43	351	0.14	0.06	0.02	0.44
4-8 weeks	666	0.13	0.07	0.01	0.49	322	0.12	0.06	0.02	0.29
8-12 weeks	612	0.12	0.06	0.00	0.43	294	0.12	0.06	0.01	0.32
12-16 weeks	594	0.11	0.07	0.00	0.79	270	0.10	0.06	0.00	0.61
16-20 weeks	587	0.12	0.13	0.00	1.08	254	0.09	0.05	0.01	0.29
20-24 weeks	572	0.11	0.08	0.00	1.22	238	0.11	0.05	0.01	0.40

Table 3. Actual means, standard deviations (SD) and ranges for carcass traits and meat compositions in Qassim experiment

Trait	Mean	SD	Minimum	Maximum
Carcass traits:				
Pre-slaughter weight, kg	26.455	6.170	22.345	42.650
Hot carcass weight, kg	12.561	3.048	11.2	20.6
Dressing percentage	47.4	2.6	41.5	54.0
Giblet weight, kg	0.739	0.156	0.604	1.071
Chemical composition of meat:				
Moisture (MP), %	75.3	11.4	72.4	78.2
Dry matter (DM), %	24.7	1.4	22.2	27.4
Crude protein (CP), %$^{+}$	78.8	3.7	76.2	80.4
Ether extract (EE), %$^{+}$	16.8	4.0	12.8	18.2
Ash content, %$^{+}$	4.3	0.7	3.6	5.4

Giblet = heart + liver + kidneys + spleen.
$^{+}$ On dry matter basis.

The average pre-slaughter weight of the kids of this project was 26.455 kg and ranging from 22.345 to 42.650 kg. Hot carcass weight ranged from 11.2 to 20.6 k*g* with an average of 12.561 k*g*. Reviewed estimates for carcass traits for the same breed indicated that carcass weights are varied from one country or region to another. The dressing out percentage averaged 47.4% across all genetic groups (Table 3). This percentage is higher to that recorded by El-Gallad *et al.* (1988) in Egypt for Zaraibi goats. They found that dressing percentage in Zaraibi goats ranging from 39.4 to 46.4, with an average value of 44.2%. For meat compositions, the results gave an impression to that kids' meat could be appreciated for its high nutritional and dietetic properties since ether extract in the lean was low (16.8 %) and protein content was high (78.8%) on dry matter basis.

Additive and common litter environmental effects

Estimates of heritability for body weights and gains are moderately heritable in most cases; ranging from 0.12 to 0.36 for body weights and from 0.12 to 0.24 for gains in weight in Jouf experiment and from 0.15 to 0.26 and from 0.11 to 0.25 in Qassim experiment, respectively (Table 4). From the genetic point of view, moderate genetic potentiality could be achieved in body weights or gains through selection. Numerous studies on genetic parameters have shown that body weights and gains are moderately heritable suggesting considerable potentiality for genetic improvement of meat goats (Bigham et al, 1993; Gebrelul *et al*, 1994; Tahir *et al*, 1995; Roy *et al*, 1997; Mourad and Anous, 1998; Fahmy and Shrestha, 2000; Shrestha and Fahmy, 2007b). Heritability estimates observed in this study are within the range of those reported for tropical goats, e.g. Mavrogenis et al. (1984a,b) for Damascus goats; Das *et al*. (1994,2001) for Tanzanian blended goats; Al-Shorepy et al. (2002) for Emirati goats; Portolano *et al.* (2002) for Silican Girgentana goats, and Mugambi *et al* (2006) for native goats in Kenya. Niekerk et al (1996) and Mourad and Anous (1998) reported similar results in Adelide Boer and common African and Alpine crossbred goats, respectively.

Nahardeka *et al*. (2001) reported moderate heritability estimates for body weights at 6, 9 and 12 months in Assam local goats and crosses with the Beetal breed. Mugambi *et al* (2006) found that heritability estimates were 0.13, 0.16, 0.16, 0.24 and 0.10 for birth weight, weaning weight, yearling weight, pre-weaning and post-weaning average daily gains, respectively. Common litter effects for most growth traits were slightly higher than the respective heritabilities since the estimates for body weights and daily gains in weight ranged from 0.08 to 0.20 and 0.11 to 0.34 in Jouf experiment and from 0.07 to 0.20 and 0.08 to 0.14 in Qassim experiment (Table 4). It is important to say that common litter effects appeared to have strong effects on kid's growth even up to late age. In Boer goats and using a comparable model, Schoeman *et al*. (1997) reported lower heritability to be 0.18 and common litter effects to be 0.07. However, Al-Shorepy *et al* (2002) reported that inclusion of maternal litter environmental effects provided reasonable estimates of heritability for growth traits since the estimates of heritability ranged from 0.16 to 0.32 for body weight traits, and 0.09 to 0.42 for daily body gains. Heritabilities given in Table 5 for pre-slaughter weight (0.26) and hot carcass weight (0.34) were moderate, while the estimate for dressing percent was low (0.10). All meat composition traits were not significantly different from zero (Table 5). Unfortunately, little available literature was reported on heritabilities of carcass traits and meat composition of carcass (Fahmy and Shrestha, 2000; Shrestha and Fahmy, 2007b).. The common litter effects for carcass traits and meat compositions were always higher than their respective heritabilities and they ranged from 0.16 to 0.23 for carcass traits and from 0.02 to 0.12 for meat compositions (Table 5), suggesting that common litter effects appeared to have strong effects on kid's growth even up to late age. The common litter effects were low but significantly different from zero for moisture, dry matter and ash contents in the lean.

Crossbreeding effects

Direct additive genetic effects

In both experiments, all the estimates of direct additive effects for body weights at 0, 4, 8, 12, 16, 20 and 24 weeks of age were significantly positively in favour of Damascus goats by 0.260, 1.504, 1.337, 1.505, 1.324, 1.873 and 2.080 kg in Jouf experiment and by 0.548, 1.876, 2.168, 2.975, 2.739, 3.594 and 4.046 kg in Qassim experiment, respectively (Table 6). For daily gains in weight, the same trend was observed where the estimates of direct additive effects for gains at interval of 0-4, 4-8, 8-12, 12-16, 16-20, and 20-24 weeks of age were significantly positively in favour of Damascus goats by 47, 31, 7, 30, 33, and 28 g in Jouf experiment and by 47, 35, 22, 42, 18, and 32 g in Qassim experiment, respectively (Table 6). The differences in direct additive effects obtained here for body weights and gains between the two breeds lead to state that Damascus goats could be used in crossbreeding programmes in Saudi Arabia and other hot climatic countries. Mugambi *et al* (2006) with crossing Toggenburg (T), Anglo-Nubian (N), Small East African (E) and Galla (G) breeds found that superiorities of exotic bucks over the indigenous breeds in weaning weight, daily gain in weight and yearling weight were in line with heavy mature body weights and high milk yield in their crosses, which provide favourable maternal influence to the kids.

In both experiments, all estimates of direct additive effects for growth traits were significantly high and in favor of Damascus goats by 7.7 to 18.0 % for body weights and 6.0 to 26.3 % for daily body gains in Jouf experiment relative to the founder breeds, while the respective estimates ranged from 16.7 to 25.3 % and from 18.0 to 38.8 % in Qassim experiment (Table 6). The positive additive effects of the Damascus breed in average body weights and daily gains indicate that its effects are of maternal origin that is expressed in their crosses during nursing period after which they are unable to express their potential under harsh environmental conditions. Mugambi *et al* (2006) in Kenya stated that the positive effect of Galla native goat above exotic breeds in birth weight and post-weaning average daily gains reflects the ability of the breed to survive in harsh environmental conditions and poor quality feeds, during gestation.

Table 4. Proportions of the phenotypic variance due to genetic additive effects (h^2) and to common litter non-additive effects (C^2) and random error (e^2) with their standard errors (±SE) for growth traits in Jouf and Qassim experiments

Trait	Jouf experiment			Qassim experiment		
	h^2±SE	C^2±SE	e^2±SE	h^2±SE	C^2±SE	e^2±SE
Body weight:						
0-week	0.20±0.082	0.20±0.045	0.60±0.070	0.15±0.129	0.20±0.078	0.65±0.102
4-week	0.31±0.099	0.11±0.037	0.58±0.090	0.21±0.186	0.12±0.104	0.68±0.114
8-week	0.31±0.125	0.12±0.046	0.47±0.090	0.21±0.179	0.11±0.108	0.68±0.111
12-week	0.22±0.023	0.09±0.047	0.67±0.025	0.22±0.118	0.11±0.093	0.67±0.176
16-week	0.12±0.093	0.15±0.055	0.73±0.083	0.26±0.161	0.10±0.109	0.64±0.100
20-week	0.36±0.017	0.08±0.053	0.56±0.019	0.15 ± 0.165	0.10 ± 0.093	0.75 ± 0.134
24-week	0.24±0.111	0.12±0.059	0.64±0.095	0.19 ± 0.189	0.07 ± 0.098	0.74 ± 0.151

Table 4. cont.

Daily gain in weight:						
0-4 weeks	0.19±0.084	0.11±0.044	0.70±0.077	0.19±0.098	0.13±0.134	0.68±0.166
4-8 weeks	0.18±0.128	0.11±0.054	0.71±0.106	0.14±0.120	0.14±0.122	0.72±0.166
8-12 weeks	0.12±0.082	0.12±0.045	0.76±0.086	0.19±0.171	0.12±0.087	0.69±0.140
12-16 weeks	0.24±0.085	0.21±0.053	0.55±0.043	0.21±0.145	0.12±0.111	0.67±0.186
16-20 weeks	0.21±0.069	0.26±0.059	0.53±0.061	0.25±0.174	0.08±0.099	0.67±0.146
20-24 weeks	0.20±0.078	0.34±0.066	0.46±0.055	0.11±0.182	0.10±0.114	0.79±0.150

Table 5. Proportions of the phenotypic variance due to genetic additive effects (h^2) and to common litter non-additive effects (C^2) and random error (e^2) with their standard errors (±SE) for some carcass and meat composition traits in Qassim experiment

Trait	h^2±SE	C^2±SE	e^2±SE
Carcass traits:			
Pre-slaughter weight, kg	0.26±0.051	0.22±0.029	0.52±0.018
Hot carcass weight, kg	0.34±0.054	0.23±0.028	0.43±0.021
Dressing percentage	0.10±0.047	0.16±0.029	0.74±0.023
Giblet weight, kg	0.32±0.062	0.18±0.024	0.50±0.022
Meat chemical composition			
Moisture (MP), %	0.02±0.024	0.08±0.023	0.90±0.022
Dry matter (DM), %	0.02±0.022	0.10±0.025	0.88±0.024
Crude protein (CP), %	0.02±0.019	0.02±0.031	0.96±0.023
Ether extract (EE), %	0.02±0.018	0.02±0.023	0.97±0.025
Ash content, %	0.01±0.001	0.12±0.027	0.86±0.026

Table 6. Estimates of direct additive effects and their standard errors (D^I±SE) for body weight and daily body gains in Jouf and Qassim experiments

Trait	Jouf experiment $D^I=(D^I_D-D^I_A)$			Qassim experiment $D^I=(D^I_D-D^I_A)$		
	Estimate	SE	D^I %[+]	Estimate	SE	D^I %[+]
Body weight (*kg*):						
0-week	0.260*	0.124	7.8	0.548**	0.162	16.7
4-week	1.504**	0.316	18.0	1.876**	0.480	25.3
8-week	1.337**	0.434	11.0	2.168**	0.82	21.0
12-week	1.505**	0.252	10.2	2.975**	1.086	21.4
16-week	1.324**	0.378	7.7	2.739**	1.513	17.0
20-week	1.873**	0.482	9.5	3.594**	1.54	18.7
24-week	2.080**	0.434	9.0	4.046**	1.645	18.0
Daily body gains (*kg*):						
0-4 weeks	0.047**	0.006	26.3	0.047**	0.004	32.3
4-8 weeks	0.031**	0.005	19.0	0.035**	0.010	30.0
8-12 weeks	0.007*	0.004	6.0	0.022**	0.005	18.0
12-16 weeks	0.030**	0.008	22.0	0.042**	0.004	38.8
16-20 weeks	0.033**	0.006	22.2	0.018**	0.002	19.1
20-24 weeks	0.028**	0.005	18.7	0.032**	0.004	26.4

[+] D^I%= [D^I in units / (average of purebreds)] x 100.
*= P<0.05; **= P<0.01.

With cross-bred kids between Alpines and Nubian breeds, Gebrelul *et al*. (1994) reported estimates of direct additive effects of 0.24, 1.9 and 19.8 kg for birth weight, weaning weight and daily gain in weight, respectively. In most cases, the estimates of direct genetic effects for carcass traits were significant and in favour of Damascus kids by 6.2 kg for pre-slaughter weight, 4.2 kg for hot carcass weight, 4.8 % for dressing out percentage, and 0.173 kg for giblet weight (Table 7). In the corresponding percentages relative to the average of parents, direct genetic effects were moderate and in favour of Damascus kids by 22.1, 29.1, 10.2, and 28.1 %, respectively. Also, the estimates for meat compositions traits were somewhat low and in favour of Damascus kids (Table 7). Damascus kids had slightly more direct additive effects for dry

matter content in the lean by 4.4% and ether extract by 3.4% than for Saudi Ardi kids.

Table 7. Estimates of direct additive effects and their standard errors (D^I±SE) for carcass and meat composition traits in Qassim experiment

Trait	$D^I=(D^I_D-D^I_A)$		
	Estimate	SE	D^I %+
Carcass traits:			
Pre-slaughter weight, kg	6.2**	0.74	22.1
Hot carcass weight, kg	4.2**	1.6	29.1
Dressing percentage	4.8**	1.2	10.2
Giblet weight, kg	0.173**	0.063	28.1
Meat chemical composition:			
Moisture (MP), %	0.14	0.16	0.1
Dry matter (DM), %	1.18	0.68	4.4
Crude protein (CP), %	0.32	0.44	0.4
Ether extract (EE), %	0.62	0.42	3.4
Ash content, %	0.16	0.28	3.5

+ D^I%= [D^I in units / (average of purebreds)] x 100.
**= P<0.01.

Direct (H^I) and maternal (H^M) heterosis

The actual heterotic increments in body weights of Jouf experiment were 0.205, 0.362, 0.729, 0.481, 0.497, 0.435 and 0.712 kg at 0, 4, 8, 12, 16, 20 and 24 weeks of age, while the respective significant heterotic increments in Qassim experiment were 0.212, 0.551, 0.711, 1.125, 1.497 0.797 and 0.875 kg, respectively (Table 8). For daily body gains, heterotic increments were 18, 55, 45, 60, 45, and 60 g in Jouf experiment and 15, 85, 40, 40, 40, 50 g in Qassim experiment during age intervals of 0-4, 4-8, 8-12, 12-16, 16-20 and 20-24 weeks, respectively (Table 8). The estimates for direct heterosis reported here for growth traits are lower than those reported by Gebrelul *et al.* (1994) who found estimates of 0.24, 1.9 and 19.8 kg for birth weight, weaning weight and daily gain between birth and weaning, respectively, in cross-bred kids between Alpines and Nubian breeds. However, El Fadili and Leroy (2001) reported low estimate of heterosis for weaning weight (0.03 kg) in crosses between two indigenous Moroccan breeds. This may be caused by loss of co-adaptive gene combinations in the crossbreds between divergent breeds or due to environmental conditions where the animals were reared. Mugambi *et al* (2006) found that direct heterosis had positive effects on birth weight (+0.05 kg), yearling weight (+0.36 kg) and post-weaning average daily gains (+3.04 g/day) but negative in pre-weaning traits. The study shows that the developed KDPG composites have not optimized on the positive dominance effects; an effect due to retained recombination loss caused by lack of selection during breed development. They concluded also that the KDPG composites are still segregating and have not stabilized into a new breed as was the aim of the breeding programme. In both experiments, all estimates of direct heterosis expressed as percentages relative to the founder breeds for all body weights and gains were positive and significant (Table 8). The estimates for body weights were ranging from 3.2 to 6.1 % in Jouf experiment and 3.9 to 9.3 % in Qassim experiment. For daily gains in weight, the estimates were ranging from 3.0 to 4.7 % in Jouf experiment and 3.7 to 11.4 % in Qassim experiment relative to the founder breeds. In practice, the positive and significant estimates of direct heterosis for growth traits (Table 8) indicate that the benefits of heterosis were realized after weaning when the favourable maternal common environmental effects are diminished.

Table 8. Estimates of direct heterosis and their standard errors (H^I±SE) for body weights and daily body gains in Jouf and Qassim experiments

Body weight	Jouf experiment			Qassim experiment		
	Units	SE	H^I %+	Units	SE	H^I %+
Body weight (*kg*):						
0-week	0.205*	0.049	6.1	0.212*	0.14	6.4
4-week	0.362*	0.059	4.3	0.551*	0.21	7.4
8-week	0.729*	0.071	6.0	0.711*	1.045	6.9
12-week	0.481*	0.086	3.2	1.125**	0.485	8.1
16-week	0.497*	0.103	4.3	1.497**	0.686	9.3
20-week	0.435*	0.099	3.7	0.797*	0.815	4.1
24-week	0.712*	0.132	3.6	0.875*	0.465	3.9
Daily weight gain (kg):						
0-4 weeks	0.018*	0.003	4.7	0.015**	0.002	7.2
4-8 weeks	0.055*	0.002	3.3	0.085**	0.005	7.2
8-12 weeks	0.045*	0.003	3.1	0.040**	0.008	11.4
12-16 weeks	0.060*	0.002	4.4	0.040*	0.006	3.7
16-20 weeks	0.045*	0.029	3.0	0.040*	0.002	4.2
20-24 weeks	0.060*	0.032	4.0	0.050*	0.001	4.1

+ H^I%= [H^I in units / (average of purebreds)] x 100.
*= P<0.05; **= P<0.01.

The actual maternal heterotic increments in body weights of Jouf experiment were 0.130, 0.202, 0.219, 0.288, 0.305, 0.300 and 0.554 kg at 0, 4, 8, 12, 16, 20 and 24 weeks of age, while the respective significant heterotic increments in Qassim experiment were 0.165, 0.859, 2.135, 1.421, 1.375, 1.286 and 1.298 kg, respectively (Table 9). For daily body gains, maternal heterotic increments during age intervals of 0-4, 4-8, 8-12, 12-16, 16-20 and 20-24 weeks were 7.5, 4.5, 6.5, 2.0, 3.5 and 3.0 g in Jouf experiment and 33.5, 31.5, 7.0, 15.0, 10.0, and 5.0 g in Qassim experiment, respectively (Table 9). In both experiments, all estimates of maternal heterosis for body weights and gains expressed as percentages relative to the founder breeds were positive, significant and ranging from 1.5 to 3.9 % in Jouf experiment and 5.0 to 20.7 % in Qassim experiment (Table 9). For daily gains in weight, the estimates were ranging from 1.4 to 5.6 % in Jouf experiment and 1.0 to 27.0 % in Qassim experiment. These moderate estimates of maternal heterosis for body weights and gains indicate that crossbred dams had moderate heterotic maternity over their purebred dams in these growth traits. Estimates of direct heterosis for most carcass traits were significant (Table 10). This notation implies that dominance effects on these traits were of considerable importance. Pre-slaughter weight, hot carcass weight, and giblet weight showed favorable positive estimates of direct heterosis of 1.35 kg, 0.8 kg, and 103 g, respectively. Crossbred kids showed considerable improvements in the carcass performance since the estimates of direct heterosis were mostly significant and ranging from 2.4 to 22.1 %. The estimates of direct heterosis for meat composition traits were mostly negative and non-significant (Table 10), although crossbred kids were associated with reductions in moisture, ether extract and ash in meat of the carcass.

Table 9. Estimates of maternal heterosis and their standard errors ($H^M \pm SE$) for body weights in Jouf and Qassim experiments

	Jouf experiment			Qassim experiment		
	Units	SE	H^M %$^+$	Units	SE	H^M %$^+$
Body weight (*kg*):						
0-week	0.130*	0.084	3.9	0.165*	0.032	5.0
4-week	0.202*	0.096	2.4	0.859**	0.045	11.6
8-week	0.219	0.092	1.8	2.135**	0.067	20.7
12-week	0.288	0.082	1.9	1.421**	0.092	10.2
16-week	0.305	0.136	1.7	1.375**	0.156	8.5
20-week	0.300	0.214	1.5	1.286*	0.234	6.7
24-week	0.554*	0.248	2.4	1.298*	0.267	5.7
Daily weight gain (kg):						
0-4 weeks	7.5*	0.8	4.2	33.5**	1.1	23.0
4-8 weeks	4.5*	0.9	2.7	31.5**	1.8	27.0
8-12 weeks	6.5*	0.7	5.6	7.0*	2.2	5.7
12-16 weeks	2.0	0.6	1.4	15.0**	2.6	13.8
16-20 weeks	3.5*	2.1	2.3	10.0	2.2	1.0
20-24 weeks	3.0	2.8	2.0	5.0*	1.2	4.1

$^+$ H^M%= [H^M in units / (average of purebreds)] x 100.
*= P<0.05; **= P<0.01.

Table 10. Estimates of direct heterosis and their standard errors ($H^I \pm SE$) for carcass and meat composition traits in Qassim experiment

Trait	Estimate	SE	H^I %$^+$
Carcass traits:			
Pre-slaughter weight, kg	1.35*	0.34	4.8
Hot carcass weight, kg	0.8*	0.13	5.5
Dressing percentage	1.15^{NS}	0.32	2.4
Giblet weight, kg	0.103*	0.051	22.1
Meat chemical composition:			
Moisture (MP), %	-0.6^{NS}	0.23	-0.7
Dry matter (DM), %	0.9^{NS}	0.36	3.3
Crude protein (CP), %	0.7^{NS}	0.56	0.8
Ether extract (EE), %	-0.45^{NS}	0.64	-2.5
Ash content, %	-0.27*	0.05	-6

$^+$ H^I%= [H^I in units / (average of purebreds)] x 100.
NS = Non-significant; *= P<0.05; **= P<0.01.

Direct recombination effects

The estimates of direct recombination losses in heterosis for growth traits were mostly not-significant (Table 11). These favourable estimates gave an impression to indicate that crossbred dams including Damascus genes could be effective to improve growth performance by crossing Damascus with native Saudi Ardi goats. Information in the literature concerning estimates of direct recombination effects for growth performance of crossbreeding experiments in goats are scarce and most of the available results are contradicted. Pre-slaughter weight, hot carcass weight, dressing percentage and offal weights showed losses of genetic recombination (Table 11). Also, the estimates of direct recombination effects for meat composition

traits were non-significant. For all traits, the estimates were mostly with different parameters relative to those estimates of direct heterosis. This notation implies that dominance effects on these traits were of little importance.

Table 11. Estimates of direct recombination effects (R^I) and their standard errors (SE) for growth performances in Jouf and Qassim experiments

Trait[+]	Direct recombination losses in units ± SE	
	Jouf experiment	Qassim experiment
Body weight (kg):		
0-week	0.032±0.013 ^{NS}	0.034±0.011 ^{NS}
4-week	0.035±0.048^{NS}	0.038±0.036^{NS}
8-week	0.059±0.056^{NS}	0.039±0.027^{NS}
12-week	0.068±0.089^{NS}	0.057±0.027^{NS}
16-week	0.067±0.056^{NS}	0.059±0.046^{NS}
20-week	0.194±0.034*	0.234±0.042*
24-week	0.384±0.045*	0.245±0.051*
Daily gain in weight (g/d):		
0-4 weeks	8.2±0.084 ^{NS}	5.6±0.076 ^{NS}
4-8 weeks	6.5±0.057^{NS}	2.8±0.043^{NS}
8-12 weeks	4.2±0.064^{NS}	3.2±0.064^{NS}
12-16 weeks	5.8±0.044^{NS}	6.8±0.041^{NS}
16-20 weeks	8.6±0.034*	6.2±0.028*
20-24 weeks	9.2±0.054*	7.2±0.034*
Carcass traits:		
Pre-slaughter weight, kg		0.198±0.076*
Hot carcass weight, kg		0.192±0.066*
Dressing percentage		2.34±0.72*
Giblet weight, kg		0.256±0.089*
Meat quality:		
Moisture (MP), %		-0.42±0.45 ^{NS}
Dry matter (DM), %		0.52±0.72 ^{NS}
Crude protein (CP), %		0.14±0.87 ^{NS}
Ether extract (EE), %		0.34±0.52 ^{NS}
Ash content, %		0.046±0.098 ^{NS}

Conclusions

1) Direct additive effects for most traits studied were in favour of Damascus breed relative to the Ardi breed.
2) Crossing Damascus breed with local goats in hot climatic countries could produce crossbred kids (**½D½A** and **¾D¼A**) with reasonable growth performances.
3) The favourable estimates of direct and maternal heterosis obtained for most growth traits would be an encouraging factor for the goat producers in hot climate countries to use crossbred does and dams on commercial scale.
4) Insignificant direct recombination losses for most growth traits conclude that epistatic recombination losses for these traits in crossbred kids were negligible, and therefore, there is a potential advantage to use crossbred dams and sires including Damascus genes to develop parental lines (maternal and paternal) having more available heterosis to be used in crossbreeding stratification systems in Saudi Arabia and the other hot Arabian countries.

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Antioxidant and Protective Effect of Total Extract of Black cumin (*Nigella Sativa L.)* seed on Carbontetrachloride Induced Rat Liver and kidney Injuries and lipid peroxidation.

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Abstract

The aim of this study was to investigate the protective effect of *Nigella Sativa L.* seed total extract (*N.S.T.E.)* against the injury of carbontetrachloride (CCl_4)-induced liver and kidney functions and lipid peroxidation in rats. The used treatments were: untreated (control), N.S.T.E. (control), CCl_4 (control), CCl_4 before N.S.T.E., CCl_4 with N.S.T.E. and CCl_4 after N.S.T.E. administration. CCl_4 administration induced elevation in serum alanine transaminase (ALT), aspartate transaminase (AST), lactic dehydrogenase (LDH), gama gutamyl transferase (G-GT), total bilirubin and alkaline phosphatase (ALP) levels and increased oxidative stress in the liver. Also, CCl_4 led to a significant increase in serum blood urea, creatinine and uric acid. Administration of N.S.T.E. after, with and before CCl_4 treatment decreased the harmful effect of CCl_4 and enhanced the activities of antioxidant enzymes. Also hematological parameters were increased comparing with CCl_4 group. Histopathological examination of tissues showed slight abnormalities in hepatic parenchyma and nephrotoxicity that were consistent with biochemical variations observed in CCl_4 treatment. Protective effects of N.S.T.E. were also evident on histopathology of liver anti brosis a nd kidney by reducing glomerular atrophy, tubular degeneration .It is suggested that N.S.T.E. effectively protect liver and kidneys against the CCl_4 -induced oxidative damage in rats, through antioxidant and free radical scavenging effects of flavonoids, alkaloids, terpenes, tannins and saponins present in the extract.

Key words: Nigella Sativa, carbontetrachloride, antioxidant, liver and kidney functions.

Introduction

Exogenous factors including sunlight, ultraviolet light, ionizing radiation, toxic chemicals and our body's normal cellular activities cause the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Among these reactive oxygen species (ROS) the superoxide anions, hydrogen peroxides and hydroxyl radicals are the most prominent (Cerutti, 1991). CCl_4 is one of the synthetic industrial chemicals that is used as a solvent and other synthetic products cause hepatotoxicity and nephrotoxicity in workers exposed to it as well as in experimental animals (Abraham *et al.,* 1999).Exposure to CCl_4 through inhalation, ingestion, skin absorption or intraperitoneal administration in experimental animals increases lipid peroxidation (Daniels et al., 1995; Abraham et al., 1999 and Khan *et al.,* 2009) and decreases total proteins, activity of antioxidant superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and phase II metabolizing enzymes protect lipid peroxidation in the kidney (Dogukan *et al.,* 2003 and Adewole *et al.,* 2007). Chronic carbontetrachloride (CCl_4) intoxication is a well-known model for producing oxidative stress and hepatic injury. Its biotransformation produces hepatotoxic metabolites, the highly reactive trichloromethyl free radical, which could be further converted to peroxytrichloromethyl radicals and induce oxidative damage in the cell (Williams and Burk, 1990). Herbal therapies are commonly used for the prevention and treatment of cancer, although we have little understanding of their molecular and cellular bases of action. *Nigella sativa*, or the black cumin seed, is a herb used in traditional medicine in many Middle Eastern and Asian countries to treat a broad array of diseases. Hepatoprotective effects of N.S.T.E. in carbontetrachloride (CCl_4)-induced liver fibrosis and renal disorders in rats for 8 weeks.*N.S.* relieves the deleterious effects of ischemia reperfusion injury on liver (Fahrettin *et al.*,2008). Anti-inflammatory effects of the N.S. seed extract, thymoquinone, in pancreatic cancer cells as a novel inhibitor of proinflammatory pathways provides a promising strategy that combines anti-inflammatory and proapoptotic modes of action (Chehl N. *et al*., 2009). The aim of this work was to study the protective effect of N.S.T.E. in carbon tetra chloride to examine the antioxidant and protective effect of N.S.T.E. in the rats administrated with CCl_4 induced rat liver and kidney injury.

Materials and Methods

Plant Materials

All Seeds of Turkey one N.S. *L.* (black cumin) were chosen due to its active material content. Seeds were collected from Assiut Governorate (May 2009) and were identified by Plant Taxonomy, Department of Natural Products, National Research Center, Dokki, Cairo, Egypt.

Preparation of plant extract

1.5 kg powder of N.S. was extracted in a separatory funnel with 2.0 litre of absolute methanol with refluxing for 5 hours. The extract was cooled at room temperature, filtered and evaporated under reduced pressure in rotary evaporator. It was dissolved in distilled water. Total extract was prepared according to Didry *et al.,* (1998).

Experimental animals

Weaning male albino 36 rats, strain with an average 32 days age and average weight of 80 g were obtained from the National Research Center, Dokki, Giza. Rates were housed in controlled room (25°C ± 2°C) **(**Hagsted *et al*, 1941). In individual cages with screen bottoms and water was supplied and fed on basal diet for eight days. The diet consisted of corn starch 70%, casein 10%, corn seed oil 10%, salts mixture 4%, vitamin mixture 1% and cellulose 5% (Comphell 1963). Dosage and administration of decoction: The decoction was administered at a dose of 20 mg/kg/day N.S.T.E. (Ahmed *et al* 2008), using a Sondi needle by gastric gavage method. (S.S. Iddamaldeniya et al 2006). CCl4 was dissolved in olive oil 20% v/v and administrated at dose of 2 mL/kg body weight (Domitrovi et al, 2010). Male albino rats were equally divided into 6 groups (6 rats in each group).The first group (control) was fed on the basal diet for another 8 weeks. The second group was fed on the basal diet and administered N.S.T.E. for 8 weeks. The third group was fed on basal diet, and treated with CCl4 for 8 weeks. The fourth group was fed on basal diet, and treated with CCl4 for 4 weeks, then with a dose of N.S.T.E. for another 4 weeks. The fifth group was feed on basal diet, and treated with CCl4 and in the same time with a dose of N.S.T.E. For 8 weeks. The sixth group was feed on basal diet, and treated with a dose of N.S.T.E. For 4 weeks then with CCl4 for 4 weeks.

Blood sample

At the end of experiment, animals were killed by decapitation after an overnight fast, and blood was centrifuged at 3000 rpm for 20 min, for serum preparation.

Serum analysis

Serum parameters were determined by enzymatic colorimetric methods. Total Proteins, albumin, Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Lactic dihydrogenase (LDH) and Gama glotamyl transaminase (G-GT), were determined according to Watanable et al., (1986), Doumas *et al*, (1971), Retiman and Frankel, (1957), Schmidt and Schmidt, (1963), Belfield and Goldberg, (1971), Young (1975) and Szasz (1969) respectively. Also Urea and uric acid were determined according to Caraway, (1975). Prothrombin (P.T.), Creatinine, direct bilirubin and total bilirubins were detrimend according to Rezzo, *et al* (2008) , Scirmeister, (1964) and Tietz (1963) respectively. In addition, lipids profile including Triglycerides (T.G), total cholesterol, total lipids, high density lipoproteins (HDL-C), low density lipoproteins (LDL-C) and very low density lipoproteins (VLDL-C) were determined according to Lowell *et al.,* (1973), Allian *et al.,* (1974), Knight *et al.,* (1972), and Denacherp *et al.,* (1980) respectively. Glutathione-s-transferase and malon dialdehyde activity was determined according to Habig *et al,* (1974). And Uchiymama and Mihara (1978) respectively.

Hematology

Hematology included determination of hematocrit by micro- hematocrit capillaries, hemoglobin concentration were determined by diagnostic kit produced by Biologic Products Inc. Erythrocyte and leukocyte counts, differential leukocyte count determined by extension in microscopy slides and blood clotting time by addition of calcium chloride to citrated blood was determined according to Betty (2007).

Histopathology

Rats organs were removed as rapidly as possible wiped with filter paper, weighed, chopped and kept as solution until the specimens were processed for histopathology. Histopathological sections from liver and kidney organs were stained with hematoxylin and eosin (Drury and Wollington, 1980). Liver specimens were fixed in 4% phosphate buffered formalin, embedded in paraffin and cut into 4μm thick sections and stained with hematoxylin and eosin (H&E) accordin to Ishak *et al.,* (1995).

Statistical analysis

Statistical analysis was done by Duncon's Method (SAS, 1996).

Results and discussion.

Effect of N.S.T.E. on liver functions in rats treated with CCL_4

Data reported in table (1) indicate that rats received N.S.T.E. (control) during the experiment showed significant increase in ALP and globulin while, prothrombin percentage and bilirubin concentration decreased in the serum comparing with control rats (untreated). The increment in ALP could be

attributed to *N.S.* alkaloids (Marschner 1995). Also, data show that rat administrated with CCl_4 had the highest increase in serum ALT,AST, ALP, LDH, G-GT, globulin, total and direct bilirubin, and highest decreased in serum, albumin, total proteins and prothrombin percentage. Rats fed on basal diet and received CCl_4 before, with or after administration with N.S.T.E. showed significant decrease in most serum liver functions and significant increase in albumin and prothrombin comparing with CCl_4 control. It could be suggested that N.S.T.E. had the advantage of decreasing liver enzymes level and increase liver functions due to the anti-oxidant components. (Evan et al, 1998), and to its free radical scavenging ability (De Lima *et al.,* 2007). Chronic Carbontetrachloride intoxication is a well known model for producing oxidative stress and hepatic injury. Its biotransformation produces hepatotoxic metabolites; the highly reactive trichloromethyl free radical which could be further converted to peroxytrichloromethyl radical and induces oxidative damage in the cells. Anti-oxidants of N.S.T.E. seem to be effective in treating chronic liver damage and fibrosis (Parola and Robino, 2001 and Williams and Burk, 1990). Liver is the basis of protein synthesis and CCl4 induces oxidative DNA damages, with the formation of DNA adducts, genetic mutation, strand breakage and chromosomal aberrations. (Jia *et al*., 2002).

Effect of N.S.T.E. on kidney functions and lipids profile in rats treated with CCl_4

Data reported in Table 2 show that rats administrated with N.S.T.E. had significant decrease in urea, creatinine, total lipids, total cholesterol and LDL-cholesterol and significant increase in uric acid comparing with control group. Rats administrated CCl_4 had the highest values of kidney serum parameters. Carbontetrachlorid caused significant increase in kidney functions and increase, protein content of kidney tissues, oxidative damage of proteins (Abraham *et al.,* 1999) and protein accumulation (Simeonova *et al.,* 2001) due to poor degradation by proteasomal and lyssosomal pathways causing metabolic dysfunction of kidney (Dalle-Donne *et al.,* 2006) and induce physiological imbalance in liver and kidney (Mehmetcik *et al.*,2008). Also, LDL- cholesterol, total lipids, triglycerides, total cholesterol, HDL- cholesterol and VLDL- cholesterol showed reduction in it's values. Rats treated with CCl_4 before, with and after treatment with N.S.T.E. showed reduction in urea, creatinine, uric acid and general reduction in total lipids and LDL- cholesterol, triglycerides, HDL-cholesterol and VLDL- cholesterol but showed incremeas in its values. LDL- cholesterol showed reduction in its values in the groups administrated CCl_4 with and after treatment with N.S.T.E. compared with rats administrated CCl_4. This decrease could be attributed to N.S. antioxidant (Ahmed *et al* 2008). Flavonoids, tannins, and other bioactive compounds in the total extract which could be responsible for the protective effects against the oxidative stress of CCl_4 in kidneys of rats.(Vardavas *et al.,*2006, Alpinar *et al.,*2009 and Etuk *et al.,*2009).

Effect of N.S.T.E. on antioxidant markers

Data reported in Table 2 indicate that rats administrated with N.S.T.E. had significant decrease in both glutathaion-S-tansferase and malondialdehyde concentrations compared with the control (untreated) group. Rats treated with CCl_4 showed significant decrease in glutathaion-S-tansferase and significant increase malondialdehyde concentration compared with the control group. Rats treated with CCl_4 before, with and after N.S.T.E. revealed significant increase glutathaion-S-tansferase concentration and significant decrease in malondialdehyde concentration compared with CCl_4 group. Also glutathaion-S-tansferase concentration were lower than control while malondialdehyde concentration were higher than control These results may be attributed to the exhaustion of antioxidant enzyme GST in detoxification of CCl3• free radical resulting from CCl4 toxicity via its conjugation with other antioxidant substrates as GSH. This suggestetion is confirmed by the findings of Lee *et al,* (2008).

Effect of N.S.T.E. on hematology parameters

All hematology parameters appeared to be affected by CCl_4 and N.S.T.E. (Table 3) and (Fig –C). Hemoglobin concentration, erythrocyte, hematocrit level and platelets count were significantly decreased in CCl_4 control, and could be named acute anemia (Betty 2007) but, leukocyte levels, in the same group were significantly fluctuated compared with the untreated group. Data concerning the groups which were treated before, with and after treatment with N.S.T.E. showed significant increase in hemoglobin concentration, erythrocyte, hematocrit level and platelets count, and could be named normochromic normocytic anemia. (Betty 2007) however, there was CCl_4-induced hepatotoxicity, inflammation, fibrosis and increase TLC (Simeonova *et al.,* 2001). It could be suggested that the N.S.T.E. had the advantage of decreasing liver enzymes level, which ensures the efficacy of these components to increase liver function. (Zaoui *et al.,* 2002). In this study, N.S.T.E. significantly reduced oxidative stress in the liver of CCl_4-treated rats. Hepatoprotective effects of N.S.T.E., at least in part, could originate from free radicals scavenging ability (De Lima *et al.,* 2007).

Histopathology

Data concerning liver histological studies are shown in Fig. A. Liver sections from untreated control rats show normal hepatic architecture. (Fig. A1). In the CCL_4 control group, observed spontaneous regression of fibrosis, degenerated and ballooned

hepatocytes containing acidophilic hyaline inclusions emerged within hepatic lesions (Fig. A3).for rats administered with CCl_4 then with N.S.T.E. necrotic cells have been replaced by hepatocytes, with the livers showing maintained histoarchitecture, similar to controls (Fig. A4).The cross section of livers of rats treated with CCl_4 and the same time with N.S.T.E. showed only sporadic hepatic lesions, hapatocyte ballooning and fatty degeneration (Fig. A5). The cross section of livers treated with N.S.T.E. then with CCl_4 showed massive necrosis, accompanied with micro vesicular steatosis and mild mononuclear cell infiltration. The histological result coincide with that of the chemical analysis of liver enzymes and lipid profile level of blood, which showed a decrease in enzyme percentage in the last two groups, and also the amount of the total lipids was decreased. Also, kidney histological changes are shown in Fig. B, sections of the control group showed normal histological including normal glomerular tubules, afferent arterioles, and bowman capsule. Marked histological changes were observed in cortex and medulla in kidneys of CCl_4-treated rats. Cortex was more severely affected due to the CCL_4 toxicity as compared to medullary portion. The cross sections showed tubular degeneration, tubular dilatation, interstitial fibrosis, glomerular atrophy and capillaries congestion in rats. Co-treatment with N.S.T.E. on G4, G5 and G6 markedly recovered the toxic changes near to the normal histology of kidney and showed normal glomerular tubules, reversed tubular dilatation and prevented interstitial edema and capillary congestion. However, histology of G4 treated group was more closely related with the normal group. These results confirm the chemical analysis of uric acid, creatinine and urea in blood, which showed a clear decrease in their level at the group 5 and 6. (Bamosa *et al.,* 1997). The results obtained herein suggest that N.S.T.E. Has the ability to reduce the harmful effect of CCl4 and in agreement with (Bashandy, 1996).

Table 1. Effect of CCl_4 toxicity and N.S.T.E. on liver functions parameter (sALT , sAST ALP, LDH, G-GT Albumin, total proteins, Prothrombin, total and direct bilirubin and globulin) activity in rats after 8 weeks.

Treatment	Control untreated(G1)	N.S.T.E.. control(G2)	CCL4 control(G3)	CCL4 Before N.S.T.E. (G4)	CCL4 With N.S.T.E. (G5)	CCL4 After N.S.T.E. (G6)
sALT	4.8 ^{e} ±	4.5 ^{ef} ±	34.76 ^{a} ±	12.81 ^{cd} ±	14.46 ^{c} ±	23.62 ^{bc} ±
u/l	2.41	1.51	5.90	4.25	3.96	6.51
sAST	7.3 ^{ef} ±	7.41 ^{e} ±	89.11 ^{a} ±	23.22 ^{cd} ±	19.16 ^{d} ±	39.34 ^{b} ±
u/l	1.41	0.53	5. 10	3.23	4.97	3.50
ALP	68.3 ^{f} ±	88.8 ^{e} ±	666.71 ^{a} ±	188.90 ^{cd} ±	244.44 ^{b} ±	200.91 ^{c} ±
u/l	3.15	7.35	17.32	20.22	26.19	11.92
LDH	11.9 ^{f} ±	14.2.28 ^{e} ±	41.92 ^{a} ±	23.8 ^{d} ±	31.2 ^{b} ±	29.71 ^{bc} ±
u/l	2.12	1.24	2.11	4.72	2.24	2.15
G-GT	0.80 ^{e} ±	0.71 ^{ef} ±	5.1 ^{a} ±	3.3 ^{d} ±	3.6 ^{cd} ±	4.12 ^{bc} ±
u/l	0.05	0.06	0.63	0.54	0.65	0.81
Globulin	3.55 ^{bc} ±	3.89 ^{a} ±	3.03 ^{cd} ±	2.59 ^{e} ±	3.12 ^{c} ±	3.35 ^{b} ±
g/dl	0.02	0.05	0.05	0.02	0.06	0.08
T. proteins	7.3 ^{ef} ±	7.41 ^{e} ±	4.5 ^{d} ±	5.72 ^{c} ±	6.15 ^{b} ±	5.93 ^{bc} ±
g/dl	1.41	0.53	0.31	0.21	0.19	0.31
Albumin	3.75 ^{ab} ±	3.52 ^{a} ±	1.47 ^{e} ±	3.13 ^{c} ±	3.03 ^{cd} ±	2.58 ^{d} ±
g/dl	0.18	0.21	0.32	0.17	0.20	0.16
Prothrompin percentage	100 ^{a} %	90.9 ^{ab} ± 14	31.25 ^{f} ± 7.9	55.5 ^{c} ± 12.9	40 ^{d} ± 8.33	38.5 ^{de} ± 15
T. bilirubin	0.79 ^{d} ±	0.62 ^{e} ±	4.9 ^{a} ±	3.5 ^{b} ±	3.2 ^{bc} ±	4.04 ^{ab} ±
mg/dL	0.04	0.05	0.54	0.59	0.78	1.6
D. bilirubin	0.2 ^{ef} ±	0.21 ^{e} ±	1.6 ^{a} ±	1.1 ^{cd} ±	1.3 ^{b} ±	1.12 ^{c} ±
mg/dL	0.05	0.06	0.63	0.54	0.65	0.81

A,b,c,d and e Means within column different letters differ significantly (P<0.01) from each other means followed by the same letter don't differ at the 0.01 probability level. Each value represents the mean of 6 rat's ± S.E

Table 2. Effect of CCl_4 toxicity and N.S.T.E. on total lipids, triglycerides, total cholesterol HDL-cholesterol, LDL-cholesterol, VLDL cholesterol, urea, creatinine, uric acid, Glutathaion-S-tansferase and Molondialddehyde level in rats after 8 weeks.

Treatment	Control untreated(G1)	N.S.T.E.. control(G2)	CCL4 control(G3)	CCL4 Before N.S.T.E. (G4)	CCL4 With N.S.T.E. (G5)	CCL4 After N.S.T.E. (G6)
Total lipid mg/dl	365.26 ^{a} ± 24.06	319.28 ^{b} ± 15.35	303.42 ^{bc} ± 20.19	295.45 ^{c} ± 5.82	250.27 ^{d} ± 3.96	230.63 ^{e} ± 6.51
Triglycerides mg/dl	98.25 ^{a} ± 1.95	94.5 ^{ab} ± 4.87	73.35 ^{d} ± 2.64	83.6 ^{c} ± 3.23	76.4 ^{cd} ± 4.97	94.34 ^{ab} ± 3.50
T. cholesterol Mg/dl	96.47 ^{a} ± 2.01	88.29 ^{b} ± 4.68	79.35 ^{c} ± 6.31	82.90 ^{bc} ± 2.22	70.6 ^{e} ± 6.19	73.91 ^{d} ± 3.92
HDL- cholesterol Mg/dl	41.57 ^{a} ± 0.35	39.5 ^{ab} ± 0.41	27.08 ^{e} ± 0.29	29.42 ^{d} ± 0.21	35.15 ^{c} ± 0.15	35.7 ^{bc} ± 0.31
LDL-cholesterol Mg/dl	34.75 ^{b} ± 0.18	29.25 ^{c} ± 0.21	37.07 ^{a} ± 0.32	36.13 ^{ab} ± 0.17	18.6 ^{e} ± 0.20	19.36 ^{d} ± 0.16
VLDL- cholesterol Mg/dl	19.65 ^{a} ± 0.21	18.9 ^{ab} ± 0.50	14.67 ^{e} ± 0.34	16.72 ^{c} ± 0.21	15.82 ^{d} ± 0.06	18.85 ^{b} ± 0.08
B. Urea mg/dL	41.33 ^{e} ± 5.12	33.28 ^{f} ± 4.24	63.92 ^{a} ± 7.21	42.8 ^{d} ± 6.71	44.2 ^{c} ± 4.31	46.71 ^{b} ±9.15
Creatinine mg/dL	0.80 ^{d} ± 0.04	0.69 ^{e} ± 0.05	1.3 ^{a} ± 0.24	0.85 ^{cd} ± 0.09	0.99 ^{bc} ± 0.08	1.04 ^{b} ± 0.09
Uric acid mg/dL	4.2 ^{f} ± 0.15	5.12 ^{e} ± 0.04	9.64 ^{a} ± 0.41	7.50 ^{c} ± 0.82	6.36 ^{d} ± 0.74	8.25 ^{b} ± 0.92
Glutathaion-S-tansferase u/ml	3.89 ^{ab} ± 0.35	3.68 ^{c} ± 0.35	1.91 ^{f} ± 0.03	2.99 ^{a} ± 0.03	2.23 ^{e} ± 0.03	2.52 ^{d} ± 0.04
Molondialddehyde nmol/ml	0.71 ^{e} ± 0.09	0.66 ^{f} ± 0.09	11.55 ^{a} ± 0.08	1.88 ^{d} ± 0.04	2.5 ^{b} ± 0.03	2.11 ^{c} ± 0.03

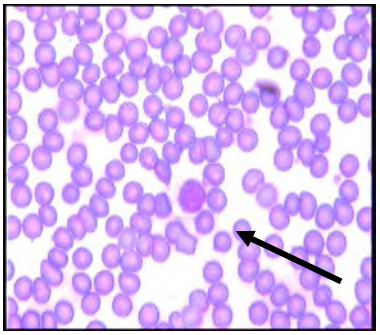
A,b,c,d and e Means within column different letters differ significantly (P<0.01) from each other means followed by the same letter don't differ at the 0.01 probability level. Each value represents the mean of 6 rats ± S.E.

Table 3. Effect of CCL4 toxicity and N.S.T.E. on hematological parameters (HB%, RBCs count Total leukocyte count and platelets count and Packed cell value (Hematocrite)) level in rats after 8 weeks.

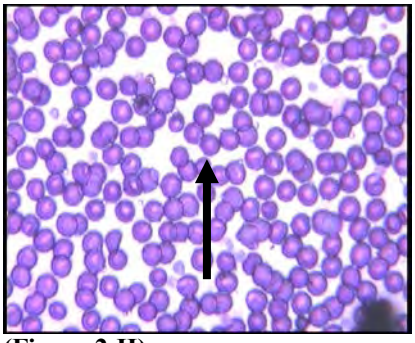
Treatment	Control untreated(G1)	N.S.T.E.. control(G2)	CCL4 control(G3)	CCL4 Before N.S.T.E. (G4)	CCL4 With N.S.T.E. (G5)	CCL4 After N.S.T.E. (G6)
HB% g/dl	13.2 ^{a} ± 1.1	13.1 ^{a} ± 1.23	8.43 ^{d} ± 1.31	9.88 ^{cd} ± 0.82	11.36 ^{bc} ± 1.49	12.78 ^{ab} ± 1.21
RBCs count (Cell x 10^{3} /mm^{3})	4.75 ^{a} ± 1.42	4.68 ^{a} ± 0.91	3 ^{d} ± 0.6	3.46 ^{c} ± 0.59	3.9 ^{bc} ± 0.7	4.31 ^{ab} ± 0.50
Packed cell value (PCV)%	44.16 ^{a} ± 7.84	44.33 ^{a} ± 5.05	27.5 ^{c} ± 5.5	32 ^{b} ± 3.0	36.33 ^{b} ± 5.67	41.16 ^{a} ± 5.84
Total leukocyte count (TLC) (Cell/mm 3)	7.51 ^{ef} ± 1.7	7.6 ^{e} ± 1.6	15.9 ^{a} ± 0.9	9.7 ^{d} ± 1.1	12.1 ^{b} ± 0.7	11.88 ^{bc} ± 1.6
Platelets count (Cell x 10^{3} /$mm^{)}$	355.83 ^{a} ± 36.2	327.66 ^{b} ± 31	120.33 ^{e} ± 2.67	260.33 ^{c} ± 3.67	234 ^{c} ± 36	173.5 ^{d} ± 11.5

A,b,c,d and e Means within column different letters differ significantly (P<0.01) from each other means followed by the same letter don't differ at the 0.01 probability level. Each value represents the mean of 6 rats ± S.E.

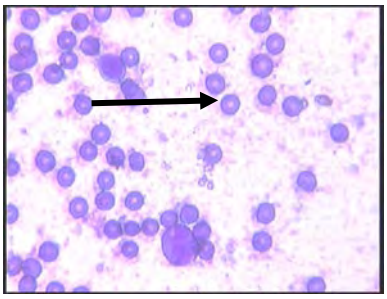
Fig. C. The influence of N.S.T.E. on Hematology of different groups of rats animal for 8 weeks.



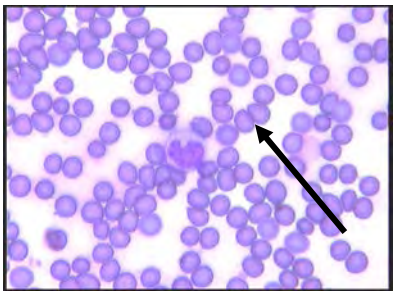
(Figure 1-H)
Smear film of hole blood on rats which untreated



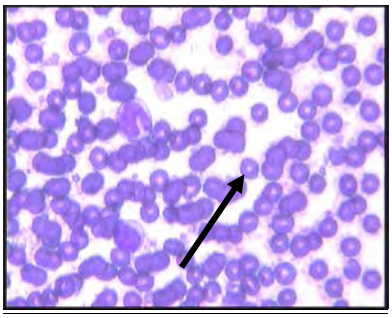
(Figure 2-H)
Smear film of hole blood on rats which treated by N.S.T.E.



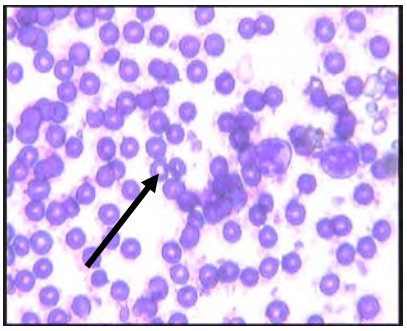
(Figure 3-H)
Smear film of hole blood on rats which treated by CCL4



(Figure 4-H)
Smear film of hole blood on rats which treated by CCL4 Before N.S.T.E.

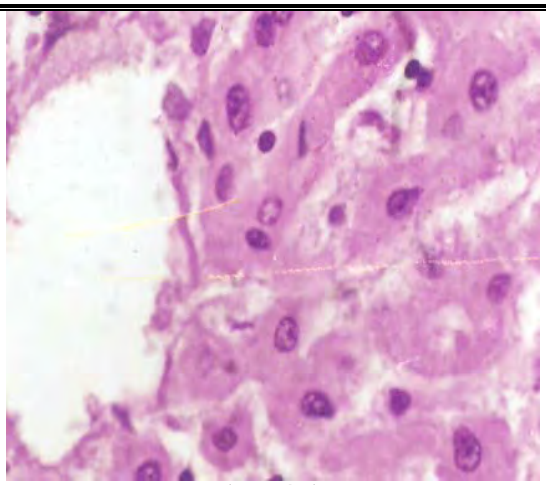


(Figure 5-H)
Smear film of hole blood on rats which treated by CCL4 with N.S.T.E.

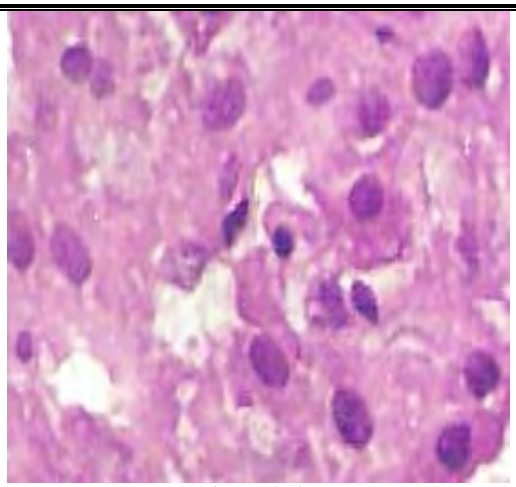


(Figure 6-H)
Smear film of hole blood on rats which treated by CCL4 after N.S.T.E.

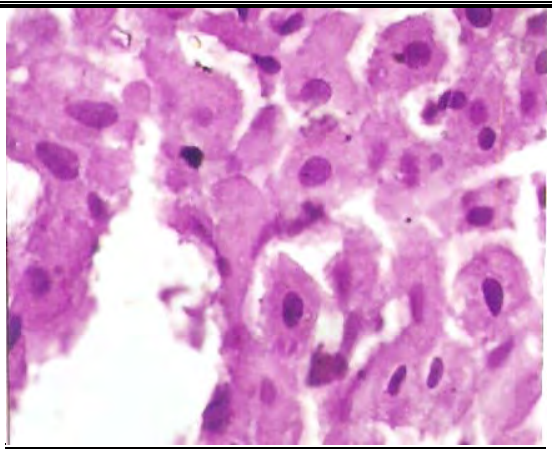
Cross sections in liver of different groups of rats.



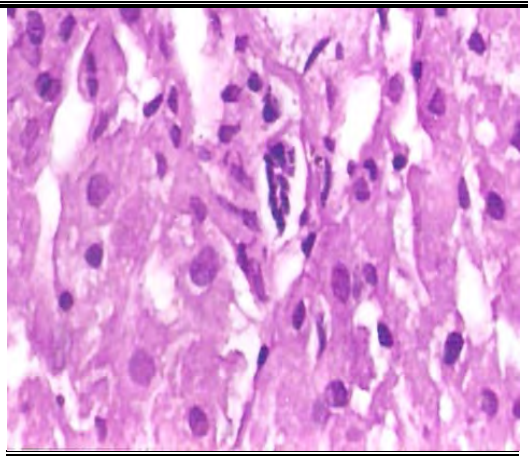
(Fig. A1)
Control untreated



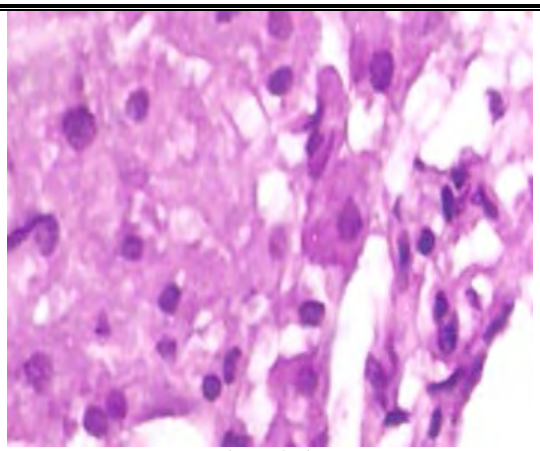
(Fig. A2)
N.S.T.E. control.



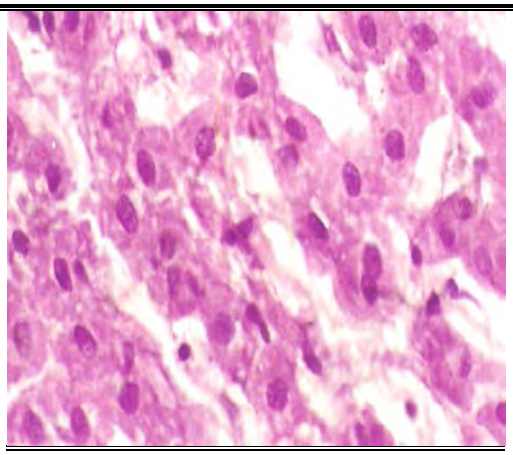
(Fig. A3)
CCL4 control.



(Fig. A4)
CCL4 Before N.S.T.E.

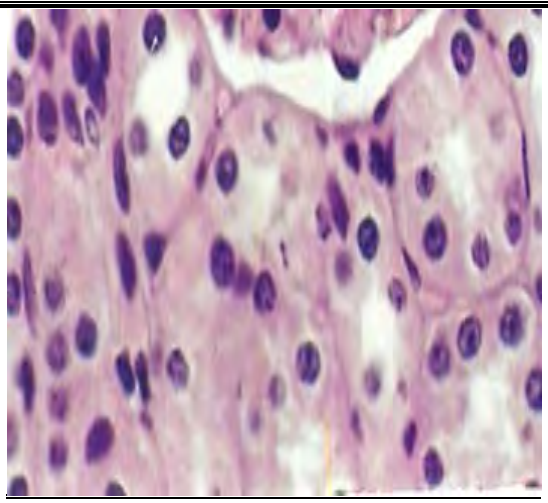


(Fig A5)
CCL4 with N.S.T.E.

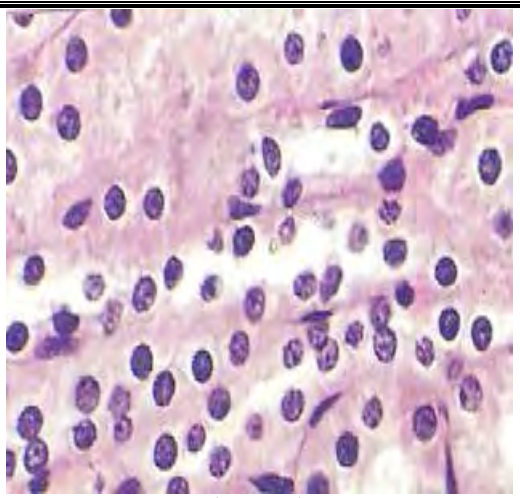


(Fig A6)
CCL4 after N.S.T.E.

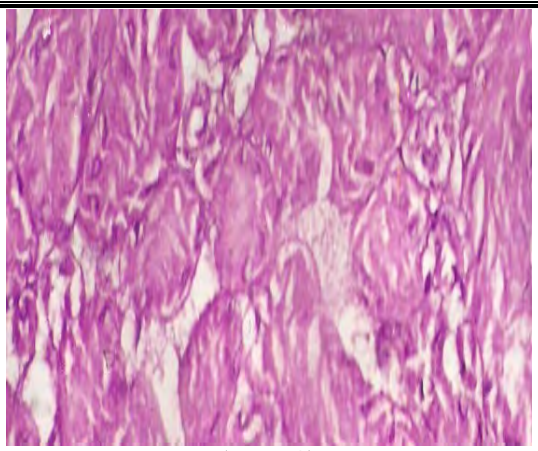
Cross sections in kidney of different groups of rats.



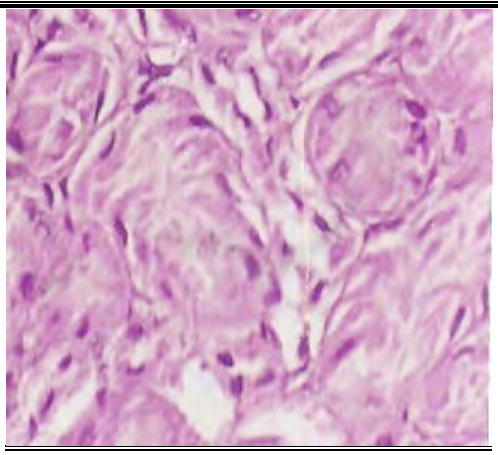
(Fig. B1)
Control untreated



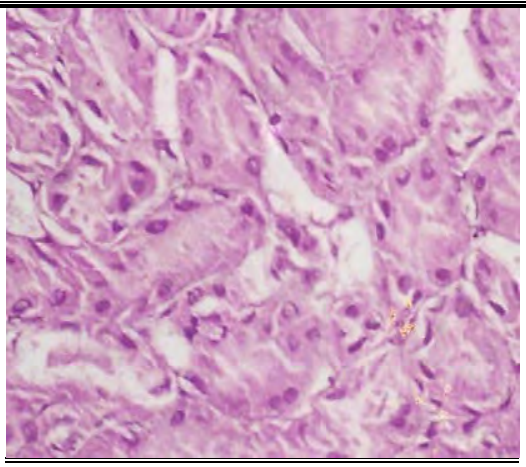
(Fig. B2)
N.S.T.E. control.



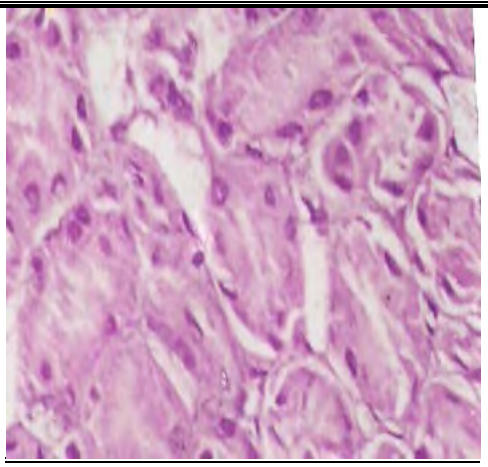
(Fig. B3)
CCL4 control



(Fig. B4)
CCL4 before N.S.T.E.



(Fig. B5)
CCL4 with N.S.T.E.



(Fig. B6)
CCL4 after N.S.T.E.

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Effect of Deoxyribonucleic acid transfer using sperm-mediated gene technique on productive and reproductive performance of Mamourah chickens

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Abstract

The present study was aimed to investigate the effect of introducing broiler hybrid chicken-DNA into embryonic cells of Mamourah chickens using spermatozoa as vectors which known as sperm-mediated gene transfer technique on productive and reproductive performance of Mamourah chickens which evaluated over two generations. A total number of 150 matured Mamourah pullets aged 8 months were randomly grouped into six groups (25 pullets per each group). Pullets of the first three groups were artificially inseminated with semen of the same strain containing spermatozoa used as vectors for transferring level 20, 25 and 30 µg of broiler hybrid-DNA (male) diluted with skim milk media in the presence of lipofectin reagent and considered as treated groups. Pullets of the other three groups were artificially inseminated with semen diluted with either media in the presence of lipofectin reagent, semen diluted with only media or untreated semen and considered as control groups. Results obtained showed that applying foreign DNA level significantly decreased the fertility percentage of eggs collected from treated pullets. The rate of decrement increased as the level of foreign DNA applied increased. The lowest average of fertility percentage was detected in pullets of the first generation (71.85%). However, it increased in birds of the second generation to reach (90.37%). No significant variations were found due to treatments applied and pullet's generation on hatchability percentage. Hatchability percentage was slightly higher (88.93%) in pullets of the first generation when compared to those of pullets of the second generation (86.85%). It was clearly obvious that mortality rate estimated at different periods during incubation process was higher in the second generation when compared to that of birds of the first one. However percent of embryonic malformation was slightly high in birds of first generation 1.58% than those of the second generation 1.44%. The embryonic mortality estimated at different times of incubation period as well as the percent of embryonic malformation increased as the level of foreign DNA introduced during artificial insemination increased to reach its highest magnitude when the level of foreign DNA reached 30 µg. Highly significantly improving was found in both average of body weight and growth rate of Mamourah transgenic chicken at the first generation than at the second generation. Applying foreign DNA at a level of 25µg showed higher averages of body weight than did by the other two levels (20 or 30 µg) and control groups.

Key words: DNA transfer, sperm-mediated gene, growth traits, fertility, hatchability and embryonic mortality, Mamourah chickens.

Introduction

In the last twenty years the foreign DNA transfer technology has been available to apply in poultry. This is technology offers potential benefits, such as providing a means to increase genetic variation, and producing genetic change more rapidly in poultry populations. The most common used for DNA transfer in animal was the direct micro-injection of DNA solutions into the pronuclear of zygotes and embryos Sang and Perry (1989), Perry *et al.* (1991) and Naito *et al.* (1992). Although, the micro-injection technique results in high success rate in mice, but it is not an efficient method when applied to livestock (Lavitrano *et al*., 2003). Thus, a logical alternative strategy to generate transgenic animals theoretically consists of the introduction of foreign DNA into male gametes before the fertilization process (Spadafora, 2002). Lavitrano *et al.*, (1989) demonstrated that for the first time that (a) the epididymal sperm of the mouse can spontaneously incorporate plasmid DNA molecules; (b) genetically modified offspring can be generated by *in vitro* fertilization procedures; (c) exogenous DNA sequences are expressed in the progenitors, and (d) that the sperm-carried exogenous DNA incorporated in the fertilized ovum, is transmitted from the parents to F1 progeny.

The role of the spermatozoa during fertilization includes the transfer of a haploid genome to the resultant zygote. This capacity has been exploited as an innovative strategy for the delivery of exogenous DNA for the production of transgenic animals (Lauria and Gandolfi., 1993; Kim *et al.*, 1997 and Perry *et al*., 1999). The sperm-mediated gene transfer (SMGT) technique in vertebrates has gone through many adaptations in the last 10 years, in different laboratories (Gandolfi, 2000).The definition and the establishment of work protocols for SMGT technique could be effectively applied to different animal species would be of high value in biotechnology (Celebi *et al*., 2002).

Nakanishi and Iritani (1993) found by using the fluorescence in situ hybridization that 51.6% of the

exogenous DNA-lipofectin-treated sperm retained the exogenous DNA. The fertility percentages of eggs were 47, 23 and 67% for the DNA incubated with sperm, electroporated sperm and the lipofectin-treated sperm, respectively. Trefil *et al.,* (1996) observed that continuous egg fertility for three weeks of hens inseminated with lipofectin-treated spermatozoa and the fertility percent reached to 52.3% during the third week.

El-Gendy *et al.* (2007) reported that the reduction in fertility of cock spermatozoa attributed to the depression in sperm viability due to the introduction of foreign DNA that was much more pronounced with the addition of lipofectin further to the relative damage in the sperm due to the incorporation with exogenous substrates (DNA and lipofectin). The aim of this study was to investigate the effect of introducing broiler hybrid chicken DNA into embryonic cells of Mamourah chicken used spermatozoa as vectors known as sperm-mediated gene transfer technique on productive and reproductive performance of Mamourah chicken which evaluated over two generations.

Materials and methods

The present study was carried out at the Poultry Research Farm, Department of Animal Production, Faculty of Agriculture, Benha University, Egypt. Genomic DNA isolation and purification was fulfilled at laboratories belonging to the Department of Genetics in the same College.

The DNA e xtraction, purification and concentration

The DNA was extracted according to the method of Sambrook *et al*. (1989) from hepatic tissue samples of broiler hybrid chickens (Hubbard Isa-30) males (genomic donor). It was taken as foreign-DNA source because of high meat producer evaluated under Egyptian conditions. Hubbard genome was transformed to Mamourah strain (Genomic recipient) being resistance to diseases and of good livability under different environmental conditions in Egypt (Abd-El-Gawad *et al.,* 1979). The DNA concentration was estimated according to (Charles, 1970) using the optical density (O.D.) readings of Spectrophotometer at wavelength of 260 nm.

Experimental groups

Semen was collected individually from 12 adult Mamourah chicken males aged 9 months using dorsal-abdominal massage method according to Lake (1957). The pooled semen was equally divided into three parts and diluted with skim-milk media then incubated with three levels of broiler hybrid-DNA (20, 25 and 30 µg) at 37°C for 2hrs in water bath in the presence of Lipofectin reagent (purchased from Invitrogen® company, Germany) which is considered to be suitable for transfection processing of the foreign-DNA into spermatozoa according to Fellegner *et al*. (1987) and Sato *et al.* (2003), considering to manufacturer's manual processing guide.

After incubation, each sample of genetic modified semen (DNA-Lipofectin-treated semen) was used for artificial insemination of 25 Mamourah pullets which is considered as treated groups (groups 1, 2, and 3). While other three groups in the same number artificially inseminated with semen diluted with media in the presence of lipofectin reagent, semen diluted with media and untreated semen which are considered as control groups (groups 4, 5 and 6). The treatments applied are listed in the following Table 1:

Group	Treatment					
	Semen	Media	Lipofectin	20µg	25µg	30µg
1	√	√	√	√		
2	√	√	√		√	
3	√	√	√			√
4	√		√			
5	√	√				
6	√					

(√) means that this item was applied

Eggs laid by treated pullets of all experimental groups were collected for 10 days after artificial insemination. A number of 90 eggs were collected from each experimental group with total number of 540 were incubated in forced draft incubator at optimum incubation conditions of heating temperature , humidity, turning and ventilation during all over the incubation period.

Parameters estimated and data collection

The performance of hatched chicks resulted from the previous experimental groups were evaluated by estimating the following parameters over two generations of transgenesis.

Fertility

Fertility was calculated as a percent of fertile eggs to a total of incubated eggs according to the following equation:

$$\textbf{Fertility (\%)} = \frac{\text{Fertile eggs}}{\text{Total incubated eggs}} \times 100$$

Hatchability and embryonic mortality:

Hatchability was calculated as a percent of hatched chicks to fertile eggs according to the following equation:

$$\textbf{Hatchability (\%)} = \frac{\text{Hatched chicks}}{\text{Fertile eggs}} \times 100$$

Embryonic mortality was estimated during three periods during the process incubation to find out the early from (1-7d), mid (8-18d) and late mortality from (19-21d).

Body weight (g) and rate of growth (%)

Live body weight for birds of 1st and 2nd generation was weighed at hatch, then at monthly intervals up to sexual maturity. Measurement was also performed at the peak of egg production and at molt. The rate of growth was individually calculated according to the following formula suggested by Brody (1945).

$$\text{Rate of growth \%} = \frac{W_2 - W_1}{(W_2 + W_1)/2} \times 100$$

Where: W1 and W2 are body weight at the two successive periods.

Statistical analysis

Analysis of variance was carried out using SAS procedure guide (SAS, 2004) for traits of fertility, hatchability, embryonic mortality, body weight and rate of growth according to the following linear model:

$$X_{ijk} = \mu + G_i + T_j + GT_{ij} + e_{ijk}$$

Where: X_{ijk} = the k^{th} observation.
μ = overall mean
G_i= effect of the i^{th} generation
T_j = effect of the j^{th} treatment
GT_{ij} = the interaction between i^{th} generation and j^{th} treatment
e_{ijk} = the experimental error.

Results and discussion

Fertility percentage

It was clearly evident that treatments applied had highly significant effect ($P<0.001$) on the percentage of fertility as found in Table 2. Inseminating birds with natural semen alone or with natural semen + media applied resulted in relatively higher fertility percentage which mounted a value of 92.22 and 92.78%, respectively without any significant difference between them. However, adding lipofectin to semen + media lowered the fertility percentage to reach an average of 84.44%. This indicates that adding media to semen slightly improved fertility percentage while adding lipofectin to semen + media had relatively bad effect. On the other hand, inserting foreign DNA to semen + media + lipofectin decreased the fertility percentage of eggs collected from treated pullets. The rate of decrement increased as the level of foreign DNA applied increased to reach its lowest level when applying foreign DNA at a level of 30µg (67.78%). The lowest level of decrease in fertility was observed when foreign DNA reached 25µg (78.34%) when compared to pullets treated with foreign DNA at a level of 20µg (71.11%) (Table 2). These results may lead to conclude that insertion of foreign DNA to semen inseminated may modify the genome of the resulted zygote which result in a degree of genetic performance that affect the biological activity which is reflected as a decrease in fertility percent which depends on a series of biological reactions during the process of fertilization. These may include the biological effects on the gamones (either on androgamones or gynogamones) that have a pronouncing effect on the success of fertilization.

The obtained results go in agreement with those obtained by El-Gendy *et al*. (2007) who reported that the reduction in fertility of cock spermatozoa which used as a vector to transfer the foreign DNA in the present of lipofectin reagent was attributed to the depression in sperm viability due to the introduction of foreign DNA that was much more pronounced when lipofectin was added. They disagree with the findings of Nakanishi and Iritani (1993) who stated that lipofectin added to chicken sperm did not affect fertility, although it slightly reduced mortality.

Concerning to the effect of bird's generation, the lowest fertility percentage was detected in pullets of the first generation (71.85%). However, it increased in birds of the second generation to reach an average mounted 90.37%. This may indicate an occurrence of rearrangement of the genetic constitution in favor of coordination between the biological "reactions related to fertility" of the genome of the second generation birds. This coordination is reflected as relatively better enzymatic performance related to gametic formation and efficiency in fertilization process.

The interaction effect between treatment applied and pullet's generation was found to be of highly significant value.

Hatchability percentage

Inspecting the obtained data listed in Table 3 revealed no significant variation in the average of hatchability percentage due to either treatments applied or bird's generation as well as the interaction between them. However, the higher hatchability percent average was obtained in incubated eggs collected from pullets of control group inseminated with semen (92.18%) which differed significantly from hatchability percent averages of other treated groups which ranged from 89.22% in egg of pullets inseminated with semen + media to 83.59% in eggs of pullets inseminated with semen + media +lipofectin + 30µg foreign DNA. These results may enable to suggest that insertion of foreign DNA with different levels applied may affect the formation of the pronucleoli during the process of early stage of embryonic development and correspondingly be the main cause of failure in the forming of the blastocyte and incidence of cleavage process. This may be logic cause of decreasing the percentage of hatchability. The previously mentioned results agree with those obtained by Ali, 2001; El-Garhy, 2004; El-Khalea, 2005 and Ahmed, 2006.

Hatchability percentage was slightly higher (88.93%) in pullets of the first generation when compared with those of pullets of the second generation (86.85%). This may lead to state that insertion of foreign DNA as well as insemination with sperm + media or with semen + media + lipofectin may interfere in the process of early division of zygote during the first stages of embryonic development. These results disagree with results reported by Mohamed (2009) who found that although the introducing MD-gene (40 µg) in fertile eggs of Brown and Golden quail induced high hatchability percentage 51.43 and 60.0 %, respectively during the first generation. Hatchability percentage was increased to (85.24%) during second generation.

Table 2. Fertility percentage for eggs collected from Mamourah pullets of different experimental groups during two successive generations (least-square means± standard error).

Treatment (Artificial inseminated with)	Fertility% (Mean±SE)		Treatment (Mean ± SE)
	1st generation	2nd generation	
Semen	92.22^{a} ± 2.10	92.22^{a}± 2.10	92.22^{a} ±1.49
Semen+ media	92.22^{a}± 2.10	93.33^{a} ±2.10	92.78^{a} ±1.49
Semen+ media+ lipofectin	77.78^{b}± 2.10	91.11^{a} ±2.10	84.44^{b} ±1.49
Semen+ media+ lipofectin +20 µg-DNA	54.44^{d} ± 2.10	87.78^{c} ±2.10	71.11^{d} ±1.49
Semen+ media+ lipofectin +25 µg-DNA	67.78^{a}± 2.10	88.89^{a} ±2.10	78.34^{c} ±1.49
Semen+ media+ lipofectin +30 µg-DNA	46.67^{e}± 2.10	88.89^{a} ± 2.10	67.78^{d}±1.49
Generation (Mean ± SE)	71.85^{b} ±0.86	90.37^{a}±0.86	

Means of treatments within each generation and those of different treatments or generations having different superscripts are significantly different.

Table 3. Hatchability percent for eggs collected from Mamourah pullets of different experimental groups during two successive generations (least-square means± standard error).

Treatment (Artificial inseminated with)	Hatchability % (Mean ± SE)		Treatment Mean ± SE
	1st generation	2nd generation	
Semen	95.20^{a}±2.90	89.16^{ab}±2.90	92.18^{a}±2.05
Semen+ media	90.30^{ab}±2.90	88.13^{ab}±2.90	89.22^{ab}±2.05
Semen+ media+ lipofectin	89.96^{ab}±2.90	86.54^{ab}±2.90	88.25^{ab}±2.05
Semen+ media+ lipofectin +20 µg- DNA	85.83^{ab}±2.90	86.13^{ab}±2.90	85.98^{ab}±2.05
Semen+ media+ lipofectin +25 µg- DNA	90.08^{ab}±2.90	86.18^{ab}±2.90	88.13^{ab}±2.05
Semen+ media+ lipofectin +30 µg- DNA	82.22^{b}±2.90	84.95^{b}±2.90	83.59^{b}±2.05
Generation (Mean ± SE)	88.93±1.18	86.85 ±1.18	

Means of treatments within each generation and those of different treatments or generations having different superscripts are significantly different.

Embryonic mortality and abnormality

It was clearly obvious that mortality rate estimated at different periods during incubation process was higher in the second generation when compared to that of birds of the first one. However percentage of embryonic malformation was slightly high in birds of first generation 1.58% than those of the second generation 1.44% (Table 4).

Results obtained in Table 4 revealed that the embryonic mortality estimated at different times of incubation period as well as the percentage of embryonic malformation increased as the level of foreign DNA introduced during artificial insemination increased to reach its highest magnitude when the level of foreign DNA reached 30µg. However, applying media or media + lipofectin in semen inseminated seemed to have no significant effect on the average of embryonic mortality estimated during incubation period.

No significant variation in the average of embryonic mortality percentage was found due to the bird's generation, treatments applied and the interaction between them.

These results agreed with those obtained by Perry *et al.* (1991), Naito *et al.* (1992), Ali (2001), El-Garhy (2004), El-Khalea (2005) and Ahmed (2006) who all found that inserting foreign DNA into embryonic cells decreased the hatchability percent of incubated eggs and increased the embryonic mortality percent at the different time of the incubation period and it mainly increase at the early embryonic stage of the embryonic development. This may be due to genetically unbalanced chromosome arrangement during embryonic development. The increase in early embryonic mortality may be occurred as a result of

the incidence of abortive membranes that an embryo was lacking (Thorne *et al*., 1991).

Live body weight

Data presented in Table 5 showed highly significantly increased in average body weight of Mamourah chicks hatched from eggs laid by artificially inseminated pullets using genetic modified sperms (semen treated with different levels of broiler hybrid-DNA) all over the times of estimation compared to controls.

Table 4. Average of embryonic mortality and embryonic malformation (%) for different experimental groups during two successive generations (least-square means± standard error).

Treatment (Artificial inseminated with)	1st generation					
	Embryonic mortality (%)					Abnormal shape (%)
	Early (1-7day)	Mid (8-18day)	Late (19-21day)	Failed egg	Total	
Semen	0.00^{b} ± 1.28	1.19^{ab} ±1.16	2.38^{a} ±1.35	1.23 ±1.41	4.80^{b} ±2.57	0.00 ±1.48
Semen+ media	2.42^{ab} ± 1.28	3.61^{ab} ±1.16	2.42^{ab} ±1.35	1.23 ±1.41	9.67^{ab} ±2.57	0.00 ±1.48
Semen+ media+ lipofectin	4.30^{ab} ±1.28	0.00^{b} ±1.16	1.33^{ab} ±1.35	1.45 ±1.41	7.08^{ab} ±2.57	2.97 ±1.48
Semen+ media+ lipofectin +20µg-DNA	3.94^{ab} ±1.28	4.08^{a} ±1.16	2.22^{ab} ±1.35	1.85 ±1.41	12.08^{ab} ±2.57	2.08 ±1.48
Semen+ media+ lipofectin +25µg-DNA	4.92^{a} ±1.28	1.67^{ab} ±1.16	0.00^{b} ±1.35	1.67 ±1.41	8.25^{ab} ±2.57	1.67 ±1.48
Semen+ media+ lipofectin +30µg-DNA	5.00^{a} ±1.28	0.00^{b} ±1.16	5.00^{a} ±1.35	5.00 ±1.41	15.00^{a} ±2.57	2.77 ±1.48
Generation (Mean ± SE)	3.43 ±0.52	1.76 ±0.47	2.23 ±0.55	2.07 ±0.58	9.49 ±1.05	1.58 ±0.60
Treatment (Artificial inseminated with)	2nd generation					
	Embryonic mortality (%)					Abnormal shape (%)
	Early (1-7day)	Mid (8-18day)	Late 19-21day)	Failed egg	Total	
Semen	2.42^{ab} ±1.28	1.19 ±1.16	3.61 ±1.35	2.42 ±1.41	9.65 ±2.57	1.19 ±1.48
Semen+ media	3.57^{ab} ±1.28	3.57^{ab} ±1.16	1.19^{ab} ±1.35	2.38 ±1.41	10.72^{ab} ±2.57	1.15 ±1.48
Semen+ media+ lipofectin	3.66^{ab} ± 1.28	2.38^{ab} ±1.16	4.85^{a} ±1.35	1.28 ±1.41	12.18^{ab} ±2.57	1.28 ±1.48
Semen+ media+ lipofectin +20µg-DNA	3.80^{ab} ±1.28	2.57^{ab} ±1.16	2.47^{ab} ±1.35	3.80 ±1.41	12.64^{ab} ±2.57	1.23 ±1.48
Semen+ media+ lipofectin +25µg-DNA	3.76^{ab} ±1.28	2.47^{ab} ±1.16	2.57^{a} ±1.35	2.47 ±1.41	11.26^{ab} ±2.57	2.57 ±1.48
Semen+ media+ lipofectin +30µg-DNA	5.03^{a} ±1.28	1.28^{ab} ±1.16	3.75^{ab} ±1.35	3.75 ±1.41	13.82^{a} ±2.57	1.23 ±1.48
Generation (Mean ± SE)	3.87^{a} ± 0.91	2.24 ±0.47	3.07 ±0.55	2.67 ±0.58	11.71 ±1.05	1.44 ±0.60

Means of treatments within each generation and those of different treatments or generations having different superscripts are significantly different.

Improving average of body weight of Mamourah transgenic chicken was higher at the first generation than at the second generation.

Body weight of Mamourah transgenic chicken was steadily increased, as chicks grew older. However, the rate of increment differed due to the level of introduced foreign DNA. Applying foreign DNA level of 25µg showed higher averages of body weight than did at either the level of 20 µg or 30 µg.

These results agree with those reported by El-Naggar (2002); Ali (2001); El-Garhy (2004); El-Tahawy (2005); El-Khalea (2005); Ahmed (2006) and Mohamed (2009) who all found that introducing foreign DNA into chicken embryonic cells significantly increased body weight of transgenic chicks.

However, these results disagree with those of El-Fiky and Mehana (1998) who reported that injection of Bacterial DNA from *Streptococcus agalactia* into the eggs of Gimmizah eggs did not caused any significant differences in hatch body weight and up to 8weeks of age.

Rate of growth

Highly significant variation was mostly found in growth rate of Mamourah transgenic chicken due to either introducing of broiler hybrid-DNA level or the generation.

Table 5. Average body weight (g) for different experimental groups during two successive generations (least-square means± standard error)

Treatment (Artificial inseminated with)	1st generation								
	Body weight (g) at								
	Hatch	1st m	2nd m	3rd m	4th m	5th m	S.M	Peak	Molt
Semen	35.67^{c} ±0.48	206.7^{cd} ±7.12	575.7^{cd} ±12.21	946.1^{c} ±26.21	1296.2^{cd} ±26.76	1719.2^{cd} ±29.67	1940.9^{edf} ±29.76	2089.9^{b} ±29.93	2224.3^{c} ±30.00
Semen+media	35.66^{c} ±0.49	206.5^{cd} ±7.31	573.7^{cd} ±12.54	951.6^{c} ±26.89	1294.0^{cd} ±27.46	1718.0^{cd} ±30.46	1940.7^{edf} ±30.54	2090.0^{b} ±30.72	2224.3^{c} ±30.79
Semen+media+lipofectin	37.72^{a} ±0.54	208.2^{cd} ±7.98	568.3^{cd} ±13.67	926.6^{c} ±29.35	1265.9^{d} ±29.96	1676.2^{ed} ±33.23	1925.2^{ef} ±33.32	2083.8^{b} ±33.52	2207.9^{c} ±33.59
Semen+media+lipofectin+20µg-DNA	35.85^{bc} ±0.66	228.6^{bc} ±9.77	593.7^{bcd} ±16.74	967.1^{abc} ±35.94	1367.9^{bc} ±36.69	1800.2^{c} ±40.70	2050.9^{bc} ±40.81	2151.2^{b} ±41.05	2350.0^{b} ±41.14
Semen+media+lipofectin+25µg-DNA	36.12^{abc} ±0.58	276.6^{a} ±8.54	640.1^{a} ±14.63	1060.1^{a} ±31.41	1535.0^{a} ±32.07	2064.9^{a} ±35.56	2192.9^{a} ±35.66	2383.6^{a} ±35.87	2605.4^{a} ±35.95
Semen+media+lipofectin+30µg-DNA	37.57^{ab} ±0.72	249.4^{b} ±10.71	607.7^{abc} ±18.34	965.3^{abc} ±39.37	1418.6^{b} ±40.20	1908.6^{b} ±44.58	2111.1^{abc} ±44.71	2170.0^{b} ±44.97	2438.6^{b} ±45.07
Generation (Mean ± SE)	36.43 ±0.2	229.3^{a} ±3.5	593.2 ±6.1	969.9 ±11.8	1365.3 ±12.0	1814.5^{a} ±14.7	2026.9 ±14.8	2161.4 ±14.9	2341.8 ±14.9
Treatment (Artificial inseminated with)	2nd generation								
	Body weight (g) at								
	Hatch	1st m	2nd m	3rd m	4th m	5th m	S.M	Peak	Molt
Semen	35.59^{c} ±0.51	166.5^{e} ±7.51	558.5^{d} ±12.88	960.1^{bc} ±27.64	1301.4^{cd} ±28.22	1625.8^{ed} ±31.30	1896.1^{f} ±31.39	2095.1^{b} ±31.57	2229.2^{c} ±31.65
Semen+media	35.83^{bc} ±0.51	168.2^{e} ±7.51	560.9^{d} ±12.88	969.0^{abc} ±27.64	1304.2^{cd} ±28.22	1631.4^{ed} ±31.30	1901.7^{f} ±31.39	2100.7^{b} ±31.57	2234.8^{c} ±31.65
Semen+media+lipofectin	37.54^{ab} ±0.52	167.4^{e} ±7.74	555.8^{d} ±13.25	928.5^{c} ±28.46	1266.4^{d} ±29.05	1575.7^{e} ±32.22	1875.7^{f} ±32.31	2084.0^{b} ±32.51	2206.7^{c} ±32.58
Semen+media+lipofectin+20µg-DNA	36.15^{abc} ±0.54	188.3d^{e} ±8.04	576. 9^{bcd} ±13.78	967.5^{abc} ±29.58	1386.3^{bc} ±30.20	1709.8^{cd} ±33.50	2011.9^{cde} ±33.59	2158.1^{b} ±33.79	2359.7^{b} ±33.86
Semen+media+lipofectin+25µg-DNA	36.17^{abc} ±0.53	237.7^{b} ±7.92	620.6^{ab} ±13.56	1055.9^{ab} ±29.12	1526.9^{a} ±29.73	1953.4^{b} ±32.97	2130.3^{ab} ±33.06	2373.4^{a} ±33.26	2596.1^{a} ±33.33
Semen+media+lipofectin+30µg-DNA	37.29^{abc} ±0.56	203.9^{cd} ±8.24	594.2^{bcd} ±14.12	938.7^{c} ±30.32	1406.4^{b} ±30.96	1786.8^{c} ±34.34	2037.8^{bcd} ±34.44	2146.6^{b} ±34.64	2422.9^{b} ±34.71
Generation (Mean ± SE)	36.4 ±0.2	188.7^{b} ±3.2	577.8 ±5.5	969.5 ±13.0	1362.9 ±13.3	1713.8^{b} ±13.3	1975.6 ±13.4	2159.7 ±13.4	2341.6 ±13.5

Means of treatments within each generation and those of different treatments or generations having different superscripts are significantly different.

Where; S.M = Sexual Maturity Peak = Peak of egg production m = month.

Table 6. Average growth rate (%) for different experimental groups during two successive generations (least-square means± standard error).

Treatment (Artificial inseminated with)	1st generation							
	Growth rate(%) at							
	1st m	2nd m	3rd m	4th m	5th m	S.M	Peak	Molt
Semen	138.0^{bc} ±2.31	89.9^{cd} ±2.84	34.6^{b} ±2.16	49.8^{a} ±1.16	28.9^{d} ±0.79	10.4^{e} ±0.39	8.7^{b} ±0.22	6.0^{c} ±0.22
Semen+ media	136.9^{bc} ±2.37	93.5^{bcd} ±2.93	46.3^{a} ±2.22	32.8^{def} ±1.19	29.2^{d} ±0.82	12.5^{d} ±0.40	7.6^{cd} ±0.23	6.4^{c} ±0.23
Semen+ media+ lipofectin	134.3^{c} ±2.59	93.9^{bcd} ±3.18	47.2^{a} ±2.42	31.5^{f} ±1.30	28.2^{d} ±0.89	13.9^{c} ±0.44	8.0^{bc} ±0.25	5.8^{c} ±0.25
Semen+ media+ lipofectin +20µg-DNA	142.5^{b} ±3.17	89.4^{cd} ±3.89	47.0^{a} ±2.96	34.7^{cdef} ±1.59	27.5^{d} ±1.09	13.2^{cd} ±0.54	4.8^{e} ±0.31	8.9^{b} ±0.30
Semen+ media+ lipofectin +25µg-DNA	152.6^{a} ±2.77	79.5^{e} ±3.41	49.0^{a} ±2.59	37.0^{bc} ±1.39	29.3^{d} ±0.95	6.1^{g} ±0.47	8.4^{b} ±0.27	9.0^{b} ± 0.27
Semen+ media+ lipofectin +30µg-DNA	144.0^{b} ±3.47	84.7^{de} ±4.27	44.3^{a} ±3.25	38.7^{bc} ±1.74	29.8^{d} ±1.19	10.2^{e} ±0.59	2.8^{f} ±0.34	11.8^{a} ± 0.33
Generation (Mean ± SE)	141.4^{a} ±1.15	102.5^{a} ±1.28	49.2^{a} ±0.97	37.4^{a} ±0.58	40.9^{a} ±0.36	14.6^{a} ±0.18	9.1^{a} ±0.10	8.1^{a} ±0.10

Table 6. cont.

Treatment (Artificial inseminated with)	2nd generation							
	Growth rate(%) at							
	1st m	2nd m	3rd m	4th m	5th m	S.M	Peak	Molt
Semen	122.2^{d} ±2.44	107.9^{a} ±2.99	49.8^{a} ±2.28	32.5^{ef} ±1.22	40.9^{ab} ±0.84	15.9^{b} ±0.42	10.2^{a} ±0.24	6.4^{c} ±0.24
Semen+ media	123.3^{d} ±2.45	107.0^{a} ±3.02	50.7^{a} ±2.29	31.7^{f} ±1.23	41.2^{ab} ±0.84	15.8^{b} ±0.42	10.2^{a} ±0.24	6.3^{c} ±0.24
Semen+ media+ lipofectin	118.6^{d} ±2.51	108.9^{a} ±3.09	49.6^{a} ±2.35	31.4^{f} ±1.26	39.4^{bc} ±0.86	17.6^{a} ±0.43	10.6^{a} ±0.24	5.7^{c} ±0.25
Semen+ media+ lipofectin +20µg-DNA	130.9^{c} ±2.61	102.2^{ab} ±3.21	49.3^{a} ±2.44	36.2^{cde} ±1.31	37.9^{c} ±0.89	16.4^{ab} ±0.44	7.1^{d} ±0.25	9.0^{b} ±0.25
Semen+ media+ lipofectin +25µg-DNA	145.2^{ab} ±2.57	89.6^{cd} ±3.16	51.7^{a} ±2.39	36.8^{bcd} ±1.29	43.2^{a} ±0.88	8.9^{f} ±0.44	10.9^{a} ±0.25	9.0^{b} ±0.26
Semen+ media+ lipofectin +30µg-DNA	132.3^{c} ±2.67	99.2^{abc} ±3.29	44.2^{a} ±2.49	40.4^{b} ±1.34	42.8^{a} ±0.92	13.3^{cd} ±0.46	5.3^{e} ±0.26	12.2^{a} ±0.26
Generation (Mean ± SE)	128.8^{b} ±1.04	88.5^{b} ±1.41	44.7^{b} ±1.07	34.8^{b} ±0.52	28.8^{b} ±0.39	11.17^{b} ±0.19	6.7^{b} ±0.11	8.0^{b} ±0.11

Means of treatments within each generation and those of different treatments or generations having different superscripts are significantly different.

Where; S.M = Sexual Maturity Peak = Peak of egg production m = month

Mamourah chicken showed higher growth rate in birds of the first generation than in those of the second one. Moreover, chicks hatched from eggs resulted from pullets artificially inseminated with genetic modified semen had higher growth rate than controls.

Applying foreign DNA at level of 25µg improved growth rate than did the other two levels (20 and 30 µg) applied at the 1st, 3rd and 5th month of age (Table 6).

It was clearly evident that growth rate was gradually decreased as birds grew older reached its minimum level at the peak of egg production. This goes in agreement with the general growth pattern in fowl. It is well known that growth rate depends mainly on the rate of biological action of either growth hormone and hormones related to metabolic activity in general and those sharing in the anabolic processes in particular. All of them play a part in attaining growth that is higher during the early age and diminished as bird reach sexual maturity or the age of sexual hormones activity that control productivity during the period of egg production.

It could be concluded that the increasing occurred in average body weight, body weight gain and rate of growth of Mamourah transgenic birds may be attributed to the successful transfer of some transmitted genes that closely related to the enzymatic activity which optimizing the growth performance. This statement was previously emphasized by Ali (2001).

These results agree with those obtained by Ali (2001), El-Naggar (2002), El-Garhy (2004), El-Khalea (2005), Ahmed (2006) and Mohamed (2009) who all reported that introducing of foreign DNA level into embryonic cells of chicken fertilized eggs significantly increased average body weight, body weight gain and rate of growth of transgenic chicken estimated at different ages of birds.

Conclusion

It could be concluded that introducing the foreign DNA level of broiler chicken into the embryonic cells of Mamourah chickens by using sperm mediated gene technique increased the fertility percentage, embryonic mortality as well as the percentage of embryonic malformation which increased to reach its highest magnitude when the level of foreign DNA reached 30µg. However, the hatchability percentage and growth performance were improved due to the foreign DNA applied.

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Effect of heavy metals pollution on histopathological alterations in muscles of *Clarias gariepinus* inhabiting the Rosetta branch, River Nile, Egypt

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Abstract

The heavy metals (iron, copper, lead, manganese, zinc and cadmium) concentrations in the water of El-Kanater El-Khyria and El-Rahawy drain sites as well as the accumulation of these metals in skin and muscles of the bony fish *Clarias gariepinus* living in that water were seasonally estimated during the year 2009. Also, the histological alterations of the skin and muscles of the same fish species were studied. The obtained results showed that the heavy metals concentrations in water and their accumulation in fish muscles obtained from El-Rahawy drain region during the four seasons were higher than those obtained from El-Kanater El-Khyria station. The histopathological study of fish skin and muscles showed marked signs of necrosis, accumulation of hemosidren pigment, hemorrhage, hemolysis and presence of parasitic forms. It has been noticed that skin and muscles of fish living in El-Rahawy drain water suffered from much more severe damage than those living in El-Kanater El-Khyria water which may be due to input of sewage at El-Rahawy drain in the Rosetta branch. It could be recommended that treatment of the sewage and domestic wastes before their discharge into the River Nile is a necessary process to protect the fish and the citizens from the danger of pollution, especially heavy metals pollutants.

Key words: Heavy metals, River Nile, Muscles, Histopathology, Necrosis, Degeneration, Hemorrhage.

Introduction

Aquatic systems are very sensitive to heavy metal pollutants. Determination of trace metal concentration in natural water system has received increasing attention for monitoring environmental pollution, due to the fact that some metals are not biodegradable and their presence in the food chain through a number of pathways may be accumulated in different organs of human beings or animals (Korai *et al.*, 2008). Therefore, in order to maintain the quality of aquatic product, it is important to regularly monitor and evaluate the pollution levels in fish. Water reservoirs are collectors of all materials spread by human, sewage, industrial and agricultural activities, and these penetrate into water reservoirs via atmosphere, drainage, soil waters and soil erosion (Staniskiene *et al*., 2006). As the concentration of heavy metals in the environment increases, the metals inevitably enter the biogeochemical cycle and can cause damage health or even death at highly concentrations (Bialy *et al.*, 2005; Weher, 2008 and Olgunoğlu and Olgunoğlu, 2011). Wastes discharged into the River Nile are extremely complex. The most remarkable types of these wastes are toxic due to non-microbial pollution and pathogens as a result of microbial pollution that present in water at dangerous amounts than permissible (Chiu *et al*., 2010). Many metals, particularly heavy metals are toxic at high doses, but some heavy metals such as iron and copper are essential for the growth and well-being of living organisms including man. Other elements such as lead and cadmium are not essential for metabolic activities or growth and exhibit toxic properties and inhibited the photosynthesis, phytoplankton and fish growth at low concentrations (FAO, 1992). Trace elements may be accumulated in fish lived in polluted water via their food chain, thus possibly endangering human health (Mahmoud, 2002). Several works were conducted to study the effect of water pollution on fish organs histology and to estimate the amount of accumulated heavy metals on different fish organs (Ibrahim and Mahmoud, 2005; Sitohy *et al.*, 2006; Bayomy and Tayel, 2007; Tayel *et al.*, 2008; Yacoub *et al.*, 2008; El-Naggar *et al.*, 2009; Mol *et al*., 2010; Barbieri *et al*.,2010; Olgunoğlu and Olgunoğlu, 2011; Abdel-Warith *et al.*, 2011 and Abdel-Tawwab *et al*., 2011). The concentration of heavy metals in different organs and tissues are varied considerably with regard to seasons and sites (Abdel-Baky *et al*., 1998). Although muscles are the most edible part of fish body, it is the most exposed part to be damaged by several types of pollution (Tayel, 2003; El-Serafy *et al.*, 2005 and Sitohy *et al.*, 2006). The epidermis has been shown to contain naturally bacteriolytic substances and to contain a continuous reacting immune system. Thus the affected epidermis lost its protective function as well as its efficiency as an osmotic barrier. If fish have epithelial lesion in polluted water, they would probably be invaded by micro-organisms which cause severe epidermal pathology resulting in skin and underlying musculature destruction (Bayomy and Tayel, 2007). El-Naggar *et al*., (2009) studied the bioaccumulation of some heavy metals and histopathological alterations in liver of Oreochromus niloticus in relation to water quality at different localities along the River Nile. They showed that heavy metals accumulations in fish liver at area under investigation were detected in following descending order: Fe> Cu>

Zn> Mn> Pb> Cd. Also histological study indicated that the liver of Oreochromus *niloticus* collected from the same investigated area showed several pathological alterations including: degeneration, fatty degeneration, necrosis and edema. Also congestion, branching (anastimosis), hemorrhage, hemolysis, hemosidrin accumulation and parasitic forms were seen in blood vessels. It was noticed that the liver of fish collected from Shoubra El-Khema and El-Rahawy drain showed much more damages than that collected from the other stations as these sites receives more drainage water that is much more loaded with industrial and sewage wastes than the other stations.

The present study was mainly conducted to assess the heavy metals accumulation in *Clarias gariepinus* skin and muscles and their concentrations in the surrounding water to elucidate the effect of these elements on the histology of skin and muscles.

Material and methods

Water samples and the selected fish *Clarias gariepinus* were collected from studied area (El-Kanater El-Khyria and El-Rahawy drain down stream) during the four seasons of 2009 to carry out the present study.

Measurement of heavy metals in water

A quantity of twenty ml of concentrated nitric acid was added to 500 ml of the water sample in a beaker and boiled on a hot plate until complete digestion. The remaining volume was made up to 100 ml with deionized distilled water. A portion of this solution was used for the determination of heavy metals. The metals (iron, copper, lead, manganese, zinc and cadmium) were determined using Graphite Atomizer (G.A.Z.) atomic absorption spectrophotometer (Hitachi model 170-30) and the readings were compared to a standard curve. The results are expressed in µg / l.

Determination of heavy metals accumulation in fish skin and muscles

The collected skin and muscle samples of the selected fish were transferred to weighing beakers and placed in a drying oven thermostatically regulated at 105 ^{0}C overnight. Dried samples (1 g of each sample) were taken and digested according to the method described by Goldberg *et al.,* (1963) in which concentrated nitric and perchloric acids, with 1 : 1 ratio, were used. The digested solutions were cooled and made up to 25 ml using deionized distilled water. The concentrations of heavy metals (iron, copper, lead, manganese, zinc and cadmium) in solutions were determined as described above. The results were expressed in µg dry weight/g body weight.

Histological studies on skin and muscles of fish samples

Other part of skin and muscles from each fish sample were carefully removed and fixed in 10% formalin, dehydrated in ascending grades of alcohol and cleared in xylene. The fixed tissue was embedded in paraffin wax and sections of 5 micrometers were cut, using Euromex Holland Microtome and stained according to Harris Hematoxylin and Eosin method (Kadry, 1985). Then the sections were examined microscopically and photographed by a microscopic camera.

Statistical analysis

Statistical analysis of data obtained was carried out by applying the computer program, SAS (2002). Differences between means were tested for significance ($P< 0.05$) according to Duncan's multiple range test as described by Duncan (1955).

Results and discussion

Heavy metals in water

The concentrations of heavy metals (iron, copper, lead, manganese, zinc and cadmium) in the water of El-Kanater El-Khyria and El-Rahawy drain down stations during 2009 were tabulated in table (1). Iron concentration at the two studied sites recorded the highest values were 1017.1 µg/l ±0.1 at El-Rahawy drain down and 597.1 µg/l ±0.1 at El-Kanater El-Khryai during summer and spring (hot seasons) respectively. The elevated iron concentration of El-Rahawy drain is attributed to the release of iron from the sediment during the dissolution of iron (Kadry *et al*., 2003 and Tayel *et al*., 2008). While the lowest values were 876.1 µg/l ±0.1 and 464.1 µg/l ±0.1 during winter at El-Rahawy drain down and El-Kanater El-Khryai (cold season).This might be attributed to the increase in dissolved oxygen which leads to oxidation of iron. This finding is in agreement with the results obtained by Abdel-Satar (2001) and Bayomy and Tayel (2007).

Copper concentration ranged between 4.15 ±0.08 µg/l and 9.45 ±0.08 µg/l at El-Kanater El-Kahyria during summer and autumn, while at El-Rahawy drain down it ranged from 21.45 ±0.08 µg/l to 30.75 ±0.08 µg/l during winter and spring season. The high concentration of copper at El-Rahawy drain in this study may be due to domestic sewage discharges where the domestic sources were the major contributors of copper in the environment or may be due to $CuSO_4$ herbicide to prevent the infection of man with bilharzial diseases (Massoud *et al.,* 1994; Abdel-Satar, 2005 and Tayel *et al*., 2008). The correlation matrix for copper showed significant positive with Fe (r= +0.941) as shown in table (3).

Lead concentration showed that the lowest value was obtained during autumn (34.35 ±0.07 µg/l) at El-Kanater El-Khyria and the highest value during winter (72.2 ±0.07 µg/l) at El-Rahawy drain down.

The elevation of lead concentration in this study may be due to the increase in density of boats and ships which discharge their effluent directly to the River Nile containing high amount of lead in both the dissolved and particular phase (Moon *et al.,* 1994; Ibrahim, 2007 and Bayomy and Tayel, 2007).

Table 3. Correlation coefficient matrix of some heavy metals in the water during four seasons in the studied areas during 2009

	Fe	Cu	Pb	Mn	Zn	Cd
Fe	1.0000					
Cu	0.941	1.0000				
Pb	0.433	0.258	1.0000			
Mn	0.45	-0.031	0.229	1.0000		
Zn	0.886	0.941	0.448	-0.013	1.0000	
Cd	0.982	0.962	0.328	-0.019	0.888	1.0000

The results of manganese values varied from 78.5 ±0.065 to 180.0 ±0.065 µg/l during winter and autumn and 121.0 ±0.065 to 174.0 ±0.065 µg/l during summer and autumn at El-Kanater El-Khyria and El-Rahawy drain down, respectively. The high concentration of manganese in the present study may be due to the breakdown of organic matter and dead micro-organisms with subsequent release of the metal into water (Prince, 1994). Massoud *et al.*, (1994) and Ibrahim (2007) reported that the concentration of manganese is higher in the presence of suspended organic matter, while the low concentration of manganese may be related to uptake of manganese by phytoplankton.

The values of zinc concentration ranged between 12.5 ±0.34 µg/l at El-Kanater El-Khyria and 81.7 ±0.34 µg/l at El-Rahwy drain down during spring season. The high concentration of zinc at El-Rahawy drain may be attributed to adsorption of zinc hydroxide which binds to organic matter (Abdel-Satar, 1998 and Ibrahim, 2007). The correlation coefficient matrix showed that, a highly significant positive with iron and copper (r= +0.886 and +0.941).

The values of cadmium fluctuated within a wide range 2.1 ±0.09 to 2.9±0.09 µg/l at El-Kanater El-Khyria during autumn and spring seasons, whereas they were between 7.45 ±0.09 and 9.0 ±0.09 µg/l at El-Rahawy drain down during winter, autumn and summer seasons. The high concentration of cadmium in the present study may be due to the direct input of sewage and agriculture wastes (Ibrahim, 2007 and Tayel *et al*., 2008). The correlation coefficient matrix showed a highly significant positive with iron and copper (r= +0.982 and +0.962).

Bioaccumulation of heavy metals in muscles of *Clarias gariepinus*

Bioaccumulation of heavy metal does not only depend on the structure of the organ but also on the interaction between metals and the target organs (Sorensen, 1991). The relationship between the concentrations of trace metals in water and their accumulation in *Clarias gariepinus* skin and muscles was indicated in the present study. It can be noticed that fish accumulates trace metals in the skin and muscles with lower quantities than those found in ambient water.

The concentration of heavy metals (iron, copper, lead, manganese, zinc and cadmium) in the skin and muscles of the *Clarias gariepinus* which were obtained from El-Kanater El-Khyria and El-Rahawy drain stations during 2009 were shown in table (2).

The values of iron accumulation in skin and muscles of *Clarias gariepinus* ranged between 115.1 ±0.24 µg/g dry wt. at El-Kanater El-Khyria and 268.2 ±0.24 µg/g dry wt. at El-Rahawy drain down stream which were higher than the permissible limit (30.0 µg/g, recorded by FAO, 1992). The increase of iron concentration in skin and muscles of the selected fish might be attributed to increase of total dissolved iron in Nile water causing increase the free metal iron concentration and accordingly increased iron uptake by different fish organs (Ibrahim and Mahmoud, 2005).

Copper concentrations of skin and muscles of the selected fishes in the present study were found to be 3.9 and 13.9 ±0.5 µg/g at El-Kanater El-Khyria and from 4.9 µg/g to 23.0 ±0.5 µg/g at El-Rahawy drain down stream. These results agree with those obtained by El-Serafy *et al*., (2005), who revealed that this increase is anticipated to drainage and sewage effluents.

The concentration of lead and manganese in skin and muscles showed that the lowest values were obtained during summer (16.6 µg/g ±0.2 and 18.8 µg/g ±0.25) at El-Kanater El-Khyria and the highest values during winter were 46.3 µg/g ±0.2 and 77.1 µg/g ±0.25 at El-Rahawy drain down stream.

The obtained values of lead concentration in skin and muscles of selected fish in the present study were higher than the permissible limit (2.0 µg/g) and the values of manganese concentration were higher than the permissible limit (30.0 µg/g) at El-Rahawy drain down, recorded by FAO (1992). The high level of lead may be attributed to high lead concentration in water of the area under study (Tayel *et al*., 2008). The high concentration of manganese in skin and muscles may be due to large amounts of sewage discharged at El-Rahawy drain. The correlation matrix for lead and manganese showed significant positive with iron (r= +0.729), Cu (r= +0.798) and lead (r= +0.867) as shown in table (4).

The results indicated that zinc concentrations in skin and muscles ranged between 36.7 µg/g and 87.8 ±0.13 µg/g at El-Kanater El-Khyria and El-Rahawy drain down during autumn season, respectively. At El-Rahawy drain down, zinc levels in the investigated fish skin and muscles were higher than the permissible limit (50.0 µg/g) recorded by FAO (1992). The change of zinc accumulation in muscles during the period of study related to its fluctuation in water during the same period. The present results are in accordance with Gomaa (1995) and Tayel (2007).

The correlation matrix for zinc showed significant positive with iron (r= +0.947) in table (4).

Table 4. Correlation coefficient matrix of some heavy metals in the muscles of *Clarias garipinus* during four seasons in the studied areas during 2009

	Fe	Cu	Pb	Mn	Zn	Cd
Fe	1.0000					
Cu	0.597	1.0000				
Pb	0.729	0.798	1.0000			
Mn	0.471	0.662	0.867	1.0000		
Zn	0.947	0.513	0.663	0.407	1.0000	
Cd	0.654	-0.182	0.223	0.04	0.669	1.0000

Cadmium concentrations ranged from 4.7 µg/g to 15.75 µg/g ±0.15 at El-Kanater El-Khyria and El-Rahawy drain down during winter and summer. Cadmium level were generally higher than the permissible limit (5.0 µg/g), as recorded by FAO, 1992. The obtained high level of cadmium accumulation in skin and muscles of selected fish in the present study may be due to its strong binding with cystine residues of metallothionein (Abu El-Ella, 1996 and Tayel *et al*., 2008). The correlation coefficient matrix showed that, a highly significant positive with zinc (r= +0.669).

The increments of iron, copper, lead, manganese, zinc and cadmium level in the water and fish may be due to input of sewage and agricultural wastes at El-Rahawy drain in the Rosetta branch.

Histological studies of skin and muscles

Normally, the skin is composed of, dermal and hypodermal layers. The skin covers the muscles layer which is composed chiefly of segmental myomeres. Each myomere is regarded as muscle and its fibers are parallel the long axis of the body (Bayomy and Tayel, 2007). In the present study, the major morphological and histological changes in skin and muscles of *Clarias gariepinus* caught from the two studied areas during the four seasons are illustrated in figures (1 and 2).

The morphological changes

Some specimens of *Clarias gariepinus* especially these obtained from El-Rahawy area showed abnormal skin with white or dark spots and destroyed dorsal fin. Also, the skin of some specimens was covered with mucous fluid.

The histological changes

Several changes were observed in skin and muscles of the collected samples such as: Hypertrophy in mucous cells, degeneration, necrosis, hemosidrin accumulation and hemorrhage in collagen bundles. Degeneration in connective tissues layer, parasitic form, degeneration, necrosis, hemorrhage, hemosidrin, edema in muscles fiber. These malformations appeared in severe conditions in fish specimens caught from El-Rahawy region during hot seasons. Meanwhile, these results are in accordance with those obtained by El-Mansy (2005) and Bayomy and Tayel (2007).

The morphological and histological changes of skin and muscle specimens observed in from the fish caught from the El-Rahawy area were of higher degree than those obtained from the same fish species caught from El-Kanater El-Khyria area. Also, these changes appeared more severe during hot seasons in the two regions than those obtained during cold seasons. This might be due to high level of heavy metals concentrations in water and hence these heavy metals have been accumulated in fish skin and muscles. These results are in agreement with those obtained by Mabrouk (2004); El-Mansy (2005); El-Serafy *et al.* (2005); Sitohy *et al*. (2006) and Tayel (2007).

Conclusion

The present study revealed that water was contaminated at El-Rahawy drain region with some heavy metals which accumulate in skin and muscles of *Clarias gariepinus*. This caused some morphological and histopathological changes in skin and muscles of the studied fish.

Recommendation

Finally, according to the obtained results, it could be recommended that, firstly, treatment of sewage wastes before entrance to the River Nile is a necessary procedure to protect the fish and the people from the dangerous effects of such pollution. Secondly, preventing the discharge of sewage wastes and dead animal bodies into the Nile. Thirdly, cultivation of special kinds of algae which have the ability to absorb heavy metals and culturing fishes feeding on weeds.

Table 1. Seasonal variations of heavy metals concentration (µg/l) in the River Nile water at El-Katanter El-Khyria and El-Rahawy drain down during, 2009

Seasons	Winter		Spring		Summer		Autumn		±SE
Site / Parameters	Mean		Mean		Mean		Mean		
	I	II	I	II	I	II	I	II	
Iron µg/l	464.1^{h}	876.1^{d}	597.1^{e}	988.0^{b}	592.0^{f}	1017.1^{a}	488.5^{g}	918.1^{c}	±0.1
Copper µg/l	6.25^{f}	21.45^{d}	4.55^{g}	30.75^{a}	4.15^{h}	27.0^{b}	9.45^{e}	24.85^{c}	±0.08
Lead µg/l	37.35^{g}	72.2^{a}	72.1^{b}	66.35^{c}	48.5^{e}	50.5^{d}	34.35^{h}	44.0^{f}	±0.07
Manganese µg/l	78.5^{h}	145.0^{f}	177.85^{b}	157.1^{e}	170.0^{d}	121.0^{g}	180.0^{a}	174.0^{c}	±0.65
Zinc µg/l	15.75^{f}	62.75^{b}	12.5^{h}	81.7^{a}	15.35^{g}	51.0^{c}	19.55^{e}	47.55^{d}	±0.34
Cadmium µg/l	2.55^{e}	7.45^{c}	2.9^{d}	8.8^{b}	2.9^{d}	9.0^{a}	2.1^{f}	9.0^{a}	±0.09

I: El-Kanater El-Khyria II: El-Rahawy drain down

* Data are presented as means ± standard error (SE). * Means followed by different letters in each column are significantly different ($p<0.001$).

Table 2. Seasonal variations of heavy metals accumulation in muscles (µg/g dry wt.) of *Clarias garipinus* inhibiting at El-Katanter El-Khyria and El-Rahawy drain down during, 2009

Seasons	Winter		Spring		Summer		Autumn		
Site / Parameters	Mean		Mean		Mean		Mean		±SE
	I	II	I	II	I	II	I	II	
Iron µg/g	115.1^{h}	194.0^{c}	155.6^{e}	226.4^{b}	119.4^{g}	190.5^{d}	130.4^{f}	268.6^{a}	±0.24
Copper µg/g	13.9^{c}	21.7^{b}	10.75^{f}	13.45^{d}	3.9^{h}	4.9^{g}	10.9^{e}	23.0^{a}	±0.5
Lead µg/g	23.45^{e}	46.3^{a}	18.6^{f}	27.1^{d}	16.6^{h}	28.45^{c}	17.0^{g}	41.0^{b}	±0.2
Manganese µg/g	27.3^{e}	77.1^{a}	30.1^{d}	37.8^{c}	18.8^{g}	34.35^{d}	21.0^{f}	38.0^{b}	±0.25
Zinc µg/g	41.4^{g}	61.9^{d}	61.3^{e}	69.9^{b}	47.5^{f}	63.4^{c}	36.7^{h}	87.8^{a}	±0.13
Cadmium µg/g	4.7^{h}	8.2^{e}	8.0^{f}	15.0^{b}	11.1^{d}	15.75^{a}	5.8^{g}	12.75^{c}	±0.15

I: El-Kanater El-Kahyria II: El-Rahawy drain down

* Data are presented as means ± standard error (SE). * Means followed by different letters in each column are significantly different ($p<0.001$).

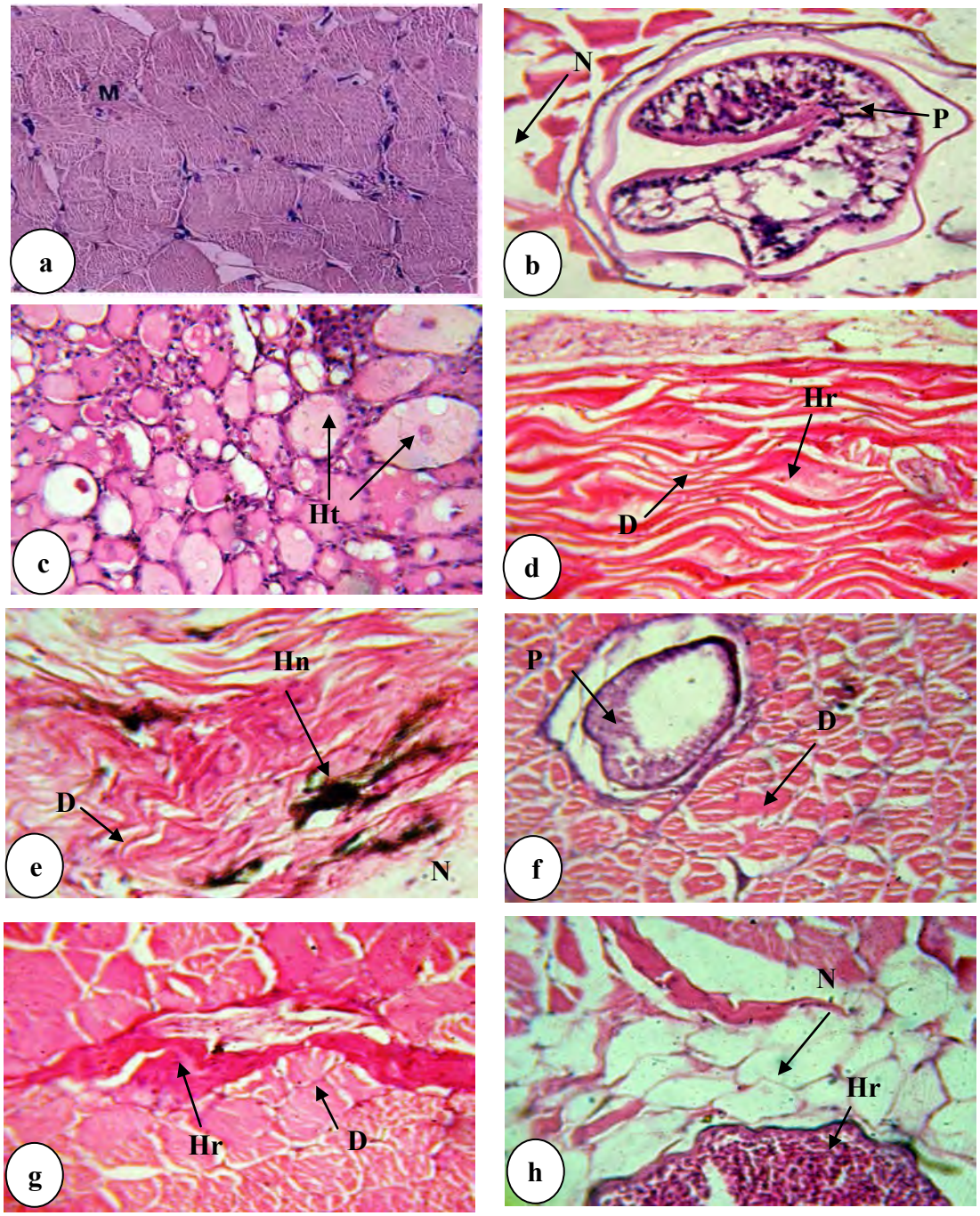

Figure (**1**).V.S. of skin and muscles of *Clarias gariepinus* obtained from El-Kanater El-Khyria region (Bouin's –H&E X400) showing:

(**a**): Normal skin and muscles of *C. gariepinus* showing muscle fiber (M)
(**b**): Parasitic cyst (P) and necrosis (N) in muscle fibers.
(**c**): Hypertrophy (Ht) in mucous cells.
(**d**): Degeneration (D) and hemorrhage (Hr) in collagen bundles.
(**e**): Degeneration (D), hemosidrin (Hn) and necrosis (N) in collagen bundles.
(**f**): Parasitic cyst (P) and Degeneration (D) in muscle fiber.
(**g**): Degeneration (D) and hemorrhage (Hr) in muscle fiber.
(**h**): Hemorrhage (Hr) and necrosis (N) in muscle fiber.

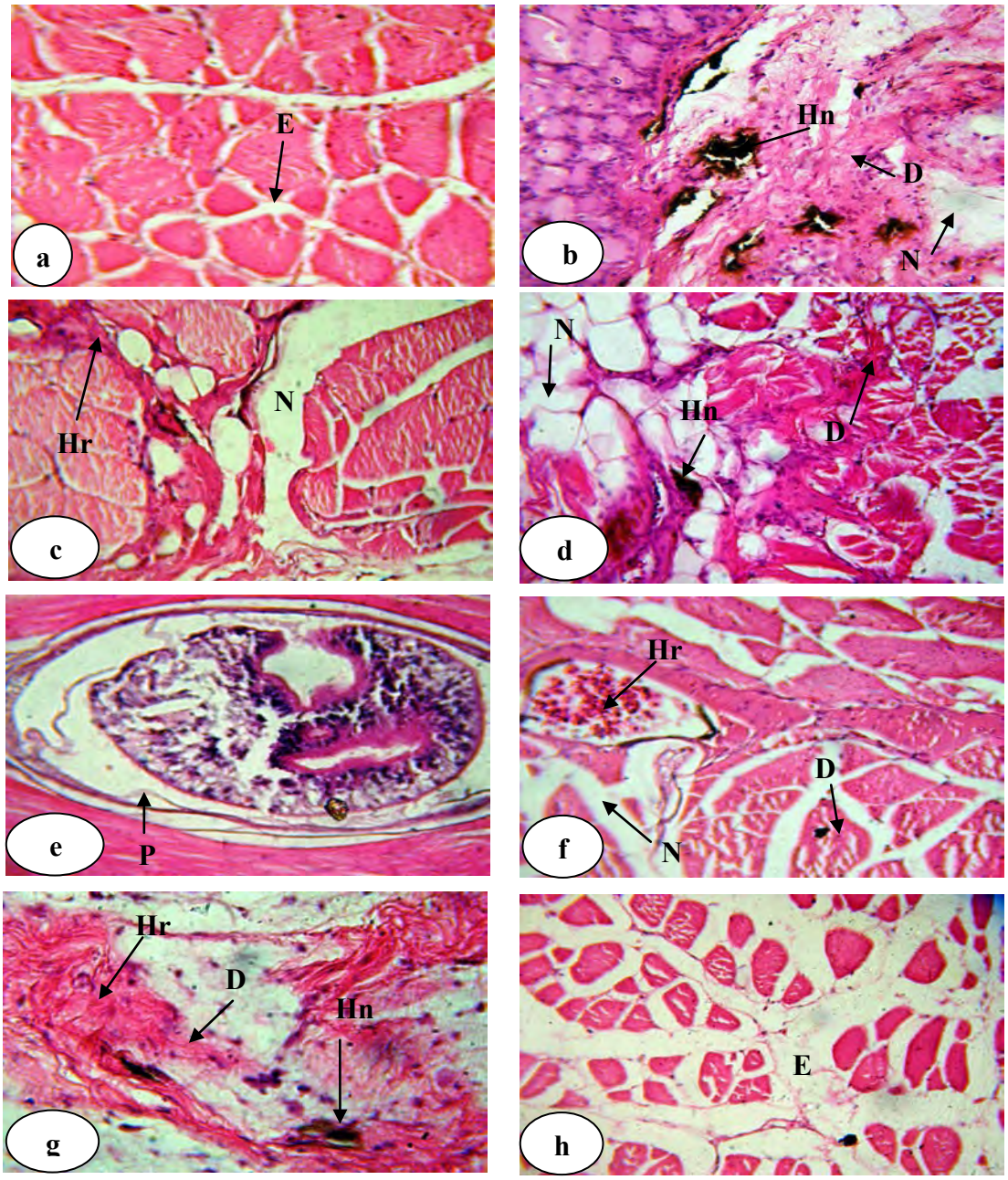

Figure (**2**). V.S. of skin and muscles of *Clarias gariepinus* obtained from El-Rahawy drain region (Bouin's – H&E X400) showing:
(**a**): Edema (E) in muscle fiber.
(**b**): Degeneration (D), hemosidrin (Hn) and necrosis (N) in collagen bundles.
(**c**): Hemorrhage (Hr) and necrosis (N) in muscle fiber cells .
(**d**): Degeneration (D) in muscle fiber and necrosis (N) and hemosidrin (Hn) in connective tissue layer
(**e**): Parasitic form (P) in muscle fiber.
(**f**): Degeneration (D) and hemorrhage (Hr) in muscle fiber.
(**g**): Degeneration (D) hemorrhage (Hr) and hemosidrin (Hn) in collagen bundles.
(**h**): Edema (E) in muscle fiber.

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Effect of superovulation on rabbit's ovarian response, normal embryos production and *in vitro* development of embryos post-thawed

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Abstract

Forty-one of multiparous New Zealand White rabbits were used as embryo donors, allotted in three groups. Does of the 1st group were treated with intramuscular injection of 20 µg GnRH/doe for induction of normally ovulation and artificially inseminated (control). Embryo donors in the 2nd and 3rd groups were administered intramuscularly with 20 or 40 PMSG IU/kg live weight (superovulation), 68 h before artificially insemination. Immediately prior to insemination, the does injected intravenous with hCG at 40 IU/kg live weight. All does were slaughtered at 48-50 h after insemination. Embryos were recovered, counted, evaluated for their stage of development, morphological appearance then vitrified. Post 48 h storage in LN2, embryos were thawed and cultured *in vitro* with Ham's F10 medium+ 20% FCS for two days. Results showed that donors treated with 20 or 40 IU PMSG/kg live weight produced significantly ($P<0.01$) more CLs, haemorrhagic follicles and yielded three to four folds embryos/doe when compared with GnRH normally ovulated ones. Embryos recovered from normal ovulated does reflected a higher fertilization rate (96.6%) than those recovered from does treated with 20 (89.4%) or 40 (84.1%) IU PMSG/kg live weight. The percentage of morphological normal embryos recovered from donors injected with 20 or 40 IU PMSG/kg live weight (84.9 or 77.0%) was significantly ($P<0.05$) lower than that obtained from the normally ovulated does (97.4%). Treating donors with 40 IU PMSG/kg live weight retard the rate of embryonic development and reduced their diameters more than those injected with 20 IU PMSG/kg live weight compared to GnRH normally ovulated ones. In 40 IU PMSG treated donors-vitrified embryos group, only 61 from 147 embryo vitrified-thaw reached blastocysts (41.5%), whereas 112/186 (60.2%) in 20 IU PMSG treated does compared to 74/111 (66.7%) in control group. The present study demonstrates that treating rabbit does with 20 IU PMSG/kg live weight provides superior embryos survival rates over the 40 IU for rabbit embryos production manipulation studies and gene bank.

Key words: Rabbit, superovulation, ovarian response, embryo donors, vitrification.

Introduction

Ovulation in rabbit does can be induced by exogenous GnRH administration (intramuscularly and intravaginal, Rebollar *et al.*, 2012). Superovulation of rabbits is commonly used to synchronize estrus and to increase the number of oocytes or embryos for various reproductive and embryo manipulation studies. A single injection of PMSG at dosages from 40 to 150 IU has been frequently used for the induction of superovulation (Schmidt *et al.*, 1992) for collection of embryos for *in vitro* studies. For most domestic animals, the responses to superovulation treatments are not controlled as a consequence of the lack of knowledge on exogenous gonadotrophins effects on the ovarian function (Salvetti *et al.*, 2007). Structurally intact embryos and high morphological quality grade may be essential components for successful rabbit embryo cryopreservation. The induction of ovulation in mammals by administration of exogenous gonadotrophin may be responsible for an increased incidence of chromosomal abnormalities in the resulting embryos. Fujimoto *et al.* (1974) noted slight increases, but statistically insignificant, in chromosomal abnormalities in rabbit blastocysts and intratubal embryos obtained after superovulation, when compared with a control group. Only the PMSG dose was varied because the work of Shaver (1970) had indicated that variation in HCG dosage apparently did not affect the chromosome constitution of rabbit embryos. Therefore, the aim of this study was to compare the ovary response, the fertilizing aptitude, the morphological normal embryo production and their developmental stage pre and post vitrification of multiparous does having received 20 or 40 IU of PMSG/kg live weight, in comparison with a control group (GnRH injection)

Materials and methods

This experimental work was carried out at the laboratory of Reproduction and Biotechnology, Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt. Forty-one of multiparous New Zealand White rabbits were used as embryo donors in the present work. Animals were housed in individual metal cages for 3 weeks prior to hormonal treatments to eliminate the chances of pseudopregnancy. The animals were kept under controlled 16h light: 8h dark photoperiod and fed *ad-libitum* with a commercial pelleted diet contained 17.5 % crude protein, 13.6% crude fibers

and 2.5 % fat. Fresh potable water was made available all times through stainless nipples.

PMSG donors treatments, embryo collection and evaluation

Embryo donors, allotted in three groups. Does of the 1st group were treated with intramuscular injection of 20 µg GnRH/doe for induction of normally ovulation and artificially inseminated. Embryo donors in the 2nd and 3rd groups were administered intramuscularly with 20 or 40 PMSG IU/kg live weight (Folligon, Intervet, International B.V. Boxineer-Holland), 68 h before artificially insemination. Immediately prior to insemination, the does injected intravenous with hCG (Pregnyl, Organon, Nile Co., Egypt) at 40 IU/kg live weight. The females were inseminated with semen collected from a male of proven fertility from the same breed. All does were slaughtered at 48-50 h after insemination. The reproductive tracts (oviducts and uterine horns) were removed and embryos were recovered by flushing twice with 5 mL of Dulbecco´s Phosphate Buffered Saline (®DPBS, (PBS: Gibco, Cat. No 21300-017, UK) supplemented with $CaCl_2$ (0.132 g/L), 0.2% of bovine serum albumin (®BSA, Sigma Chemical Co., St. Louis, Mo, USA) and antibiotics (10,000 IU Penicillin G potassium+ 10 mg streptomycin sulfate/ml, Sigma) at room temperature (20-25 ºC). After recovery, embryos and oocytes were washed twice in fresh DPBS supplemented with 10% FCS and antibiotics, counted and evaluated for their stage of development, morphological appearance and their physical measurements under stereoscopic microscope. Embryos with no abnormalities in mucin coat, *zona pellucida* and with homogenous blastomeres were scored as excellent and good embryos (grade 1 or 2) according to International Embryo Transfer Society classification. Embryo diameters, excepting ZP, were measured from the same images on the screen of the monitor using scale bar micrometer, which was previously calibrated on a ×40 objective and ×10 eyepieces.

Vitrification and warming of embryos

Morphologically normal embryos (grade 1 or 2) were vitrified using procedure described by Vicente *et al.* (1999). The cryoprotective solution was a 1:1:2 solution (v/v/v) of dimethyl-sulfoxide (3.5 M DMSO, Sigma), ethylene glycol (4.4 M EG, Sigma), in DPBSCa (DPBS supplemented with 0.132 g $CaCl_2$/L) supplemented with 0.2 (w/v) BSA per liter of cryoprotective solution. Vitrification was carried out in two steps. First, embryos were pipetted into 0.2 ml of PBS medium and placed in a culture dish and then 0.2 ml of the cryoprotective solution was added and agitated. Embryos were kept in this medium for 2 minutes. In the second step, 0.6 ml of the cryoprotective solution was added and quickly agitated. Then, embryos suspended in the final vitrification solution were loaded into 0.25 ml plastic straws (IMV, L'Aigle, France), sealed with polyvinyl-alcohol sealing powder and plunged directly into liquid nitrogen. The exposure time of embryos to the final vitrification solution did not exceed 1 minute. The two vitrification steps were carried out at 20 ºC. The straws contained three sections separated by air bubbles. The first consisted of PBS in the cotton plug, the second section contained the embryos suspended in vitrification medium (0.1 ml) and the third section consisted of PBS. The straws were sealed and identified. Each straw held between 5 to 8 normal embryos. The straws were stored in liquid nitrogen for two days. Devitrification was performed by immersing the second and third sections of the straws in a water bath at 20 ºC for 10-15 sec. The cryoprotective solution was removed from the embryos in a two step dilution procedure at room temperature (20-25 ºC). Embryos suspended in the final vitrification solution were released into a culture dish containing 1 ml of 0.33 M sucrose in PBS medium. After 5 minutes, embryos were washed twice in fresh PBS medium and morphologically scored before culture. The warmed embryos were cultured in a standard *in vitro* culture condition for 48 h in 50 µl microdrops of Ham's F10 medium+ 20% FCS (Sigma) under mineral oil (Sigma) at 38.5 °C in an incubator containing 5% CO2 and humidified air.

Statistical analysis

Chi-squared test was used with data in the percentages. Data presented in means values were analyzed using GLM procedure in SAS® Program (1998). Significance of the differences in the results was tested by Duncan's New Multiple Range Test (Duncan, 1955). Differences between groups at $P<0.05$ were considered as significant.

Results and discussion

All does treated with 20 or 40 PMSG+40 hCG IU/kg live weight (Superovulated does) were ovulated compared to 18 only from 21 GnRH treated does (85.7%, Table 1). Results also showed that the number of haemorrhagic follicles (HFs) increases according to the PMSG amount injected. PMSG significantly ($P<0.01$) increased the number of haemorrhagic follicles as they were nearly four times in 20 IU/kg live weight (6.7±0.52) and six times as many in 40 IU/kg live weight (10.3±0.46) compared with GnRH normal ovulated group (1.61±0.38). These results are accordance with earlier studies by Garcia-Ximenez and Vicente (1990) who observed that superovulation treatment cause the ovulation of a high number of abnormal haemorrhagic and cystic follicles. Confirming Kennelly and Foote (1965) observations, the gonadotrophins stimulations of the ovaries which led to an increase of the number of hemorrhagic follicles may be attributable to the supra-physiological amount of gonadotrophins

administrated, involving the precocious apoptosis of the granula's cells and leading to the follicles atresia. Moreover, Stradaioli *et al.* (1997) reported that PMSG treatment increased significantly (P<0.01) HFs (9.3±3.5 and 17.8±3.3 in rabbits injected with 20 or 100 IU PMSG compared to 4.3±3.1 in the control group). Gosselin *et al*. (2000) explained that the administration of exogenous gonadotrophins decreases the frequency and the amplitude of the pulsatile endogenous LH secretion linked with an increase of estradiol and progesterone plasmatic concentrations. As the estrogen concentration increases, the LH concentration decreases and would become inadequate to activate the positive feedback of the estradiol on the LH secretion by the hypothalamus during the preovulatory period. Further, the absence of estrogens' positive feedback was underlined by Bakker and Baum (2000) to explain the absence of spontaneous ovulation in induced ovulators. Thus, the larger amount of estradiol could inhibit the preovulatory pulse of LH and the ovulation mechanism.

Donors treatments with 20 or 40 PMSG IU/kg live weight produced significantly (P<0.01) more CLs upon visual inspection (26.4 or 29.1) and yielded three to four folds embryos/doe (21.9 or 19.1) when compared with GnRH normally ovulated does (6.56 CLs and 6.33 embryos, Table 1). These results are comparable to those reported by Salvetti *et al*. (2007) who found that the number of corpora lutea and the number of embryos produced were significantly higher (P<0.001) in superovulated does than in control group (27.1 vs 11.9 corpora lutea and 20.3 vs 9.6 embryos produced), however, gonadotrophins administrations led to defaults of ovulation when compared with untreated does. In our study embryo and ova recovery rates were adversely affected by dose of PMSG hormonal treatment (92.8 vs 78.0% with 20 and 40 PMSG, respectively). Embryos recovered from GnRH normally ovulated does was significantly (P<0.05) reflected a higher fertilization rate (96.6%) than those recovered from does treated with 20 (89.4%) or 40 (84.1%) IU PMSG. Thus, it is evident that injection does with 20 or 40 PMSG IU/kg live weight has increased the total number of recovered ova/embryos by induction of oocyte detachment and migration to the oviduct, but on the other hand, a concomitant lower fertilization rate was resulted. The percentage of morphologically normal (verifiable) embryos recovered from the GnRH normal ovulated does (control group) was significantly (P 0.05) higher (97.4%) than that obtained from 20 or 40 IU PMSG+hCG treated donors (superovulated group, 84.9 and 77.0%, Table 1). These findings suggested that the effect of PMSG may be due to an over stimulation of ovarian follicle owing to its long half-life. In general, embryo recovery and morphologically normal rate were negatively affected mainly by dose of hormonal treatment. These results are in accordance with the findings of Rebollar *et al*. (2000); Vicente *et al.* (2003); Meshreky and Salama (2005) and Fahim (2008) in rabbits and Holtz *et al*. (2012) in goats. In earlier study, Carney and Foote (1990) gained 19.9 zygotes per donor after superovulation and 6% of which were degenerated and reported that the abnormal embryos from superovulated does had lower mitotic indices, but no obvious differences between control and superovulated does regarding embryo gross appearance were observed. Theau-clement *et al*. (2008) found that the percentage of the ovulation rate and the fertilizing rate and rabbit embryo survival increases with eCG dose (0, 8 or 25 IU injected 48 h before insemination.

Table 1. Effect of PMSG doses (superovulation) treated for female rabbit on ovarian response, morphologically normal embryos recovered 48-50 h post artificial insemination and *in vitro* development post-thawing.

Item	GnRH normal ovulation (control)	PMSG doses (IU/kg live weight)	
		20	40
No. of artificial inseminated donors	21	10	10
Ovulated donors, n (%)	18 (85.7)	10 (100)	10 (100)
Haemorrhagic follicles (±SE)	1.61^{c}±0.38	6.7^{b}±0.52	10.3^{a}±0.46
No. of CLs /doe (±SE)	6.56^{b}±1.4	26.4^{a}±2.3	29.1^{a}±2.3
Embryos+ova recovered, n (%)[1]	118 (100^{a})	245 (92.8^{ab})	227 (78.0^{b})
Embryos recovered (fertilization rate), n (%)[2]	114 (96.6)	219 (89.4)	191 (84.1)
No. of embryos/doe (±SE)	6.33^{b}±1.3	21.9^{a}±2.1	19.1^{a}±2.2
Morphologically normal embryos, n (%)[3]	111 (97.4^{a})	186 (84.9^{ab})	147 (77.0^{b})
Embryos recovered at >16 cell stage, n (%)[4]	79 (71.2^{a})	105 (56.5^{ab})	61 (41.5^{b})
Embryos recovered at 16 cell stage, n (%)[4]	32 (28.8^{b})	81 (43.5^{ab})	86 (58.5^{a})
Embryos diameter at ≥16 cell stage (±SE)	137^{a}±3.2	128^{b}±2.1	122^{c}±2.0
In vitro development to blastocysts stage post-thaw, n (%)[4]	74 (66.7^{a})	112 (60.2^{a})	61 (41.5^{b})

[1] Percentage based on the number of CLs.

[2] Percentage based on the total number of embryos and ova recovered.

[3] Percentage based on the total number of embryos recovered.

[4] Percentage based on the total number of morphologically normal embryos recovered.

[a, b] Values with different superscripts in the same row differ significantly (P<0.05).

Embryos recovered at 48-50 h post insemination from GnRH normally ovulated does indicated 71.2% of embryos in >16-cell stage (early morulea) and 28.8% were in 16-cell stage compared to 56.5 and 43.5%, respectively for embryos in the donor treated with 20 PMSG IU/kg live weight and 41.5 and 58.5%, respectively for embryos in the donor treated with 40 PMSG (Table 1). The results reflected that PMSG treated and dose had a significant ($P<0.01$ or 0.05) effect on embryo development rate. It is thus indicated that superovulation resulted in retardation of embryo development. These results are in accordance with the findings of Garcia-Ximenez and Vicente (1992) in superovulated does and Peir *et al*. (2004) who found that almost 76% of embryos recovered from normally ovulated high line does were in early morulae and 24% in 8-16 cell stage. Also, he stated that the formation of the zygote and the activation of the embryonic genome occur between 25 and 48 h of gestation. The transition from maternal to zygotic control had been described at the 8-16 cell stages rabbit embryo (around 40 h after mating). At this stage, all cells were reported to be transcriptionally active and could synthesize both mRNA and rRNA. Furthermore, the transcriptionally active nucleoli appear during early morulae (Kanka, 2003). In earlier report Hawk (1988) mentioned that one possibility is that superovulation accelerates embryo transport rate resulting in their premature arrival at the uterus. In addition, the deleterious effect of transferring oviductal-stage rabbit embryos to the uterus before their normal time of entry is a well-documented phenomenon (Adams, 1979). In our work, embryo diameters, excepting ZP recovered from 40 IU PMSG/kg live weight treated does had significantly ($P<0.05$) smaller diameter, followed by those injected with 20 IU PMSG/kg live weight in comparison with those recovered from GnRH normally ovulated does (Table 1). These results are in harmony with those reported by Kauffiman *et al.* (1998). Furthermore, Meshreky (1999) observed that average oocyte diameter collected from PMSG treated does was lower than that recovered from untreated does. Generally, treating rabbit does with PMSG/hCG (superovulation) retard the rate of embryonic development and reduced quality scores and diameter measurements. Several reports have compared qualitative and quantitative characteristics of embryos produced from superovulated and control does. Foote and Ellington (1988) postulated that in inducing superovulation embryos might be of normal morphological appearance; nevertheless, exhibit lower developmental potential than embryos from non-superovulated donors. Furthermore, Carney and Foote (1990) found that superovulation reduced rabbit embryo cell number (slower rates of cell division) and size compared with embryo recovered from normally ovulated donors, nevertheless, the developmental potential of embryo recovered from superovulated donors does differ from that of non-superovulated females.

In vitro development of embryos vitrified-thaw to blastocysts stage were harmfully ($P<0.05$) affected by superovulation, mainly with increase dose of PMSG hormonal treatment (Table 1). In 40 IU PMSG treated does-vitrified embryos, only 61 from 147 embryo vitrified-thaw reached blastocysts (41.5%), whereas 112/186 (60.2%) in 20 IU PMSG treated does compared to 74/111 (66.7%) in control group. These results are in harmony with those reported by Rebollar *et al.* (2000) who reported that cryopreservation of rabbit embryos led to a higher rate of morphological damage when recovered from superovulated does compared with untreated one. In addition, Leoni *et al.* (2003) state that the higher sensitivity of embryos recovered from superovulated does to low temperatures leads to a decrease in their subsequent potential capacity for development after vitrification. However, recently Viudes De Castro *et al*. (2009) reported that the developmental potential of frozen-thawed rabbit embryos obtained from superovulation (rhFSH alone or rhFSH+5% rhLH) and control groups was similar, with an 83.5% *in vitro* development rate until the expanded blastocyst stage. The different between these reports and our work may be due to the different hormones and dose used.

It is concluded that treated rabbit does with 20 IU PMSG/kg live weight provides superior embryos survival rates over the 40 IU for rabbit embryos production manipulation studies and gene bank.

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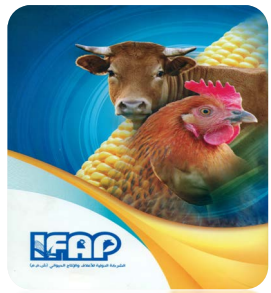

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2. Bio-Fertilizer Techniques


Bio-fertilization and efficient productivity of maize grown on a newly reclaimed saline sandy clay soil

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Abstract

A field experiment was conducted on*Zea mays* (cv Triple hybrid 310) at Sahl- Eltina – Galban village. North Sinai , Egypt, in 2011 assessing application of compost: none 0 (C_0) and 9.5 Mg ha^{-1} (C_1),; bio-fertilization:no inoculants (B_0), and inoculation with either N_2-fixing *AzospirillumbrasilenseNo. 40*), (salt-tolerant plant-growth producing rhizobacteria "PGPR")(B_1), *Bacillus megatherium* (P-dissolving bacteria "PDB")(B_2)or a mixture of B_1+B_2. and N- fertilizer (0,70,105, and 140 kg N ha^{-1},as urea)(N_0, N_1, N_2, and N_3)on productivity of a newly-reclaimed saline sandy clay soil - EC (paste extract) = 12.5 dSm^{-1}.Iirrigation was by El-Salam Canal water (a mixture of Nile water mixed and drain water, 1:1 of EC between 1.03 to 1.65 dSm^{-1}..Treatment receiving none $N_0C_0B_0$ gave lowest of 0.96 Mg grains ha^{-1} and the $N_1C_1B_2$ treatment gave 427% increase i.e. highest of 5.06 Mg ha^{-1} . Average increases of 48.1, 50.8, and 46.5% were given by N_1, N_2, and N_3 respectively. Bio-fertilization increased yield by 43.5 ,33.6 , and 46.8% averages due to B_1,B_2, an B3 respectively. Compost gave 41.8% yield increase. Soil EC after harvest decreased by treatments and the C_0 N_0B_0 treatment showed of 9.73 dSm^{-1} while soils given materials showed from 9.66 (C_0 N_1B_0) to 5.33 dSm^{-1} (C_1N_2 B_3). Available NPK after harvest were greater by fertilization. Bio-fertilization +105 kg N ha^{-1} along with compost ha^{-1}would improve soil fertility and enhance its productivity for maize in saline soils.

Key word: Bio-fertilizers, mineral fertilizer, compost, Soil salinity, Zea maize productivity.

Introduction

Under salinity condition, the most important problem facing agricultural production in irrigated arid and semi arid areas is how to prepare a suitable root zone. Fertilization plays an important role in promoting plants to tolerate salt stress and toxicity, (Ghoulam et al .2002).Bio-fertilizers especially the plant-growth promoting rhizobacteria (PGPR) which fix N_2were suggested by Mantripukhri, (2006) to reduce the use of mineral fertilizer. Ahmed (2007) noted that organic matter in soil plays an important role in building up soil aggregates and improving soil. El-Fayoumy and Ramdan (2002) reported that application of readily-soluble nitrogen fertilizer and bio-fertilization slightly affected soil salinity. Maize is one of the most important food crops in Egypt and is also used as animal feed-stuff. Shaban, et al.(2006) noted a significant decrease in soil salinity by increasing application of N applied as urea along with *Azospirillum* biofertilization. Hassan, (2004) showed that application of organic fertilizers either alone or in combination with chemical fertilizers caused a substantial increase in soil available N, P and K. Ashmaye et al. (2008) reported increases in N, P and K content in maize grown on a saline soil due to bio-fertilization in combination with organic manure application. Mohamed et al. (2001) used *A.braislense* +144 kg N $ha^{-1}$and obtained marked increase in maize yield (straw and grains). Tariq, et al (2010) found that P content in maize increased upon applying organic manure and attributed this to the increased P availability. El-Tantawy and Mohamed (2009) applied bio-fertilizers to soil under peanuts and found that P increased in the soil (available P) as well as in plant (P- uptake) . Abou El-Yazed and Abou – Aly (2011) observed phosphate solubilization in soil where P-dissolving bacteria was used. The aim of the present investigation was to study the use of biofertilization application of N and compost on maize production in saline soils.

Materials and methods:

A field experiment was conducted on maize (*Zeamays* - L cultivar ,Triple-hybred, 310) at, Sahl-El-tina Plain ,North Sinai (Msp1) during the summer of 2011. The El-Tina plain lies in the north-western Mediterranean coast of Sinai, between 32°_ 350 and 32°_ 450 E and 31°_ 000 and 31°_ 250 N,

Map 1: Sahl-El-tina Plain, North Sinai

The soil is saline sodic sandy clay low in organic matter and plant nutrients (Table 1). The area is irrigated with El-Salam Canal water (Nile water mixed with Agriculture drainage water at a ratio of 1 : 1), with EC of 1.03 to 1.65 dSm^{-1} (Table 2).The experiment was aimed at assessing fertilization with organic manure , chemical soluble N fertilizer, and bio-fertilization (singly or in different combination) on maize productivity grown on the soil .which is a newly reclaimed .The design of the experiment was a "randomized complete block , factorial involving 3 factors as follows: (1): Organic manuring using compost : two treatments " none" and "9.5 Mg compost ha^{-1}" , designated C_0, and C_1 respectively; (2): N-fertilization : four treatments :0, 70, 105, and 140 kg N ha^{-1}"as urea" , designated N_0, N_1, N_2, and N_3 respectively; (3): Biofertilization through bacterial inoculation: none , *Azospirillum braislense*No.40, "salt tolerant PGPR",*and Bacillus megatherium* "P-dissolving bacteria" , and a mixture of the two designated B_0, B_1, B2,and B_3, respectivly. The bacteria inogula were in liquid suspensions form supplied by the Microbiology Department of the Soil, Water and Environment Research Institute (Egypt). Inoculation was done to seeds before seeding and also to the soil three times: 21 , 42 , and 62 days after seeding as recommended by Shaban and Omar (2006) .Seeding was done on 12-5-2011.The experiment field received 16 kg P (as Ca-superphosphate :6.8% P) + 40 kg K(as K-sulphate: 40% K) ha^{-1} . Compost (Table 2) was added 25 days before seeding as described by Shaban (2005) with half the amount incorporated into the soil during ploughing and the other half was added on the soil surface with thorough mixing with the 5-cm soil surface to act as mulching. El-Salam water was used for irrigation , Table 3 shows analysis of the irrigation water. Maize was harvested on the 1st October 2011and yield was recoded following air drying for 7 days (reaching moisture of about 12% in grains). Plant samples were taken for analysis following oven-drying at 75^0C for analysis of contents of N, P, and K in grains (Chapman and Pratt 1961). The 1000-grain weight was recorded. Soil samples were taken after harvest and soil analyses were done according to methods cited by Piper (1952), and Black et al., (1965). The obtained data were statistically analyzed according to Steel and Torrie (1960)

Table 1. Properties of soil of the study.

C.sand(%)	F.sand(%)	Silt (%)	Clay (%)	Texture	O.M (g kg^{-1})	$CaCO_3$ (g kg^{-1})
5.6	72.0	7.4	15.0	Sandy clay	0.45	109

pH (1:2:5)	EC(dS/m)	Soluble ions $mmol_c$ L^{-1} (no CO^{--}_3 detected)						
		Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO^{-}_3	Cl^{-}	SO^{--}_4
8.12	12.5	10.2	20.4	93.5	0.9	7.5	80.0	37.1

Available nutrients (mg kg^{-1})						
NO_3.N	P	K	Fe	Mn	Zn	Cu
35	4.42	181	3.10	1.70	1.05	0.008

Available nutrient extracted by NH_4Ac-DTPA

Table 2. Chemical analysis of compost.

Moisture content %	EC dSm^{-1}	pH	C/N	O.M	N	P	K	Fe	Mn	Zn	Cu
				……..…….	……g kg^{-1}			….(mg kg^{-1})……..			
25%	4.21	7.2	9.1	330	28.6	8.0	1.57	230	80	115	44

Table 3. Chemical analysis of El-Salam canal irrigation water during winter and summer 2010/ 2011- 2011.

Property	Month							
PH	Oct.	Dec.	Feb.	Apr.	May	July	Aug.	Sep.
	8.14	8.17	8.21	8.10	8.22	8.26	8.31	8.26
EC (dS m^{-1})	1.03	1.18	1.25	1.20	1.27	1.30	1.65	1.52
SAR	4.22	4.31	4.41	4.29	4.56	4.62	4.72	4.64
NO_3- N(mg/L)	7.25	7.44	8.20	8.41	9.41	9.59	10.17	9.48
NH_4-N (,,)	12.78	13.28	13.45	13.41	14.16	14.35	15.13	14.86
P (,,)	3.69	4.55	5.71	5.60	4.93	5.23	5.66	5.38
K (,,)	6.21	6.35	6.51	6.46	7.19	7.29	7.14	7.18
Fe (,,)	1.96	2.33	2.47	2.31	3.16	3.22	3.31	3.28
Mn (,,)	1.16	1.46	1.52	1.27	1.68	1.77	1.83	1.80
Zn (,,)	0.90	0.96	0.98	1.03	1.23	1.27	1.22	1.20

Results and discussion
Soil pH.

Data in Table 4 show that the soil pH tended to decrease upon applying the treatments. The decrease was marked particularly when N and compost along with bio-fertilization were combined. Soil which did not receive any of N fertilizer or manure or fertilization (the C_0 N_0B_0treatment) showed a pH of 8.10 while soils of the other treatments showed values ranging from 8.09 (C_0 N_1B_0) to 7.37 (C_0 N_2 B_2) .The combination of fertilizer N with organic manure and bio-fertilization led to a marked decrease in soil pH which gives an indication of the combined effect of fertilization and organic manuring in decreasing soil alkalinity. The decrease upon applying N was progressive and the main effect shows pH of 8.05 for the no N (N_0) decreasing to7.98 for the highest rate of N. The main effect of compost shows pH of 8.00 for the no compost treatment and 7.46 for the treatment receiving compost. The main effect for the bio-fertilization treatment shows less marked effect regarding soil pH although the trend of decrease was evident and average pH values for the bio-fertilization treatments were 8.04,8.00,7.91, and 7.96 for B_0, B_1, B_2,and B_3, respectively. The activity of microorganism in the soil particularly where organic substrates are present , would lead to producing organic acids during decomposition of organic matter. Mantripukhri, (2006) and Ahmed (2007) observed a decrease in soil pH in soils treated with organic matter..Shaban and Omar (2006) and Bassiony and Shaban (2010) found that application of fertilizer nitrogen with bio-fertilizers led to dehydrogenase activity and production of hydrogen ions in the rhizosphere of maize.

Soil EC

Data in Table 5 show that soil EC decreased upon applying the treatments. Soil which did not receive any of N fertilizer or manure or fertilization (the C_0 N_0B_0treatment) showed an EC (dS m^{-1}) of 9.73 while soils receiving any or more of the amendments showed values ranging from 9.66 (C_0 N_1B_0) to 5.33 (C_1N_2 B_3). This indicates that applying fertilization with organic, bio-feritilizers, and N singly or in any combination enhanced plant root growth and therefore decreased salinity of the soil. Each of compost addition, N application, and bio-fertilization, showed significant decrease in soil salinity. The average decreases (i.e. the main effects) for compost is a decrease of 20.1% . Average decreases due to N are 3.7 ,10.3 , and 24.1% due to N_1, N_2, and N_3 respectively; whereas those due to bio-fertilization were 8.6 ,8.1 , and 12.1% for B_1, B_2 and B_3, respectively. There were interaction effects involving response to N and compost. Where compost was present, the decrease in salinity due to N was more pronounced , and where N was applied , the compost was more effective in decreasing salinity.The positive effect of compost in decreasing soil salinity was prominent where the highest rate of N was given. Compost was also of marked effect where the two bio-fertilizers were combined. Another interaction was shown by bio-fertilization affecting response to N.Bio-fertilization was most effective in decreasing salinity when N was present but slightly effective in absence of N. Also N decreased salinity more in presence than in absence of bio-fertilizers. The decrease in salinity caused by N was very little where no bio-fertilization was done , but it was very marked where bio-fertilizers were applied particularly where both .*A.braislense* and *B .megatherium* were applied together. This is an indication of the positive effect of the two inocula in causing greater plant growth an root system, therefore decreasing soil salinity. Nasef et al., (2009) suggested that besides improvement in soil aggregation caused by compost, its decomposition when combined with bio-fertilizers releases acids therefore such conditions facilitate leaching of soluble salts and decrease soil salinity.

Available N, P, and K in soil after crop harvest.

Data present in Tables 6 to 8 show that available N,P and K were considerably high in treatments which received N. The direct effect of adding fertilizer N and compost is due to their being sources of available nutrients. However the positive effect of bio-fertilization in causing higher available nutrients is shown in treatments receiving bio-fertilizers alone and in treatments which received bio-fertilizers in combination with N fertilizer or compost. The positive effect of the bio-fertilizers, although not marked in absence of both N fertilizer and compost, it may be improving soil fertility. Mahmoud and Mohamed (2008) found that the bio-fertilizer inoculation increased available content of N, P and K .Shaban et al (2009) observed enhancement in soil fertility to microorganism's activity in N fixation.

Grain yield, 1000-grain weight and NPK in grains

Data of Tables 9 and 10 show the effect of treatments on maize grain yield and its 1000-grain weight. Treatment not receiving any fertilizer (the $N_0C_0B_0$ treatment) gave the lowest yield of 0.96 Mgha^{-1} while that which was given the low N in combination with compost and the mixture of the two bio-fertilizers(the $N_1C_1B_2$treatment), gave the highest yield of 5.06 Mg ha^{-1}, 427% increase. The main effect of N shows that although all treatments which received N gave increase in grain yields the differences between the 3 rates of N was not very high. In absence of bio-fertilization the effect of adding N was more pronounced. Bio-fertilization increased yield giving average increases

of 43.5, 33.6, and 46.8% due to B_1,B_2, an B_3 respectively. The effect was more when combined with compost only with the low N rate in particular when increases were 53.3, 50.5, and58.6% due to B1,B2, an B_3 respectively. Hassneen et al (2009) reported that the mixture of the salt tolerant *A.brasilense* and the P-dissolving *B.megatherium* combined with compost gave increases in plant growth under salinity stress conditions.The pattern of effect regarding the 1000-grain weight was similar to that of the yield. Data present in Table 10 show a weight of 174 g for the treatment not receiving any of the three materials (the $N_0C_0B_0$ treatment) increasing to 343g for that of the low N in combination with compost and the mixture of the two bio-fertilizers(the $N_1C_1B_2$treatment), 97.1% increase. The main effect of compost shows 41.8% due to its addition.That of the N addition shows increases of 48.1, 50.8, and 46.5% due to N_1, N_2, and N_3 respectively.The main effect of bio-fertilization shows increases of 22.0 , 7.6 , and 11.9% due to B_1, B_2, and B_3 respectively. Abou El-Yzeid and Abou- Aly (2011) who reported that application of *Bacillus megaterium* increased plant growth as well as photosynthesis. Hedge et al. (1999) stated that bio-fertilizers play an important role in enhancing crop productivity through nitrogen fixation, phosphate solubilization, plant hormone productivity, and help plant to resist some diseases. Mostafa and Abo-Baker (2010) tested bio-fertilization using *A.brasilense+ B.megatherium* in combination with soluble N with positive results.

The effect on NPK in grains (Tables 11 to 13) shows increases due to application of fertilizers and compost alone. The highest values of N and P were with N_2C_1 B_2 , which received fertilizer N along with compost . K content in grains increased with increased rates of N fertilizer combined with or without compost. El-Quesni et al. (2010) reported that *A.brasilense* with or compost increased all growth parameters of plants under salt stress conditions.

Conclusion

It could be concluded that bio-fertilizer *Azospirillum brasilense No. 40* **(**Salt Tolerant) combined with *Bacillus megatherium* and fertilizer urea nitrogen at a moderate rate of 105 kg N ha^{-1} along with 9.5 Mg compost ha^{-1}would improve soil fertility and enhance its productivity for maize under the conditions of salinity.

Table 4. Soil pH after harvest as affected by N-fertilizer, compost and bio-fertilizers to maize on a saline sandy clay soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B)				Mean
		None	BN	BP	BN+BP	
	none	8.10	8.07	8.08	8.05	8.08
0	with	8.05	8.03	8.02	8.01	8.03
	mean	8.08	8.05	8.05	8.03	8.05
	none	8.09	8.02	8.03	8.00	8.04
70	with	8.04	7.93	7.89	7.84	7.93
	mean	8.07	7.96	7.96	7.92	7.98
	none	8.00	8.03	5.37	7.98	7.35
105	with	8.01	7.96	7.92	7..89	7.95
	mean	8.00	7.99	6.65	7.74	7.65
	none	8.05	8.04	8.05	8.00	8.04
140	with	8.00	7.94	7.92	7.88	7.94
	mean	8.03	7.99	7.99	7.94	7.99
G.mean		8.04	8.00	7.66	7.96	7.92
	Means compost	8.00				
	none	8.06	8.04	7.38	8.00	7.87
	with	8.03	7.96	7.94	7.91	7.96

LSD :0.05: ns

N:Fertilizer N addition C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN: *A.braislense*; BP: *B. megatherium*

Table5. Soil EC (dSm^{-1}) after harvest affected by N-fertilizer, compost and bio-fertilizers to maize on a saline sandy clay soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B) None	BN	BP	BN+BP	Mean
	none	9.73	9.42	9.45	9.32	9.48
0	with	7.58	7.41	7.36	7.16	7.38
	mean	8.65	8.42	8.41	8.24	8.43
	none	9.66	8.21	8.30	7.86	8.43
70	with	7.46	7.92	7.89	7.84	7.78
	mean	8.56	8.07	8.10	7.85	8.11
	none	9.51	8.10	8.17	7.83	8.40
105	with	7.25	6.24	6.28	5.33	6.28
	mean	8.38	7.17	7.23	6.58	7.34
	none	9.48	8.34	8.35	8.13	8.58
140	with	7.17	6.38	6.51	6.12	6.55
	mean	8.33	7.36	7.43	7.13	7.57
	G. mean	8.48	7.75	7.79	7.45	7.87
			Means compost			
	none	9.60	8.52	8.57	8.29	8.75
	with	7.36	6.99	7.01	6.61	6.99

LSD :0.05: ; N= 0.20; C= 0.01 ; B 9.6 ; NC= 0.03 ; NB=0.04 ; BC= 0.03 ; NCB=0.06
N:Fertilizer N addition ;C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN:*A.braislense* BP: *B. megatherium*

Table 6: Available N($mgkg^{-1}$)after maize harvest as affected by N-, bio-fertilizers, and compost in the saline soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B) None	BN	BP	BN+BP	Mean
	none	49	53	52	56	52
0	with	53	58	46	60	54
	mean	51	56	49	58	53
	none	50	57	54	59	55
70	with	57	62	72	72	66
	mean	54	60	63	66	61
	none	54	57	57	60	57
105	with	58	63	61	66	62
	mean	56	60	59	63	60
	none	51	59	56	60	57
140	with	60	61	62	72	64
	mean	56	60	59	66	60
G.mean		54	59	58	63	58
			Means compost615955			
	none	51	57	55	59	55
	with	57	61	60	67	61

LSD :0.05: ; N= 1.8; C= 1.3 ; B = 1.8 ; NC= 2.6 ; NB=3.7 ; BC= ns ; NCB = 5.2
N:Fertilizer N addition C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN: *A.braislense*; BP: *B. megatherium*

Table 7 . Available P(mgkg^{-1})after maize harvest as affected by N-, bio-fertilizers, and compost in the saline soil

Fertilization kg N ha^{-1} (N)	Compost (C)	(B)Bio-fertilization None	BN	BP	BN+BP	Mean
	none	4.6	5.0	5.1	5.1	5.0
0	with	4.8	5.4	5.5	5.5	5.3
	mean	4.7	5.2	5.3	5.3	5.1
	none	4.9	5.1	5.2	5.2	5.1
70	with	5.2	6.1	6.2	6.2	5.9
	mean	5.1	5.6	5.7	5.7	5.5
	none	5.0	5.2	5.3	5.4	5.2
105	with	5.2	6.1	6.3	6.3	6.0
	mean	5.1	5.7	5.8	5.9	5.6
	none	5.0	5.1	5.2	5.3	5.2
140	with	5.4	6.1	6.1	6.2	6.0
	mean	5.2	5.6	5.7	5.7	5.6
	G.mean	5.1	5.5	5.6	5.7	
			Means compost			
	none	4.9	5.1	5.2	5.3	5.1
	with	5.2	5.9	6.0	6.1	5.8

LSD :0.05: N= 0.07; C= 0.05 ; B:0.07 ; NC= 0.09 ; NB=ns ; BC= 0.09 ; NCB = 0.20
N:Fertilizer N addition ;C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN:*A.braislense* BP: *B. megatherium*

Table 8. Available K(mgkg^{-1})after maize harvest as affected by N-, bio-fertilizers, and compost in the saline soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B) None	BN	BP	BN+BP	Mean
	none	192	198	206	197	198
0	with	195	206	213	201	203
	mean	194	202	210	199	201
	none	206	214	208	219	212
70	with	209	226	230	238	226
	mean	208	220	219	229	219
	none	208	217	215	223	216
105	with	215	230	229	239	228
	mean	212	224	222	231	222
	none	212	214	218	219	216
140	with	219	218	225	228	223
	mean	216	216	222	224	220
	G.mean	211	216	218	221	217
			Means compost			
	none	205	211	212	215	211
	with	210	220	224	227	220

LSD :0.05: N= 0.8; C=0.6 ; B=0.8 ; NC= 1.1 ; NB=1.6 ; BC1.1 ; NCB = 2.2
N:Fertilizer N addition C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN: *A.braislense*; BP: *B. megatherium*

Table 9. Maize grain yield as affected by N-fertilizer, compost and bio-fertilization of a saline sandy clay soil.

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B)				Mean
		None	BN	BP	BN+BP	
0	none	0.96	2.52	2.75	2.54	2.19
	with	1.53	2.53	2.76	3.02	2.64
	mean	1.25	2.53	2.75	2.78	2.33
70	none	3.03	4.32	4.76	4.61	4.17
	with	3.19	4.89	4.80	5.06	4.48
	mean	3.11	4.61	4.78	4.84	4.33
105	none	3.39	4.03	3.82	4.73	3.99
	with	3.78	4.83	4.80	4.94	4.59
	mean	3.59	4.43	4.31	4.84	4.29
140	none	4.27	4.00	3.91	4.62	4.20
	with	3.85	4.63	4.58	4.67	4.43
	mean	4.06	4.32	4.25	4.65	4.32
	G. mean	3.01	4.22	4.02	4.28	
			Means compost			
	none	2.92	3.72	3.81	4.13	3.64
	with	3.09	4.22	4.23	4.42	3.99

LSD :0.05: ; N= 0.007; C= 0.005 ; B = 0.007 ; NC= 0.009 ; NB=0.013 ; BC= 0.009 ; NCB = 0.018
N:Fertilizer N addition C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN: *A.braislense*; BP: *B. megatherium*

Table10. 1000- maize grain weight as affected by N-fertilizer, compost and bio-fertilizers on a saline sandy clay soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B)				Mean
		None	BN	BP	BN+BP	
0	none	174	175	176	126	163
	with	181	183	185	281	207
	mean	177	179	180	203	185
70	none	185	226	244	232	221
	with	305	325	333	343	327
	mean	245	276	288	288	274
105	none	185	232	241	256	228
	with	312	337	326	345	330
	mean	249	284	284	300	279
140	none	226	226	217	237	227
	with	316	309	314	321	315
	mean	271	267	265	279	271
	G. mean	236	288	254	264	
			Means compost			
	none	192	215	219	205	208
	with	279	288	289	323	295

LSD :0.05: ; N= 9.6; C= 6.8 ; B= 9.6 ; NC= 13.6 ; NB=ns ; BC= 13.6 ; NCB = 27.1
N:Fertilizer N addition ;C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN:*A.braislense* BP: *B. megatherium*

Table 11. N in grains (gkg^{-1}) asaffected by N-fertilizer, compost and bio-fertilizers to maize on a saline sandy clay soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B)				Mean
		None	BN	BP	BN+BP	
	none	8.6	9.4	9.0	9.8	9.2
0	with	11.2	11.8	11.5	12.4	11.7
	mean	9.9	10.6	10.3	11.1	10.5
	none	10.2	13.6	12.1	13.4	12.5
70	with	11.6	13.0	12.4	13.6	12.6
	mean	10.9	13.3	12.3	13.5	12.5
	none	10.5	13.0	12.8	14.5	12.7
105	with	12.3	13.7	13.5	14.7	13.6
	mean	11.4	13.4	13.2	14.6	13.2
	none	10.8	12.0	11.8	12.8	11.9
140	with	12.7	12.5	12.3	13.3	12.7
	mean	11.8	12.3	12.1	13.1	12.3
	G. mean	11.0	12.4	11.9	13.1	12.1
			Means compost			
	none	10.0	12.0	11.4	12.6	11.5
	with	12.0	12.8	12.4	13.5	12.7

LSD :0.05: ; N= 0.22; C= 0.12 ; B =0.22 ; NC=0.31 ; NB=0.44 ; BC= 0.31 ; NCB = ns
N:Fertilizer N addition ;C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN:*A.braislense* BP: *B. megatherium*

Table 12. P in grains (gkg^{-1}) as affected by N-fertilizer, compost and bio-fertilizers to maize on a saline sandy clay soil

Fertilization kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B)				Mean
		None	BN	BP	BN+BP	
0	none	2.2	2.6	2.9	3.0	2.7
	with	2.7	2.9	3.4	3.5	3.1
	mean	2.5	2.8	3.2	3.3	3.0
70	none	2.8	2.8	3.7	3.9	2.7
	with	3.2	3.3	4.2	4.5	3.8
	mean	3.0	3.1	4.0	4.2	3.6
105	none	3.4	3.0	3.8	4.2	3.6
	with	3.7	4.0	4.7	5.1	4.4
	mean	3.6	3.5	4.3	4.7	4.0
140	none	3.6	2.7	3.0	3.5	3.2
	with	3.9	3.2	3.7	4.1	3.7
	mean	3.7	3.0	3.4	3.8	
	G. mean	3.2	3.1	3.7	4.1	3.5
			Means compost			
	none	3.0	2.8	3.4	3.7	3.2
	with	3.4	3.4	4.0	4.3	3.8

LSD :0.05: N= 0.06; C=0.04 ; B=0.06 ; NC= 0.08 ; NB=0.11; BC=0.08; NCB = 0.16
N:Fertilizer N addition C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN: *A.braislense*; BP: *B. megatherium*

Table 13. K in grains (gkg^{-1}) as affected by N-fertilizer, compost and bio-fertilizers to maize on a saline sandy clay soil

Fertilization Kg N ha^{-1} (N)	Compost (C)	Bio-fertilization (B)				Mean
		None	BN	BP	BN+BP	
	none	11.7	12.2	12.0	12.7	12.2
0	with	17.4	17.9	18.2	18.8	18.1
	mean	14.6	15.0	15.1	15.8	11.2
	none	12.4	13.4	14.1	14.7	13.7
70	With	18.2	20.7	20.6	21.4	20.2
	Mean	15.3	17.1	17.4	18.1	17.0
	None	12.9	13.5	14.3	14.8	13.9
105	With	18.9	21.0	21.8	21.2	20.7
	Mean	15.9	17.3	18.1	18.0	17.3
	None	13.2	13.2	13.8	14.2	13.6
140	With	19.4	21.7	22.6	23.0	21.7
	Mean	16.3	17.5	18.2	18.6	17.7
	G. mean	15.5	16.7	17.2	17.6	16.8
			Means compost			
	None	12.6	13.1	13.6	14.1	13.4
	With	18.5	20.3	20.8	21.1	20.2

LSD :0.05: ; N= 0.08; C= 0.06 ; B= 0.08 ; NC=0.12 NB=0.17; 0.03 ; BC= 0.12 ; NCB=0.24
N:Fertilizer N addition ;C:compost addition (9.5 Mg ha^{-1})-B:biofertilizers :BN:*A.braislense* BP: *B. megatherium*

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Studies on *jatropha curcas* l. Cultivated under the effect of shifting aeolian deposits at Toshka region, Egypt.

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Abstract

The current work was performed to study impact of agriculture period and plant age on growth of *Gatropha curcas* grown on shifting aeolian sand deposits at Toshka region during two successive seasons. Plant age and growth period, *i.e.* period of cultivation affected some growth characters, plant height and stem diameter. Sand accumulation during the study period and among the plant ages coincided with plant characters. Vigorous increase of plant growth decreased sand accentuation. Biochemical genetic studies were carried out based on plant pratiens and isozymes variation besides molecular analysis to differentiate and identify the studied six *J-aurcas* samples. There were differences between the studied samples either with respect to period or ages.

Key word: Toshka region, J.curcas, Shifting sand, Biochemial genetic, proteins, isozymes, SDS. PAGE, ISSR.

Introduction

Sandy soils represent more than 90% of the newly reclaimed lands in Egypt, are subject to wind erosion occurs, with soil loss due to wind erosion is more than 25 Mg ha-1 yr-1**,** (Bielders et al. 2001).

In the south valley development project, sand movement represents the main environmental constrain.

Jatropha curcas L., a multipurpose, drought-resistant, perennial plant of the Euphorbiaceae family has gained importance for the production of biodiesel. The properties of the crop and its oil persuaded investors, policy makers and clean development mechanism (CDM) project developers to consider it as a substitute for fossil fuels to reduce CO_2 gas emission. Basic agronomic properties of Jatropha are not thoroughly understood and the environmental effects need more investigations. Experiments were undertaken to understand the scope of this plant for intercropping in the under storey of dominating monoculture tree stands (Prosopis, Acacia and Neem**)** (Soumit *et al.*, 2010)**.** *Jatropha curcas* is a tropical plant that can be grown in low to high rainfall areas either as a commercial crop or as a hedge to protect fields from grazing animals and to prevent erosion (Ashwani Kumar and Satyawati Sharma, 2008). Effects of age and orientation of the explants on callus induction and de novo shoot regeneration from cotyledonary leaf segments of Jatropha curcas were studied. The callus induction and shoot regeneration capacity of cotyledonary leaf segments were found significantly related to the age of the explants and their orientation in culture medium. The youngest explants, derived from the cotyledonary leaf of germinated seed showed higher regeneration response as compared to one- and two-week-old explants (Mazumdar *et al*., 2010).

Genetic markers can be classified into biochemical markers (proteins and isozymes) and molecular markers (DNA markers). Isozymes are multiple forms of enzymes arising from genetically determined differences in primary structures and not to those derived by modification of such structure, they are used as useful markers in genetic studies of many plant species. Isozymes as markers for drought, water deficits, high temperature, age, root development stages in cereal crops were thoroughly investigated, **(**Qi and Peng, 1993**)** and (Frova *et al*., 1998). Harandi (2002) used isozymes as markers for early diagnosis of drought –resistance of scots pine, water stress of palm and to differentiate between pistachio species and varieties, respectively.

The choice of a molecular marker technique depends on its reproducibility and simplicity. The most effective markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation have low cost and labour requirements and high reliability. Since 1994, molecular marker technique called inter simple sequence repeat (ISSR) was in use (Zietkiewicz *et al.*, 1994). ISSRs are semi arbitrary markers amplified by PCR in presence of one primer complementary to a target microsatellite (Bussell *et al.*, 2005). In general, physiological response to stressors can be divided into two possibilities. In one case, tolerant plants have mechanisms which maintain high metabolic activity under mild stress and reduced activity under severe stress (Osmond *et al*., 1987).

Materials and methods

This work was carried out to study the effect of agriculture season and plant age on growth of Jatropha curcas cultivated under shifting aeolian sand deposits at Toshka region during two successive seasons of 2010 and 2011.

Experimental design

A split plot design with four replicates was used in this study. The main plots were assigned to two agriculture period. Three age periods (three years, two years and one year) were randomly assigned to the sub plots.

Cultivation process

Cultivation process was performed by sowing Jatropha transplants in rows (84 m long) with a distance of 2m length x 2m width . The number of rows in the experiment was six from each of three plant ages. Jatropha trees were cultivated as shelterbelts. Cultivation process was performed by sowing of Jatropha curcas perpendicular on wind direction. Sowing of Jatropha transplants took place on March 15th in 2007, October 15th in 2007 and March 15th in 2008, October 15th in 2008 and March 15th in 2009, October15th in 2009 year. Drip irrigation system was applied in this experiment.

Sand collectors **(**Bagnold, 1971) were fixed at up and down wind of each tree treatments during 2010 year for evaluation of shelterbelt efficiency.

Determinations

A- Means of the climatic normal of Abo Sembel station during the period from

2000-2010

B- Growth characters of Jatropha plants.

Crown cover and crown volume were estimated by the method described by (Thalen, 1979).

C- Shelterbelt efficiency was calculated using the following equation:

Shelterbelt efficiency = A-Bx100 where:

A= sand accumulation (g/cm^2) in the front of shelterbelt.

B= sand accumulation (g/cm^2) behind of shelterbelt.

Genetic Analyses

Six samples of Jatropha were collected, during two growth seasons, at three different ages. Shoots and leaves of these plants were subjected for the following studies.

Biochemical Analyses

Protein Electrophoresis

Extraction of grain protein, gel preparation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used according to the method outlined by Laemmli (1970) and modified by Studier (1973).

Isozymes Electrophoresis

Powdered seed samples were extracted either in cold distilled water or in Tris-borate buffer solution.

Electrophoretic runs were performed according to Stegemann *et al.* (1985).

The staining solution of three isozymes Esterase , Acid-phosphatase and Peroxidase were performed according to Wendel and Weeden (1989) , Jonathan and Wendel (1990) and Graham et al. (1964)

Gel analysis

All gels resulted from protein and isozyme electrophoresis were scanned using Gel Doc-2001 Bio-Rad system.

Molecular analysis

Genomic DNA extraction and purification

Extraction of total DNA was performed using the method outlined by Anna *et al*. (2001). Estimation of the DNA concentration in the different samples was done by measuring optical density at 260 nm according to the following equation:

Conc. (ug/mL) = OD260 × 50 × dilution factor

Inter Simple Sequence Repeats (ISSR):

Fifteen primers for ISSR were used in the study, but only 9 primers were successful in generating reproducible and reliable amplicons for the different samples. Names and sequences of the selected primers are as shown in Table 3.

Matrix comparison

Similarity matrix produced by 9 sets of ISSR primers were compared based on the genetic distance of the NTSYS ver.2.1 the normalized Mantel statistic Z (Mantel, 1967).

Data were subjected to statistical analysis according to Snedecor and Cochran (1980).

Results and discussion

Climatologically data

The climatologically records of Abo Sembel Meteorological Station, the nearest portion from the study area, during the growth periods is shown in Table 1. From the data in Table 3 it is obvious from that the average monthly temperature varied between 16.8o in January and 35.5o in August. The rain fall is few and the relative humidity varied from 21 to 44%.The wind speed (km/h) values vary from 12.2 in December to 15.9 km/h in June of the total wind speeds. The prevailing wind directions are generally North/South.

Plant growth characters

The effect of plant age and **season** on growth characteristics of plant height, stem diameter, crown cover and crown volume are shown in Table 2. Data show that plant height and stem diameter of trees showed no significant effect due to agriculture period. However, crown cover and crown volume of *Jatropha curcas* plants were significalty increased with the increasing of agriculture period. Data in Table, 2 indicate that plant age significantly affected growth characters.

Table 1. Means of the climatic normal of Abo Sembel station during the period from 2000-2010 years.

Months	Air temperature (C^o)			Relative humidity (%)	Rain fall (mm)	Evaporation (mm/month)	wind speed (km/ h)	Wind direction
	Max	Min	Aver					
January	23.5	10.1	16.8	43	4	12.0	12.5	N
February	26.1	11.7	18.9	36	5	13.1	13.1	N
March	30.9	15.6	23.4	30	3	16.1	15.2	N.w
April	35.9	20.3	28.1	25	1	19.3	14.7	N
May	39.4	24.2	31.8	21	1	23.9	14.2	N
June	41.6	26.6	34.1	21	0	25.1	15.9	N
July	42.1	27.9	35	22	0	24.3	15.4	N
August	43.2	28.4	35.5	23	0	22.7	15.6	N
September	40.1	25.5	32.8	26	0	23.7	15.1	N
October	36.5	22.2	29.4	30	1	19.8	14.1	N
November	30	17.5	23.0	38	1	13.4	13.2	N
December	24.9	11.9	18.3	44	8	10.7	12.2	N

Service Source Nation Environmental satellite data and information (NESDIS).

N	67 %
NE	8 %
E	1 %
SE	1 %
S	2 %
SW	1 %
W	3 %
NW	15 %

Averaged Value wind speed (January 2000 - December 2010): 14.3kph
Averaged Value temperature (January 2000 - December 2010): 27.2 °C
Averaged Value temperature maximum (January 2000 - December 2010): 34.4 °C
Averaged Value temperature minimum (January 2000 - December 2010): 20 °C
Averaged Value rain (January 2000 - December 2010):2 mm
Averaged wind direction (January 2000 - December 2010): N and NW.

Table 2. Effect of agriculture period and plant ages on growth characteristic of *Jatropha curcas* plants.

Plant age (year)	Agriculture period	Plant eight (cm)	Stem Diameter (mm)	Crown cover (m2)	Crown volume (m3)
One	Spring	147	33	3.52	3.45
	Autumn	141	31	2.83	2.66
	Mean	144.0	32.0	3.18	3.06
Two	Spring	188	52	3.77	4.61
	Autumn	179	50	3.68	4.50
	Mean	183.5	49.5	3.73	4.56
Three	Spring	222	68	4.87	7.21
	Autumn	214	66	4.41	6.29
	Mean	218.0	67.0	4.64	6.25
Mean	Spring	185.66	50.0	4.05	4.75
	Autumn	178.0	49.0	3.64	4.48
L.S.D at 0.05	Agriculture period	n.s.	n.s.	0.128	0.051
	Plant age	9.091	3.272	0.112	1.171
	Agriculture Period x Plant age	n.s.	n.s.	0.198	0.008

C .Sand accumulation behind of shelterbelts.

The interaction between plant age and agriculture period on plant height and stem diameter was not significant; but regarding, crown cover and crown volume it was significant. These results were in agreements with those of Draz and Zaghloul (2007).

Data in Table 3 show that the three-year treatment gave significantly lower values than given by one year for sand accumulation **in leeward rather.** Data in Table 3 reveal that the three-year spring treatment gave the highest values in windword of sand accumulation during different determination periods. This result was expected since the studied growth characters (Table, 2) showed the lowest values in this treatment and consequently the sand transmission was increased. These results were agreements with those of Zhang *et al.* (2004).

Table 3. Effect of agriculture period and plant ages on sand accumulation (gm/cm^2) during 2011year

Plant age (year)	Agriculture period	After three months			After six months			After nine months			After twelve months		
		*	**	***	*	**	***	*	**	***	*	**	***
One	Spring	228	149	34.6	64	40	37.5	692	420	39.3	549	320	41.7
	Autumn	201	135	32.8	69	44	36.2	577	370	35.9	450	270	40.0
	Mean	214.5	142.0	33.7	66.5	42.0	36.8	636.2	395.0	37.6	499.5	295.0	40.85
Two	Spring	311	140	55.0	71	26	63.3	710	250	64.8	560	170	69.6
	Autumn	300	145	51.7	82	38	53.6	695	280	59.7	600	210	65.0
	Mean	305.5	142.5	53.65	76.0	32.0	58.4	702.5	266.5	62.2	580.0	190.0	67.3
Three	Spring	288	80	72.2	67	15	77.6	590	101	82.9	550	52	90.5
	Autumn	294	91	69.1	65	15	76.9	600	122	79.7	580	77	86.7
	Mean	291.0	85.5	70.6	66.0	15.0	75.7	595.0	111.5	81.3	565.0	64.5	88.1
Mean	Spring	215.6	123.0	54.2	67.0	27.0	59.4	667.1	257.0	62.3	553.0	180.6	67.2
	Autumn	265.0	123.6	51.3	72.0	32.3	54.6	625.1	258.3	58.4	543.3	185.7	63.9
L.S.D at 0.05	Agriculture period	6.19	n.s.	3.12	3.67	n.s.	2.62	10.58	n.s.	2.17	5.05	3.88	1.64
	Plant age	26.59	6.83	4.65	5.11	4.58	3.68	17.67	2.85	3.43	1.31	11.63	2.33
	Agriculture Period x Plant age	9.62	n.s.	11.91	5.81	n.s.	4.21	16.24	11.83	2.11	7.33	8.75	n.s.

* = Windward ** = Leeward *** = shelterbelt efficiency (%)

Concerning the parameters of windward, leeward, and **shelterbelt efficiency** (Table 3), data show that the lower values of sand accumulation recorded in the treatment of one year for the autumn period may be due to the a vigorous growth of Jatropha curca trees under such conditions. From the obtained results, it could be concluded that sand accumulation records were highest after nine months. This may be due to the higher sand movement during this period of the year. Data in Table 3 also indicate that the mean values percentages of shelterbelt efficiency were 32.8 to 72.2% after three months, 36.2 to 77.6% after six months 35.9 to 82.9% after nine months and 40.0 to 90.5% after twelve months. The highest efficiency was that of three-years in the spring. This particular treatment showed averaged between 72.2 to 90.5% after three and twelve months. These results are in agreement with Su, *et al*. (2007) who found that the increase of vegetation cover decreased sand transportation rates.

Genetic Analyses

Biochemical Analyses

In this part of the study, biochemical fingerprinting was developed to identify and differentiate six *Jutropha curcas* samples. Biochemical genetic studies were based on plant proteins and isozymes variations to distinguish such species. Polymorphism among different species on the biochemical genetic levels was scored as follows:

Differences in band intensity due to differential regulation of genes governing this protein fraction.

Absence of any given band (null phenotype) in one or more species due to the differential expression of genes governing this protein fraction.

SDS-PAGE electrophoresis:

The results of SDS-PAGE electrophoresis were estimated approximately according to premixed protein

molecular weight marker, low – range (mixture of purified proteins) from Roch – Applied Science.

The protein banding patterns based on SDS-PAGE for all the six *Jutropha curcas* samples are illustrated in Table 4 and Figure 1.

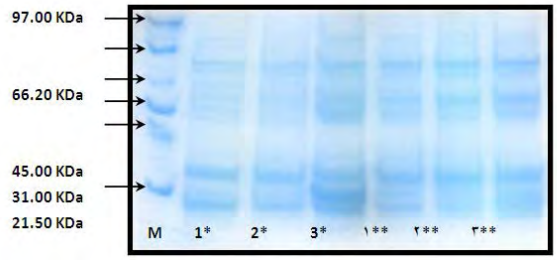

Figure 1. SDS-PAGE of protein banding patterns of the six *Jatropha curca* Samples
,2 and 3* *Jatropha curcas* samples (dry seasons).
1**,2** and 3** *Jatropha curcas* samples (wet seasons).

The bands were detected with different molecular weights ranging from 78.79 KDa to 12.86 KDa. The resulted profiles comprise 5 polymorphic bands with percentage of 55.5% (Table 5), while the remainder bands were scored as common bands with different molecular weights. The total number of bands among the six *Jutropha curcas* samples ranged from 5 bands (2 in summer season) to 7 bands in (3 in summer season). Some samples had some specific bands, which could be used to distinguish such samples among others in this season. For instance, 3 summer season had one positive specific band with molecular weight of 78.79 KDa, also (1) could be distinguished from other samples by the presence of unique band with molecular weight of 43.69 KDa. On the other hand, in winter season the samples (1 , 2 and 3) took the same genetic behavior with about 6 bands. These results are similar to those obtained by many studies which were carried out on Acacia species (Ahmed *et al.*, 2003)**,** and by (El Saied and Ahmed, 2004) on seven halophyte species they, reported that the excretive halophytes attained higher content of protein than the succulent ones. Such results could be attributed to the different adaptive responses of the studied species, which are related to their genetical characteristics and/or habitat diversity. Moons *et al*. (1995) demonstrated that plants adjusted to water stress showed a decrease in the large molecular weight fractions of the proteins and an increase in the smaller molecular weight fractions. The decrease in the number of protein fractions on desiccation due to loss of low molecular weight fractions. On the other hand, tolerant species showed an actual increase in soluble protein in spite of the decrease in the number of fractions. The tolerant species Xerophyta vislosa when desiccated and then treated with SH-compound, mercabtothanol (M.C.E), perior to elecrophoresis regained its original number of bands, with a marked increase in low molecular weight proteins. Also, Hegazi (1995) reported that plants under dry season can synthesize some stress proteins to protect themselves against the adverse conditions.

Table 4. Electrophoretic banding patterns of the six Jatropha curcas samples

Bands No.	RF.	1*	2*	3*	1**	2**	3**	MW(KD.)
1	0.12	-	-	+	-	-	-	78.79
2	0.22	+	+	+	+	+	+	56.23
3	0.26	+	+	-	-	+	-	51.08
4	0.31	+	-	-	-	-	-	43.69
5	0.37	+	+	+	+	+	+	35.00
6	0.42	-	-	+	+	+	+	29.66
7	0.68	+	+	+	+	+	+	15.87
8	0.75	-	-	+	+	-	+	13.88
9	0.80	+	+	+	+	+	+	12.86
Total		6	5	7	6	6	6	

Table 5. Number and types of bands as well as the percentage of the total polymorphism generated by total protein system of the six *Jatropha curcas* samples

Protein system	Monomorphic bands	Polymorphic bands		Total bands	Polymorphism %
		Unique bands	Non-unique bands		
Total protein	4	2	3	9	55.5 %

Isozymes analyses:-

In the present investigation, three isozyme systems; peroxidase (Prx), esterase (EST) and acid phosphatase (Acph) were studied. The zymograms are represented in Figures 2 and 3. On the other hand, polymorphic and monomorphic bands of each isozyme are tabulated in Tables 5 to 7.

Differences in band intensity were noticed between and within all of the studied samples under the two seasons. According to the presence or absence of peroxidase bands in these samples, the results reveal that the first group was scored as a common band in all samples, while the two remainder groups were polymorphic. From the table, the results cleared that the maximum gene expression was associated with 1 and 1 under wet and dry seasons, respectively, while the minimum gene expression was with the 2 dry conditions. On the other hand, the results indicated that total five bands were identified for the studied samples of esterase isozyme, which were present in some species and absent in others. The first band was scored as a common band in all species under wet and dry conditions. Four bands were scored as polymorphic bands. Also, the 2 in wet season gave the maximum gene expression of this isozyme which had 5 bands. Electrophoretic patterns of acid phosphatase isozyme indicated that a total of three bands were scored, one of them (band number 2) was polymorphic, which was present in some samples and absent in the others, while the remainder two bands were monomorphic. The results also indicate that the 1 and 1 under two season conditions gave the similar gene expression which had 2 bands; while, the other samples took the different genetic behavior under two season conditions.

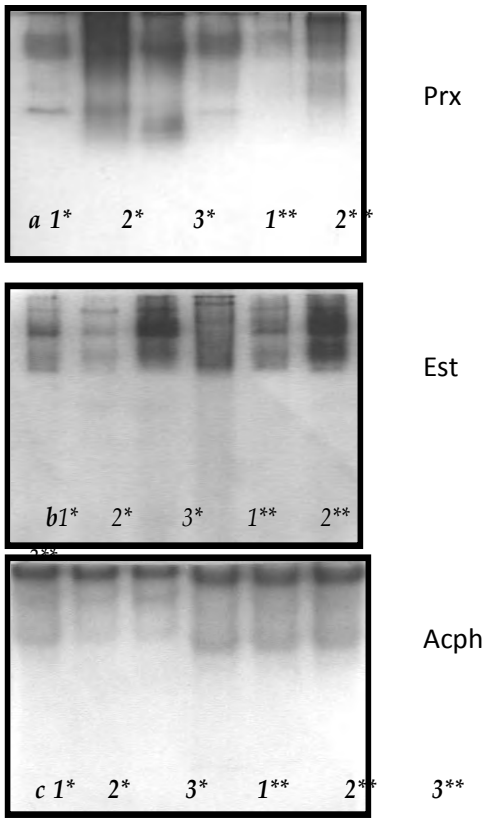

Figure 2. Electrophoretic banding patterns of three Isozymes of six *Jatropha curcas* samples

Table 5. Electrophoretic banding patterns of three isozymes; peroxidase, esterase and acid phosphatase among six *Jatropha curcas* samples

A	1 *	2 *	3 *	1* *	2 **	3* *
Prx. 1	+	+	+	+	+	+
Prx. 2	+	-	-	+	-	+
Prx. 3	+	+	+	+	-	-
Total	3	2	2	3	1	2

b	1 *	2 *	3 *	1* *	2 **	3* *
EST. 1	+	+	+	+	+	+
EST. 2	-	-	-	+	+	-
EST. 3	+	+	+	-	+	+
EST. 4	-	+	+	-	+	+
EST. 5	+	-	-	+	+	+
Total	3	3	3	3	5	4

c	**1 ***	**2 ***	**3 ***	**1* ***	**2 ****	**3* ***
Acph. 1	+	+	+	+	+	+
Acph. 2	-	+	+	-	-	-
Acph. 3	+	+	+	+	+	+
Total	**2**	**3**	**3**	**2**	**2**	**2**

Table 6. Number and types of bands as well as the percentage of the total polymorphism generated by the three isozymes of the

Isozyme	Monomorphic bands	Polymorphic bands		Total bands	% of polymrphism
		Unique bands	Non-unique bands		
Prx.	1	-	2	3	66.66 %
Est.	1	-	4	5	80.00%
Acph.	1	-	2	3	66.60%

Table 7. Matrix of the genetic similarity estimates among the

	1*	2*	3*	1**	2**	3**
1*	**100.0**					
2*	**8.0**	**100.0**				
3*	**8.0**	**100.0**	**100.0**			
1**	**54.0**	**37.0**	**37.0**	**100.0**		
2**	**8.0**	**8.0**	**8.0**	**8.0**	**100.0**	
3**	**54.0**	**8.0**	**8.0**	**8.0**	**54.0**	**100.0**

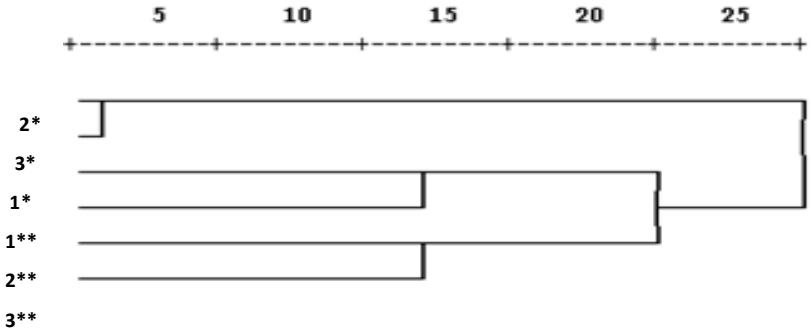

Figure 3. Dendrogram demonstrating relationship among the Isozymes

Genetic relationships among the six *Jatropha curcas* studied samples:

Based on ISSR marker polymorphisms, similarity matrix and phylogenetic dendrogram was developed by the NTSYS ver.2.1 the normalized Mantel statistic Z (Mantel, 1967). The analysis was based on the number of markers that were different between any given pair of samples to calculate the similarity matrix, Table 8 and Figure 4.

Table 8. Survey of ISSR fragments of the six Jatropha curcas samples using primers 17899B, 844B, 844A. HB10, HB11, HB12, HB13, HB8, and HB9. Where (1) means presence and (0) means absence.

Band No.	ISSR DNAmarker	1*	2*	3*	1**	2**	3**
1	17899B -3.05	0	0	0	1	1	0
2	17899B -1.59	0	1	1	1	0	0
3	17899B -1.44	1	1	0	0	1	1
4	17899B -1.29	0	1	1	1	1	1
5	17899B -1.13	1	1	1	0	1	1
6	17899B -0.84	1	1	0	0	1	1
7	17899B -0.67	1	1	1	1	1	1
8	17899B -0.24	1	1	1	1	1	1
9	17899B -0.19	1	0	1	1	1	1
	Total	6	7	6	6	8	7
10	844A -2.05	1	1	0	1	0	0
11	844A -1.87	0	0	1	1	1	1
12	844A -1.74	1	1	0	1	0	0

Table 8. cont.

13	844A -1.45	1	1	1	1	1	1
14	844A -1.24	0	1	1	1	1	1
15	844A -1.01	1	0	0	0	0	0
16	844A -0.75	1	0	0	0	0	0
17	844A -0.62	1	1	1	1	1	1
18	844A -0.49	0	1	1	1	1	1
19	844A -0.47	1	0	0	0	0	0
20	844A -0.42	0	1	1	1	1	1
21	844A -0.30	1	1	1	1	1	1
22	844A -0.20	1	1	0	1	0	0
23	844A -0.17	1	1	1	1	1	1
	Total	10	10	8	11	8	8
24	844B-0.68	1	0	1	1	1	1
25	844B-0.60	0	0	1	0	0	0
26	844B-0.56	0	0	0	1	0	0
27	844B-0.42	0	0	1	0	0	0
28	844B-0.29	1	1	1	1	1	1
	Total	2	1	4	3	2	2
29	HB8-0.70	1	1	1	1	1	1
30	HB8-0.52	1	1	1	1	1	1
31	HB8-0.21	0	0	1	1	1	1
	Total	2	2	3	3	3	3
32	HB9-0.45	1	0	1	1	1	1
33	HB9-0.42	1	1	1	1	1	0
34	HB9-0.23	1	1	1	1	1	1
	Total	3	2	3	3	3	2
35	HB10-1.12	0	1	0	1	1	1
		To be continued					
36	HB10-1.08	0	0	0	0	0	1
37	HB10-0.67	1	0	0	1	1	0
38	HB10-0.47	1	1	1	1	1	1
39	HB10-0.36	1	1	1	1	1	1
40	HB10-0.22	1	1	1	1	1	1
	Total	4	4	3	5	5	5
41	HB11-1.85	1	1	0	1	1	1
42	HB11-1.66	0	0	0	1	0	0
43	HB11-1.24	1	1	1	1	1	1
44	HB11-1.11	0	0	0	0	0	1
45	HB11-0.98	1	1	1	1	1	1
46	HB11-0.83	1	1	1	0	1	1
47	HB11-0.74	1	1	0	0	1	1
48	HB11-0.66	0	0	0	0	1	1
49	HB11-0.56	0	0	1	0	0	0
50	HB11-0.51	0	1	0	0	0	0
51	HB11-0.45	1	1	0	0	0	0
52	HB11-0.42	0	0	1	1	0	0
53	HB11-0.35	1	0	1	1	1	1
54	HB11-0.30	1	1	1	1	0	1
55	HB11-0.22	0	0	0	0	1	1
56	HB11-0.21	0	0	1	1	0	0
57	HB11-0.20	1	1	0	0	0	0
	Total	9	9	8	8	8	10
58	HB12-1.10	1	0	0	1	1	0
59	HB12-0.86	0	0	0	1	0	0
60	HB12-0.66	0	0	0	0	1	0

Table 8. cont.

61	HB12-0.64	1	0	0	0	0	0
62	HB12-0.55	1	0	0	0	0	0
63	HB12-0.27	1	1	1	1	1	1
	Total	4	1	1	3	3	1
64	HB13-0.26	1	0	1	1	1	1
65	HB13-0.20	1	1	1	1	1	1
66	HB13-0.15	0	0	0	0	1	1
67	HB13-0.10	1	0	1	1	1	1
	Total	3	1	3	3	4	4

The highest similarity matrix was scored between 2** and 3** in winter seasons (similarity of 91%), while the lowest similarity matrix scored between 1*and 3* were collected in summer season (similarity of 64.1%),

The Dendrogram (Fig 2) classified the six samples into two main clusters. The first cluster comprised only 1* and 1** were collected at two seasons, while the second cluster included all the other samples and divided into two sub clusters. The first sub-cluster was comprised 2* and 2** were collected at dry and winter conditions. However, the second sub-cluster included 3* and 3** were collected at also two conditions.

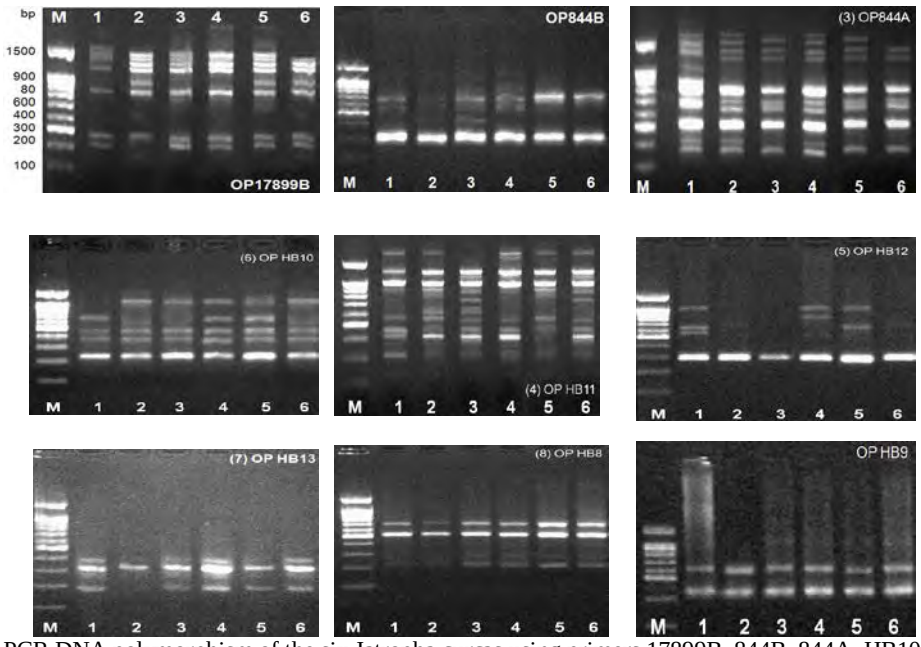

Figure 5. ISSR- PCR DNA polymorphism of the six Jatropha curcas using primers 17899B, 844B, 844A. HB10, HB11, HB12, HB13, HB8, and HB9.

Table 9. ISSR- PCR analysis from the DNAs of studied Jatropha curcas samples via 9 random primers

ISSR- PCR code	Total amplified fragments	Length range	Polymorphic fragments	Monomorphic fragments	Polymorphism%
17899B	9	3059-198	7	2	77.7%
844B	14	2052-175	10	4	71.4%
844 A	5	685-297	4	1	80%
HB8	3	706-208	1	2	33.3%
HB9	3	450-236	2	1	66.6%
HB10	6	1124-221	3	3	50%
HB11	17	1853-201	15	2	88%
HB12	6	1106-274	5	1	83.3%
HB13	4	259-104	3	1	75%
Total	67	3059-104	50	17	%75

Table 10. Species-specific markers for resulting from 9 random ISSR- PCR primers.

Samples	ISSR Species-specific markers
1*	6 + (844A -1.87\ HB8-0.21\ HB11-0.45 \ HB11-0.20) and - (844A-0.49 \844A-0.42\17899B-1.29
3* and 1**	5 - (17899B-1.44\17899B-0.84\ HB11-0.74\) + (HB11-0.42\ HB11-0.21)
2** and3**	3 + (HB11-0.66\ HB11-0.22\ HB13-0.15)

Table 11. Species-specific markers for resulting from 9 random ISSR- PCR primers.

ISSR –Primer	Sequences	Monomorphic bands	Polymorphic bands	Total bands	Polymorphism/primer %
17899B	(CA)6GG	2	7	9	77.7
844B	(CT)8GC	4	10	14	71.4
844A	(CT)8AC	1	4	5	80
HB8	(GA)6GG	2	1	3	33.3
HB9	(GT)6GG	1	2	3	66.6
HB10	(GA)6CC	3	3	6	50
HB11	(GT)6CC	2	15	17	88
HB12	(CAC)3GC	1	5	6	83.3
HB13	(GAG)3GC	1	3	4	75
Total		17	50	76	74.6

Table 12. Species-specific markers for resulting from 9 random ISSR- PCR primers

Plant ISSR	1*	2*	3*	1**	2**	3**
1*	100					
2*	75	100				
3*	66	68	100			
1**	70	71	81	100		
2**	74	72	77	97	100	
3**	71	73	79	74	91	100

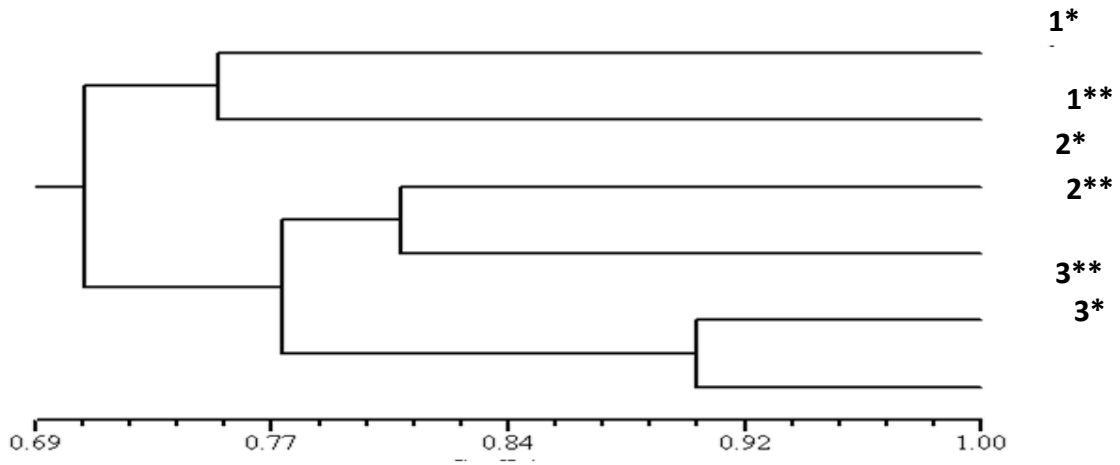

Figure 6. The genetic distances among the six *Jatropha curcas*

Samples based on ISSR analysis

In general, the relationship among the six *Jatropha curcas* samples derived from ISSR analysis was in partial agreement with some studies. In a comparable study,, Alexander (2002) successfully used ISSR marker to determine the levels and distribution of genetic relationships within and among populations of *Astragalus oniciformis*. Hassan (2005) reported that ISSR markers were offer an accurate choice for evaluation the genetic relationships between *moringa*

and *mentha* genotypes with a high accuracy. In addition, (Abdel-Tawab *et al*. 2007) used 10 ISSR primers to differentiate among four *Mentha* and three *Ocimum sp* and reported that dendrogram based on ISSR markers successfully separated the seven species into two main clusters of the two *Mentha* and *Ocimum* genera.

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Effect of some bioregulators on growth, yield and chemical composition of sesame (*Sesamum indicum* L.)

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Abstract

Two pot experiments were performed during 2007 and 2008 seasons to study the effect of foliar application with benzyladenine (BA) at 20,40 and 60 ppm, putrscine (Put) and salicylic acid (SA) at 25,50 and 100 ppm for each on growth, yield and its components as well as on chemical composition of sesame (*Sesamum indicum* (LINN.) (cv. Shandawel 3). The obtained results indicated that the foliar application of benzyladenine (40 ppm), putrscine (100 ppm) and Salicylic acid (50 ppm) significantly increased all vegetative growth characteristics, i.e. stem length, number and leaf area / plant, dry weight of stems, leaves and capsules/ plant, net assimilation rate, fruiting zone length, number of flower and capsules per plant at 75 days after sowing. In addition, foliar application of these treatments significantly increased yield and its components, i.e. number of capsules per plant, capsules weight and seeds weight (g) per plant, weight of 1000 seeds (g) and seeds yield (g) per pot as well as seed oil yield per plant. Also, foliar application of BA at 40 ppm, Put at 100 ppm and SA at 50 ppm induced an increase in photosynthetic pigments (chlorophyll a , b and carotenoids) in leaves and minerals (N, P, K, Ca, Mg, Fe and Zn), total sugars and carbohydrates, total free amino acids and crude protein concentration in sesame leaves and seeds. Also, seed oil percentage and percentage of fatty acids composition of sesame oil were increased. Besides that, foliar application with all used bioregulators decreased the percentage of total saturated fatty acids, while induced an obvious increase in the percentage of total unsaturated fatty acids and unsaturated : saturated ratios of sesame seed oil. In this concern, the foliar applications of BA at 40 and put at 100 ppm were the most effective concentrations on seed and oil yield of sesame plant. Finally, this study strongly suggests the use of BA, put and SA at 40, 100 and 50 ppm, respectively for increasing seasme growth and productivity.

Key words: Sesame, bioregulators: benzyladenine, puterscine, salicylic acid, net assimilation rate, hotosynthetic pigments, fatty acids.

Introduction

Sesame is considered as one of the most important oil crops in the world and Egypt because its seeds contain about 45-55% oil, 19-25% protein, 15-20% carbohydrates, 5% fiber and 4-6% ash (Weiss, 2000) as well as sesame oil contain about 45-50% oleic acid, 34-40% linoleic acid, 7-10% palmitic acid, 3-5%, stearic acid and 105-130 iodine value There for , its an excellent edible semi-drying oil and could be used for human consumption.

In Egypt, the local production of oil crops is insufficient for local consumption. Since, cropping composition dose not allow enough space for oil crops. Therefore, there is a need to increase its productivity by application of different factors including bioregulators or plant growth regulators (PGRs) to improve growth and development, flowering, fruit set, seed formation and yield of sesame seed oil. Many trials have been carried out for increasing or improving growth, flowering, seed and oil yield of sesame or other crops by the use of plant growth regulators (bio-regulators) by (Zaghlool *et al*., 2001&2006; Abo El-Saoud, 2005; Abd El-Dayem *et al*., 2005; and Ibrahim and Sharaf El-Deen, 2008).

Putrescine (Put) , benzyladenine and salicylic acid have been shown to be involved in several aspects of plant growth and development including cell division, somatic embryogenesis, rooting, floral initiation, development of flowers and fruits, fruit set, antisenescence, nucleic acids and protien synthesis and stabilization, ionic balance, chlorophyll content, ethylene synthesis reduction and leaf chlorophyll degradation (Smith, 1985; Evans and Malmberg, 1989; Davies, 1995; Galston and Kaur-Sawhney, 1990 & 1995; Shehata *et al*., 2000; Couee *et al*., 2004; Lester, 2000; Jain *et al*.,2005 and Papadakis and Roubelakis-Angelakis, 2005). Also, these bio-regulators enhanced chlorophyll formation and carbohydrate content and induced changes in endogenous phytohormones in plants (Sharma and Ali,1998;Shehata *et al*., 2000 & 2001) and increase enzymes activities, ion uptake, inhibit ethylene biosynthesis, reverse abscisic acid (ABA) induce stomatal closure and enhance disease resistance (Raskin, 1992). Also, Abo El-Saoud (2005) reported that foliar spray of snap bean plants with benzyladenine (40 ppm) and Put (200ppm) significantly increased number of flowers and pods/ plant, fruit setting percentage and green pods yield/plant. In addition, Abd El-Dayem et al. (2005) on soybean, observed that foliar application of BA (25 &50 &100 ppm) increased flowers number plant^{-1} and yield characteristics i.e., pod number, seed number, seed yield, seeds weight plant^{-1} and

100-seeds weight, oil percentage and the fatty acids composition, meanwhile decreased shedding percentage. Abd El-Gawad (2006) showed that BA (5 & 25 ppm) increased fruit setting, number of capsules/plant, weight of intact capsule, weight of seeds/capsule, seeds number/capsule, weight of 100-seeds and seed yield (economical yield) of Nigella sativa. Therefore, the present investigation was carried out to study the effect of foliar application with benzyladenine (BA), putrescine (Put) and salicylic acid (SA) on the growth, seeds and oil yield as well as biochemical composition of sesame plant. Also the present study aimed at studying the effect on seed yield and oil quality and content of sesame by using plant bioregulators.

Materials and methods

Two pot experiments were carried out in the green house at the Experimental Station of Agricultural Botany Department, Faculty of Agriculture, Moshtohor, Benha University during two growing successive seasons of 2007 and 2008. Seeds were obtained from the Agricultural Research Center, Giza, Egypt. Plastic pots of 30cm in diameter were filled with 7 kg of soil from mixture of clay and sand (2:1 W/W). Phosphorus and potassium were added to the soil of each before sowing at the rate of 1.4 g pot^{-1} Calcium Super Phosphate and 1.0 g pot^{-1} of Potassium Sulphate. Ten seeds were sown in each pot on 10 May in 2007 and 2008 seasons. Plants were thinned to four uniform seedlings per pot 21d from planting. After thinning, plants were fertilized as recommended with 5gm ammonium nitrate per pot in two equal doses, first after thinning and the second on at the beginning of flowering stage. The pots were arranged in a completely randomized design with 12 replicates (12 pots per treatment).
Bioregulators were used as foliar application at three concentrations as follows: Control (distilled water) 0.0, Benzyladenine (BA) at 20, 40 and 60 ppm, Putrescine (Put) at 25, 50 and 100 ppm, Salicylic acid (SA) at 25, 50 and 100 ppm. Plants of each treatment were sprayed with different bioregulators three times 30, 55 and 80 DAP.

Sampling and collecting data

Vegetative growth

Two samples of sesame plant (75 and 120 DAP) were collected from each treatment in 2007 and 2008 seasons. Twelve plants from each treatment were randomly taken for different measurements. Then the plants were separated into their organs (stems, leaves and capsules). The samples of these organs were dried in the oven at 70 C° for 48 hours till constant weight. The dried samples of different organs were weighed for dry weight estimation. The following growth characters were estimated: stem length (cm), number of leaves $plant^{-1}$, leaf area (cm^2) $plant^{-1}$, dry weight of roots (g) $plant^{-1}$, dry weight of stems (g) $plant^{-1}$, dry weight of leaves (g) $plant^{-1}$, number of flowers $plant^{-1}$, number of capsules $plant^{-1}$, net assimilation rate (NAR) ($g/cm^2/day$). Net assimilation rate was calculated between successive samples (45 and 75 days) as described by Hunt (1978) using the following equation:

$$NAR\ (g/cm^2/day) = \frac{(W2 - W1)}{(T2 - T1)} \times \frac{(LnLA2 - LnLA1)}{(LA2 - LA1)}$$

W: whole plant dry weight, LA: leaf area, T: time.

Yield and its component

At harvest, 120 days after sowing in 2007 and 2008 seasons, 12 randomly chosen plants from each treatment were taken for estimating the following yield characters: Fruiting zone length (cm) $plant^{-1}$, number of capsules $plant^{-1}$, weight of intact capsules (g) $plant^{-1}$, weight of seeds (g) $plant^{-1}$, weight of seeds (g) pot^{-1}, weight of empty capsules (g) $plant^{-1}$, weight of 1000 seeds (g), seed oil percentage, seed oil yield $plant^{-1}$, fatty acids composition in sesame seed oil.

Chemical analysis

Chemical analysis was carried out on the samples of leaves and seeds 75 DAP during 2008 only to determine chlorophyll a, b and carotenoids that were calorimetrically determined in the fresh leaves according to the method described by Wettstein (1957). Total nitrogen (Horneck and Miller, 1998), Phosphorus (Sandell, 1950), potassium (Horneck and Hanson ,1998). Calcium and magnesium were determined according to Jackson (1967). Also, Fe and Zn were determined according to (Black, *et al.*, 1965). Total soluble sugars and total carbohydrates were determined according to the method described by (Dubois *et al.*, 1956).Total free amino acid was determined in the ethanolic extract with ninhydrine, buffer, solvent and boiling time colourimetrically according to Muting and Kaiser (1963). Crude portein was calculated according to the following equation: Crude protein in seeds = Total nitrogen x 5.26 (A.O.A.C. 1990).

Determination of oil content of sesame seeds

Crude fat was estimated as percentage oil was determined using Soxlet apparatus using petrolium ether as a solvent according to A.O.A.C.(1990). Fractionation, identification and quantification of different fatty acids were carried out. The fatty acids were converted to methyl esters according to Sink *et al.*, (1964). The fatty acids methyl esters were analyzed using Gas Liquid Chromatography (GLC)/HP (6890) GC capillary equipped with flame ionization detectors and coiled glass column each (DB-23 capillary column), Dimension:60 m × 0.32 mm × 0.25 µm.

Statistical analysis

Data of growth and yield characteristics were subjected to statistical analysis according to Snedecor and Cochran (1989).

Results and discussion

Growth characteristics

The growth characteristics of sesame plants as stem length, number and leaf area per plant, dry weights of stems, leaves, capsules and net assimilation rate as well as fruiting zone length, number of flowers and number of capsules were significantly increased by foliar application with bioregulators as benzyladenine (20, 40 and 60ppm), putrescine (25, 50 and 100ppm) and salicylic acid (25, 50 and 100ppm) at 75 and 120 DAP in both seasons and are presented in Tables 1. As for stem length, number and leaf area per plant, foliar application with Put 100 ppm, BA 40 ppm and SA 50 ppm gave the highest across-season mean, respectively at 75 DAP. Regarding the total leaf area per plant it nearly behaved as the same as leaf number. Since, all foliar application treatments showed significant increase, but a maximum was also obtained using BA 40 ppm and Put 100 ppm. Increment in leaf area is of great interest because it could be reflected upon the efficiency of photosynthesis by accumulating more assimilates and high rates of their translocation specially toward formed capsules. The increment in leaf characters in response to foliar spray with bioregulators (BA, Put, SA) may be attributed to the stimulating effect of these bioregulator on cell division and enlargement in the meristems and organ primordia, increasing endogenous promoter substances (CYK, GA, IAA) levels which in turn increase leaves expansion. In this connection Shehata *et al*. (2000&2001), Ibraheem (2007), Zaghlool *et al*., (2001 and 2006), Abo El-Saoud (2005), Wanas (2007 a & b), stated that bioregulators (BA, Put and SA) increased leaves growth by (via) increasing cytokinins levels in wheat and other plants. Regarding leaf dry weight per plants, it is of interest to note that all foliar application with bioregulators significantly increased it. Also, BA 40 ppm and Put 100 ppm gave the highest mean when compared with across-season control. As for net assimilation rate at 75 days after sowing, it increased in most with different bioregulators. The exception was that SA at 100 ppm caused a reduction in this respect during 2007 season. Also, each of Put 100 ppm and BA 40ppm gave the highest values in this respect compared with the control. With regard to fruiting zone length per plant, all used bioregulators significantly increased fruiting zone length of sesame plants at 75 and 120 DAP across the two seasons. The most effective, bioregulator in this respect was the putrescine (Put) at 100ppm followed by benzyladenine (BA)at 40ppm and salicylic acid (SA) at 50ppm when compared with the control. The positive favorable effects of bioregulators (BA, Put and SA) on all previous growth characters (stems length, leaf area and dry weights of sesame organs) may be attributed to: (1) Enhancing cell division and differentiation activity, (2) Increasing endogenous growth promoters (IAA, GA and CYKs) and decreasing ABA and ethylene, Ibraheem (2007), (3) improving photosynthesis efficiency and mobilizing, (4) Enhancing the uptake of mineral nutrient assimilate translocation from source to sink, (5) Increasing leaf growth and photosynthesis capacity by increasing endogenous cytokinins. Arigita *et al.* (2005) mentioned that cytokinins promote shoot development through increasing cell division. Regulation of the cell cycle and the number of cycles which cells undergo in the meristems and organ primordials are the primary regulatory that targets cytokinins. The stimulating effect of BA, Put and SA on number of flowers and capsules/plant may be due to the critical role of these bioregulators in enhancing various processes of reproductive growth such as a) Increasing flowers bud formation (flower bud initiation differentiation and development), b) Delaying flower abscission (decrease shedding %), c) Enhancing pollination, fertilization and development of fruit, d) Increasing fruit set which in turn increasing capsules/plant, e) Enhancing transport of photoassimilates and nutrients from leaf towards fruits (sinks), which reflect on the fruits formation.

Chemical composition of leaves

Photosynthetic pigments

Data in Table 2 indicate that different photosynthetic pigments, i.e., chlorophyll a, b and carotenoids positively responded to the different foliar application with BA, Put and SA treatment 75 DAP during the two seasons. Also, BA and Put at 100ppm gave the highest values in this respect compared with the control. Moreover, the stimulation of photosynthetic pigment formation may be attributed to the vigorous growth (Table 1) obtained in increasing chlorophylls and carotenoids content, enhanced photosynthesis efficiency and increased dry matter production (Table 1). The promoting effect of bioregulators (BA, Put and SA) on chlorophyll synthesis was supported by Sudria et al. (2001) who found that cytokinins application accelerated the development and photosynthesis such as CO_2 assimilation activity of photosynthetic enzymes and consequently promoted the photosynthetic activity. The present results agreed with those obtained by Patil et al. (2002), Senthil *et al*. (2003) and Talaat *et al*. (2005). Also, Abo El-Saoud (2005) observed that foliar application of snap bean plants with BA (40 ppm) and Put (200 ppm) increased the content of photosynthetic pigments i.e., chlorophyll a, b and carotenoids in leaves. Also, Shehata *et al.* (2001) and Zaghlool *et al.* (2006)

stated that SA increased photosynthetic pigments i.e., chlorophyll a, b and carotenoids on some plants.

Minerals and some bioconstituents

Data in Table 2 clearly indicate that all foliar application treatments increased N, P, K, Ca and Mg at 75 DAP in sesame leaves during 2008 season. Also, Put at 100 ppm showed the highest content of N, P, K, Ca, Fe and Zn at 75 DAP compared with control plants and other treatments. As shown in Table 2 carbohydrates, sugars and free amino acids and protein content were increased with different foliar application of bioregulators in 2008 season at 75 DAP. Also, Put at 100 ppm and BA at 40ppm gave the highest content of total carbohydrates, sugars and free amino acids at 75 DAP, respectively when compared with the control.

The increase in total sugars, carbohydrates, proteins and amino acids of leaves in response to BA, Put and SA applications is supported by stimulation in photosynthetic pigments (Table 2) and the accumulation of dry matter in leaves at 75 days (Table 1). Also, the increase in protein and total free amino acids in leaves was accompanied by increase in total N (Table 2) parellel to growth rate, stimulation of amino acids to make protein and to the translocation of sugars and free amino acids to young leaves and developing fruits. Many investigators suggested that bioregulators BA, Put and SA not only promote photosynthetic activity, but also increased RNA and protein synthesis (Senthil *et al.*, 2003; Sood and Nagar, 2003; Abo El-Saoud, 2005; Papadakis and Roubelakis-Angelakis, 2005; and Ibraheem, 2007).

Yield and its component

Data in Table 3 indicate that foliar application with different bioregulators significantly increased yield and its components expressed as number of capsules plant^{-1}, weight of seeds (g) plant^{-1} and weight of 1000-seeds (seed index) at 120 DAP across seasons.

These increases in capsules number/plant were accompanied by increasing flower number and decreased shedding of flowers and fruits. The promotive effect of bioregulators BA at 40 ppm and Put at 100 ppm treatments on capsules numbers may be attributed to its effect on floral bud formation, multiple number of flowers on node and opposite leaf arrangement which encourages multiple flowering in the leaf axils of lower and middle nods on the main stem. The leaf arrangement is important as it controls the number of flowers formed in the axils and the number of capsules in sesame plants (Weiss, 2000). It is clear from results based on across-season mean that the highest capsule number plant^{-1} (102.05) in response to 40 ppm BA followed by 100 ppm(68.42). In this respect, Kamal *et al.* (1995) observed that application of kinetin with 5 ppm at flowering stage of soybean plants increased yield by an average of 9.3 % over the control. The yield increased was primarily due to the increase in pod number through the increase of fertile node number and number of pods per fertile node. Also, Awasthi *et al.* (1997) on faba bean plants, showed that salicylic acid treatments caused an increase in number of flowers, pod number plant^{-1}, number of seeds pod^{-1}, pod weight and seed yield plant^{-1}. Sharma and Ali (1998) working on soybean, found that foliar application of putrescine and spermine at 10-3M significantly increased number of pods per plant, 100 seed weight, seed biological and oil yield. Also, the seeds weight (g) per plant and seeds yield (g) per pot at 120 days after sowing in most cases were significantly increased.Weight of 1000 seeds (seed index) was significantly increased in most treatments with different used bioregulators.

The increase in yield and its component in sesame plants as treated with BA, Put and SA may be attributed to an increase in the vigorous growth (Tables, 1), photosynthetic pigment content (Table 2), the amount of metabolites synthesis (carbohydrates, protein and free amino acid in leaves (Table 2), absorption and translocation of nutrient elements (Table 2) and enhancing sink activity by increasing the rate of net amount of photoassimilates transport from sites of synthesis in leaf tissue source to sites of accumulation in developing seeds or storage organs (sink) and decrease shedding of reproductive organs, which reflected upon yield and its components. These results are agreed with those reported by Ibrahim *et al.* (2001), Abd El-Dayem *et al.* (2005), Abo El-Saoud (2005), Zaghlool *et al.* (2006), Wanas (2007a&b) and Ibraheem (2007) and El-Moawafy (2008).

Chemical composition of seeds (seeds quality)

Minerals and bioconstituents

Data in Table 4 and clearly show that different foliar application with bioregulators increased mineral contents N, P, K, Ca, Mg, Fe and Zn in seeds of sesame plants during 2008 season. Also, BA at 40ppm ranked the first followed by Put at 100ppm when compared with the control plants and other treatments.

As for crude protein content, it increased with different foliar application treatment. Also, SA at 50ppm treatment gave the highest content of crude protein in seed yield followed by BA at 40ppm and Put at 100ppm respectively. Also, all foliar application treatments increased total carbohydrates content in seed yield of sesame plants during 2008 season. In addition, total carbohydrates increased to reach its maximum with Put at 50 ppm followed by Put at 100 ppm during 2008 season. These stimulatory effects of BA, Put and SA on mineral contents in sesame seeds may be attributed to their enhancing effects on membrane permeability, ions uptake and increasing the rate of ion entry through

the membrane, which reflected on translocation of mineral nutrients to shoot and consequently to fruits. As well as these bioregulators treatment improved photosynthetic activity and efficiency and consequently more synthesis of sugars and increases the mobilization, translocation and partitioning of biosynthesized material (sugars) to seeds in fruits of sesame plants. Similar results were obtained by Abd El-Dayem *et al.* (2005) who found that foliar application of soybean plants with benzyladenine at 50 ppm significantly increased total soluble sugars, polysaccharides and consequently total carbohydrates, total protein, N, P, K, Ca and Mg contents in the yielded seeds. Similar responses were obtained by several investigators (Abo El Saoud (2005) on snap bean, Ibraheem (2007) on wheat plants, Ibrahim and Sharaf El-Deen (2008) and El-Mowafy (2008) on wheat plant, Zaghlool *et al.* (2001) on Phaseolus vulgaris, Zaghlool et al. (2006) on wheat plants, Wanas (2007a&b) on faba bean plants and El-Mowafy (2008) on wheat plants.

Oil percent and oil yield per plant

Data in Table 4 show that foliar application with different bioregulators increased oil percentage and yield plant^{-1} of sesame plants during 2008 season. In addition, Put at 100 ppm and BA at 40 ppm gave the highest values in oil yield (g) plant^{-1} BA at 40 ppm followed by Put at 100 ppm and SA at 50ppm during 2008. Values were 28.07 & 20.88 and 13.50 during 2008, respectively, but were 4.40 with control plants. We conclude that the foliar application of bioregulators (BA, Put and SA) caused an increase in fatty acids biosynthesis and other metabolites which reflected on an increase in the oil percentage of sesame seeds.

Fatty acids percentage

Data also in Table 4 showed the effect of foliar application with BA, Put and SA on saturated and unsaturated fatty acids of sesame seed oil in 2008 season. Percentage of saturated fatty acids were decreased by foliar application with bioregulators, on the contrary all used bioregulator treatments increased unsaturated fatty acids as well as a marked increase in unsaturated fatty acids: unsaturated fatty acids ratio of sesame seed oil when compared with the control plants and other treatments. These results are in harmony with those obtained by Sharma and Ali (1998) who showed that the soybean seed and oil yield increased by about 12.46 and 20.94% respectively with the application of 10-3 M spermine at 50% flowering stage. In addition, Abd El-Rehim *et al.* (2000) found that kinetin treatments (5,10and 20 ppm) increased the percentage of lauric, myristic, streic palmitic and oleic acids. Also, the saturated fatty acids were increased while unsaturated fatty acids were decreased in oil of datura seeds. Also, Ibrahim *et al.* (2001) indicated that kinetin treatment (50ppm) increased crude fat and unsaturated fatty acids (C18:1, C18:2 and C20:1). Meanwhile saturated fatty acids were decreased in oil of sunflower seeds. Also, Abd El-Dayem *et al.* (2005) showed that foliar application of benzyladenine (50ppm) increased oil percentage and fatty acid composition of soybean seed oil, a marked increase in the levels of unsaturated fatty acids (C18:1, C18:3 and C20:1), while linoleic was decreased. In the meantime, there was an increase in the detectable saturated fatty acids in seed oil of treated plants.

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Table 1. Effect of foliar application with bezyladenine (BA), putrescine (Put) and salicylic acid (SA) on growth traits of sesame plants at 75 and 120 DAP averaged over two 2007 and 2008.

Growth Characters		Number of leaves/plant	Leaf area/plant (cm^2)	Stem length (cm)		Dry weight			Net assimilation	Fruiting zone length		Number of flowers	Number of capsules
						stems	leaves	capsules					
Treatment		Days after sowing (DAS)											
(ppm)		75	75	75	120		75		75	75	120	75	75
Control	0.0	19.73	46.15	52.23	79.61	1.86	2.53	0.83	0.365	21.76	44.81	8.92	5.92
BA	20	21.99	64.29	61.47	116.46	3.11	3.18	1.065	0.43	26.75	76.195	10.96	6.72
	40	25.97	74.09	68.33	121.99	3.36	4.35	1.90	0.55	27.65	85.67	15.06	13.39
	60	22.08	58.18	63.85	90.95	2.65	2.92	1.34	0.42	27.36	58.91	13.14	8.95
Put	25	18.080	55.00	64.49	93.25	2.32	3.12	1.25	0.45	27.36	57.21	5.57	9.67
	50	23.55	62.36	65.38	106.08	3.6	4.56	2.82	0.75	34.14	66.76	10.31	15.79
	100	29.21	70.6	73.19	109.81	4.34	4.96	3.51	0.92	41.03	68.27	11.75	18.24
SA	25	18.61	42.87	53.55	96.99	1.89	2.76	0.87	0.36	22.71	54.45	5.89	6.72
	50	24.83	56..39	64.46	99.63	3.12	4.11	1.45	0.60	32.94	57.39	13.39	13.67
	100	21.57	50.06	50.51	89.74	1.79	2.61	0.37	0.32	20.83	48.83	5.18	6.07
LS.D at 5%		2.99	5.98	4.16	11.29	0.91	0.882	0.35	---	5.21	10.56	2.38	3.43

Table 2. Effect of foliar application with benzyladenine (BA), putrescine (Put) and salicylic acid (SA) on photosynthetic pigments, mineral contents, total carbohydrates, total soluble sugars, total free amino acids and crude protein in leaves of sesame plants at 75 DAP sowing in 2008.

Treatments (ppm)		Chlorophylls (mg/g F.W.)				Mineral contents (mg/g D.w.)					ppm		Total carbohydrates (mg/gD.W.)	Total soluble sugars (mg/g F.W.)	Total free amino acids (mg/g F.W.)	Crude protein (mg/g D.W.)
		A	b	a+b	Carotenoiss	N	P	K	Ca	Mg	Zn	Fe				
Control	0.0	1.136	0.708	1.844	0.645	34.16	4.48	24.95	23.33	2.20	49.50	4.50	331.20	20.70	3.70	213.50
	20	1.170	0.713	1.883	0.659	50.48	6.60	26.10	33.33	3.20	75.00	7.00	346.72	15.40	8.40	315.50
BA	40	1.204	0.768	1.972	0.690	53.76	6.80	28.75	36.70	3.60	91.00	8.00	372.60	33.70	9.90	336.00
	60	1.074	0.799	1.873	0.656	45.36	5.04	27.15	30.00	2.60	69.00	4.50	340.50	25.15	5.70	283.50
	25	1.277	0.506	1.783	0.624	38.64	6.80	29.40	30.00	3.20	55.00	8.50	346.40	22.60	4.40	241.50
Put	50	1.292	0.514	1.806	0.632	46.96	6.94	30.20	36.70	3.00	89.50	12.5	344.30	21.40	7.70	293.50
	100	1.308	0.742	2.050	0.718	47.84	6.98	30.40	40.00	3.60	114.50	13.5	386.90	28.00	8.20	299.00
	25	1.201	0.708	1.909	0.668	36.96	5.34	25.80	36.67	2.20	68.50	9.50	336.72	21.25	4.43	231.00
SA	50	1.245	0.737	1.982	0.694	38.96	5.48	26.80	40.00	2.80	88.50	10.5	348.20	32.95	5.00	243.50
	100	1.196	0.758	1.954	0.684	35.64	4.84	24.95	33.33	2.20	60.50	9.00	335.50	29.30	4.40	222.75

Table 3. Effect of foliar application with benzyladenine (BA), putrescine (Put) and salicylic acid (SA) on yield and its components of sesame plants at harvest time (120 days) averaged over 2007 and 2008.

Yield Characters Treatments	(ppm)	Number of capsules/ plant	Weight of intact capsules (g)/plant	Weight of seeds (g)/plant	Weight of empty capsules (g)/plant	Weight of 1000-seeds (g)	Seeds yield (g)/pot
Control	0.0	38.47	15.45	6.66	8.79	3.197	26.64
	20	85.18	45.94	19.87	26.07	3.94	79.48
BA	40	102.05	62.59	31.39	33.92	4.13	125.56
	60	54.22	25.18	11.48	13.7	3.53	45.9
	25	50.29	23.51	10.58	12.94	3.42	42.3
Put	50	54.25	29.06	13.07	15.95	3.85	52.28
	100	68.42	46.92	25.09	21.88	4.012	100.34
	25	57.66	23.21	11.14	15.22	3.55	44.54
SA	50	65.28	27.88	17.26	15.28	3.94	69.02
	100	38.75	11.69	6.62	8.17	3.46	26.48
L.S.D. at 5%		4.33	3.54	9.11	8.49	0.128	10.12

Table 4. Effect of foliar application with benzyladenine (BA), putrescine (Put) and salicylic acid (SA) on mineral contents, crude protein, total carbohydrates in defatted seeds as well as oil percentage, oil yield/plant of sesame plants during 2008.

Treatments (ppm)		Mineral contents (mg g^{-1} D.W.)					ppm		Crude protein (mg g^{-1})	Total carbohydrates (mg g^{-1} D.W.)	Oil percentage (%)	Oil yield $plant^{-1}$ (g)	Fatty acids %		
		N	P	K	Ca	Mg	Fe	Zn					Total saturated	Total unsaturated	TS:US ratio
Control	0.0	36.00	2.96	11.75	2.67	1.40	40.00	9.40	190.80	145.20	47.45	4.40	17.47	81.64	1:4.7
BA	20	41.00	3.08	12.30	2.67	1.40	51.50	11.60	217.30	149.20	51.40	16.99	15.44	84.47	1:5.50
	40	48.00	3.20	13.10	3.33	1.70	54.50	11.50	254.40	150.96	53.41	28.07	15.25	84.62	1:5.55
	60	39.00	3.08	12.15	3.00	1.60	52.50	10.50	206.70	148.24	52.30	8.51	15.38	84.53	1:5.50
Put	25	41.00	3.16	12.15	2.67	1.80	51.50	11.20	217.30	175.44	54.09	8.04	15.28	84.62	1:5.4
	50	43.00	3.00	12.30	3.33	1.50	56.00	11.70	227.90	188.88	54.10	11.03	15.204	84.59	1:5.65
	100	47.00	3.18	12.30	3.33	1.80	60.00	12.50	249.10	175.84	54.30	20.88	15.100	84.91	1:5.62
SA	25	49.00	2.80	12.00	3.00	1.50	40.00	11.10	259.70	156.40	48.80	8.35	15.41	84.32	1:5.47
	50	47.00	3.04	12.10	3.00	1.80	40.00	12.00	249.10	168.64	52.80	13.50	15.49	84.41	1:5.45
	100	37.00	2.62	12.10	2.33	1.60	38.00	11.00	196.10	145.52	9.90	3.86	15.85	83.95	1:5.30

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3. Bio-Pesticides techniques


Efficacy of two biopesticides based on entomopathogenic fungi against the honeybee ectoparasitic mite, *Varroa destructor*

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Abstract

The varroa mite, *Varroa destructor* (Anderson and Trueman) (Acari : Varroidae), is known as the most serious ectoparasitic mite on honeybee, *Apis mellifera* (Hymenoptera: Apidae) in the world. Based on the spores of entomopathogenic fungi, two commercial preparations; Bioranza (*Metarhizium anisopliae*) and Biovar (*Beauveria bassiana*) were evaluated through application into the hives against varroa mite. Data showed significant differences between treatments with Bioranza and Biovar, the results were significant after 7 and 14 days post-treatment. Mean daily fallen mite individuals was significantly different between the hives before and after the applications of the two biopesticides, and wheat flour. Also, mites' mortality was ,significantly, different between the hives before and after treatments. There were significant differences between treatments with the two biopesticides in worker's body weight. Bioranza and Biovar did not infect the honeybee in larval, prepupal, pupal and adult stages. Scanning and transmission electron microscopy images showed spores and hyphae penetration through stigma and wounds on varroa. The results suggest that Bioranza and Biovar are potentially are effective biopesticides against *V. destructor* in honeybee colonies.

Key words: *Apis mellifera, Varroa destructor*, biopesticides, *M. anisopliae , B. bassiana*, electron microscopy

Introduction

The parasitic mite *Varroa destructor* is currently a worldwide parasite on the honeybee *(Apis mellifera* L.). It feeds on the hemolymph of immature and adult bees (Harbo and Harris, 2001).This specialized blood-feeding mite species reproduces within sealed brood, showing a strong preference for drone brood over worker brood (Martin, 2001).The honeybee, *Apis mellifera* L., is considered of great economic importance, not only for honey production but also for crop pollination. A balanced host-parasite relationship is established in the sense that the host fitness loss due to parasitism is limited because mite reproduction occurs in drone brood only (Peng *et al*., 1987; Tewarson *et al*., 1992). This parasitic mite causes weight loss, malformation, a shortened life-span in honeybees (De Jong *et al*., 1982).

Several methods including biotechnical, genetic, and chemical controls have been assayed against *Varroa destructor* (Fries and Hansen 1993, Schmidt-Bailey *et al*. 1996, De Guzman and Delfmado-Baker 1996, Rinderer *et al*. 1997, El-Ghamdi and Roger 2004). Development of resistance in varroa mite populations to coumaphos was studied by Elzen *et al*. 1998; Elzen and Westervelt 2002. Control of varroa mites with organic acids often requires multiple applications to achieve a high degree of control, and pose a safety way to beekeepers (Ven *et al.,* 1998). Essential oils and their components have also been assayed (Calderone *et al.,* 1997). In previous studies, the dust or strip applications of *Metarhizium anisopliae* to honey bee hives provided satisfactory control of varroa mite under field conditions (Kanga *et al*., 2003). The fungi *Beauveria bassiana* has been used as mycopesticide to control of more than 700 arthropod species (Goettel and Johnson 1992). Meikle *et al.,* (2006) reported the discovery of several isolates of *B. bassiana* from varroa mites collected from honeybee colonies in southern France. Collecting fungal isolates from either the target environment and/or even the target pests themselves is intended to increase the probability of finding the best adapted isolates against these pests.

The present study aimed to determine the effect of two biopesticides, namely Bioranza (*M. anisopliae*) and Biovar (*B. bassiana*) against *V. destructor*, a honeybee ectoparasitic mite in Egypt. Compare between the two commercial formulations and inert powder on the fallen mites and mean weights of honeybee worker. Scanning and transmission electron microscopy images were ,also, used to show penetration of the *M. anisopliae* spores and hyphae.

Materials and methods

Biopesticides

Two commercial formulations ; Bioranza 10% W.P of dry conidia of fungus *M. anisopliae* and Biovar 10% W.P of dry conidia of fungus *B. bassiana.* A weight of 0.1 gm of each formulation was suspended in 50 ml of water to be used per each colony.

Inert materials

Both of wheat and maize flour were used to make the treatments more economical for beekeepers. Five grams of powder of maize or wheat flour were used per each colony.

Experimental honeybee colonies

The present investigation was carried out in special apiary during spring season of 2011. Fifteen colonies

of hybrid Carniolan honeybees, *A. mellifera* naturally heavily infested by varroa mites were used.

Mite collection
To test the efficacy of selected commercially formulated of entomopathogenic fungi against *V. destructor*, female mites were collected from infested frames of sealed brood taken from honeybee colonies, and additional mites were collected from nurse and adult bees on the frames. The mites were placed into glass scintillation vials (20 ml) containing two late instars honeybee larvae as food source. Twenty to thirty mites were held in each glass vial and used in the bioassays within an hour after they were collected.

Treatments
Four groups of colonies and control, all treated by spraying with fine atomizer. The 1st treated with Bioranza, the 2nd with Biovar, the 3rd with wheat flour, the 4th was treated with maize flour, and 5th is the control. Mite mortality was recorded daily for 7 days, and the experiments were repeated three times. Also, all treatments were repeated with using wheat flour. Dead mites were collected daily from the two biopesticides treatments, and tested in the following way to see if mortality was due to infection.

Electron Microscopy Examination
Mites were surface sterilized by dipping them for 1min in a sterilant-disinfectant, and rinsing them with 95% ethanol. The mites were then transferred with a camel-hair brush to Petri-dishes containing water–agar and incubated at 25° C for 4-7 days. Also, dead mites in the controls were also surface-sterilized and incubated as described above. Mites were observed daily for the presence of external fungal hyphae. The numbers of mites with external hyphae were counted; these mites were removed from the Petri dishes. Only mites that showed fungal growth were considered to have died by infection and photographed with Electron Microscopy.

Establishing the hives:
The hives used in these experiments were established by dividing larger honeybee colonies that were severely infested with *V. destructor* into 15 nucleus colonies to ensure uniform bee and mite populations. Used queens were progeny of the same mother. The nucleus colonies were placed about 1.5 m apart from each other. Twelve days after they were established, hives were ready for treatment. Honeybee workers were collected from each treatment and weighed.

Hive treatments:
The experimental started from April to October, 2011. A new set of hives was established for each experimental run. To treat the colonies, each hive was opened, and each frame (both sides) was sprayed. Data on mite mortality were recorded daily for 14 days.

Data collection
Before and after the fungal applications, the mite infestation levels in the colonies were estimated using sticky-boards (De Jong, 1990; Delaplane and Hood, 1997; Delaplane, 1998; Calderone and Turcotte, 1998). These were coated on the upper surface with a clear adhesive material the bottom board of the observation hive was removed and replaced with an 8-in mesh screen. The screen was used to prevent the bees from removing the mites and from becoming stuck to the cards. Mites that fell to the bottom of the hive passed through the screen and were trapped on the sticky board. The sticky boards were placed under each observation hive 1-7 days before the treatment in order to determine pretreatment mite fall. The mites that fell onto the boards over a period of 24 h were counted, and removed. The boards remained sticky for up to 7 days and then they were replaced by a new set of sticky boards. The collected mites were daily assessed for fungal infection by surface-sterilizing and incubating them as described above for the laboratory assays. The number of mites was then recorded.

Statistical analysis
The data was analyzed using analysis of variance (ANOVA) followed by the Student-Newman Keuls test to determine significance between different groups. These tests were performed using a computer software CoStat system for Windows, version 6.311, CoHort Software (2006), Berkeley, CA, USA.

Results
Data in Table 1 showed significant differences between treatments with Bioranza and Biovar. Results were significant after 7 and 14 days of application in the treatment with Bioranza which was more effective than Biovar which differed in its effect from 7 and 14 days. Daily mite mortality was significantly different between the hives before and after the applications of the fungus. Treating the hives with Bioranza resulted in a significant increase in mites' mortality. After treatments were initiated, mite populations were found to be significantly smaller in treated hives than in the control hives. Mites' mortality varied significantly over time within observation hives. Data in Table 2 showed significant differences between treatments with Bioranza and Biovar in workers body weights. Results were significant after treatments than before; the fungus Bioranza was the highly effective fungus, followed by Biovar. Mean workers weight was not significantly different between the hives before and after the applications of the fungus.

Table 1. Mean number of fallen *V. destructor* mites on sticky boards by using Formulations of fungi and inert materials in hives during 2011

Treatment	Mean numbers of fallen varroa /day		
	0 day	1-7 days	8-14 days
Biovar	8.2a	12.2c	11.1d
Bioranza	8.5a	12.6c	8.2e
Wheat flour	8.1a	14.4b	13.6c
Maize flour	8.2a	14.2b	15..9b
Control	8.2a	16.4a	22.4a
L.S.D.	0.463	0.588	0.613

Means followed by the same letter(s) within each vertical column are not significantly different

Table 2. Mean weights of honeybee workers before and after using formulations of fungi and Inert materials in hives during 2011

Treatment	Mean weights mg/ bee worker	
	Before	After
Biovar	227.1a	247.8a
Bioranza	226.8a	246.9a
Wheat flour	225.7a	234.5b
Maize flour	227.5a	235.4b
Control	226.2a	219.7c
L.S.D.	1.392	1.998

Means followed by the same letter(s) within each vertical column are not significantly different

Overall, *V. destructori* was found to be a suitable host for the entomopathogenic fungi, Bioranza was more virulent to varroa because it killed the host more quickly. Fungal applications did seem to affect mite infestation levels in hives. It is possible that the mites could become infected after they emerged from the brood cells if sufficient quantities of conidia are present.

Electron Microscopy Examination
Scanning Electron Microscopy (SEM)

The present scanning electron microscopy (SEM) study describes the external development of *B. bassiana* and *M. anisopliae* on the surface (cuticle) of the mite *V. destructor*. Figure 1 Illustrates the Scanning electron micrographs of the cuticle of *V. destructor* infected with *M. anisopliae* and *B. bassaina*. Conidia of *M. anisopliae* were observed adhering to all parts of the body of the mite and the fungus rapidly colonized the surface of the host's cuticle. Germ tubes grew from conidia and extended over the cuticle. The fungus was found to form germ-tubes and started to penetrate the structures of the host within 24-48 hrs after inoculation. Penetration started in the epicuticle, then to the other regions of the cuticle and thereby inside the mite's body.

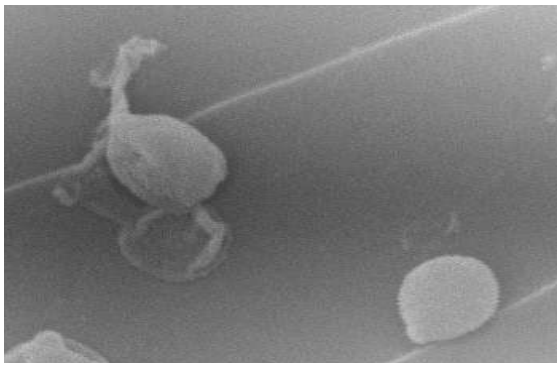

Fig. 1.1. Germ tubes emerged from a single conidia and extended over the cuticle

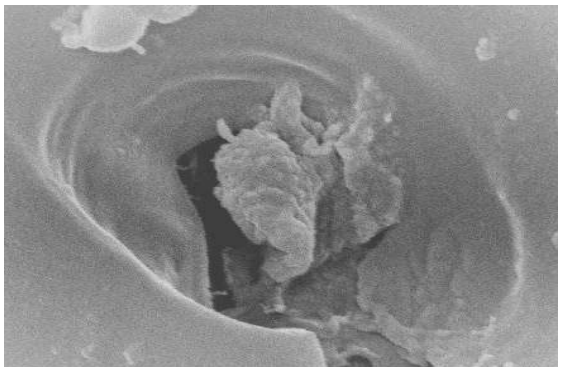

Fig. 1.2. Penetration the cuticle to find the more suitable exopenetration sites.

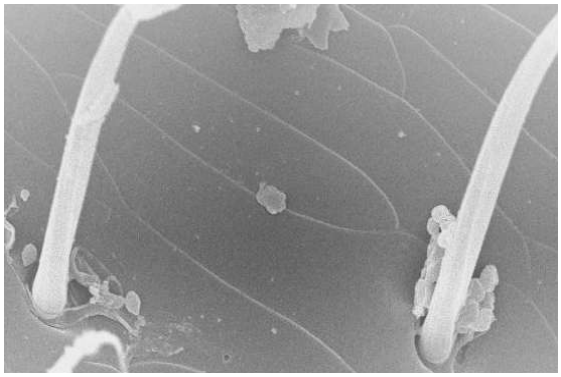

Fig. 1.3. Mycelium development on the host

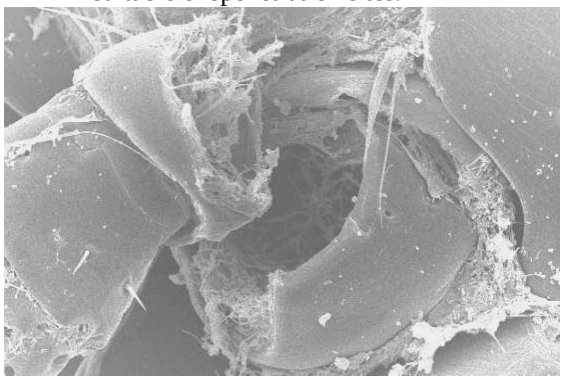

Fig. 1.4. Growth of the pathogen on and in the host

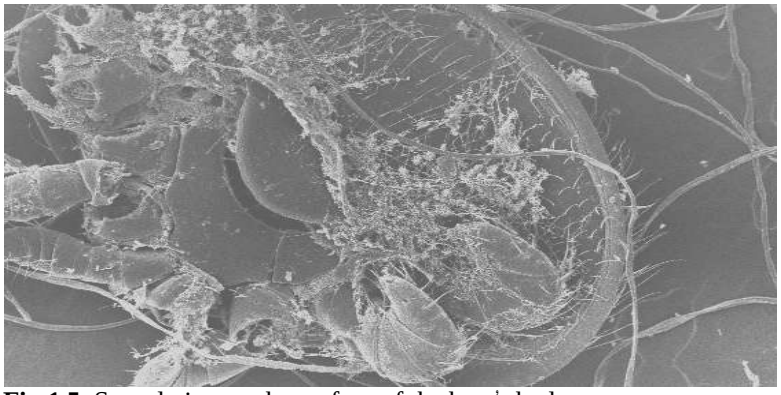

Fig.1.5. Sporulation on the surface of the host's body

The epicuticle was penetrated without formation of an appressorium-like structure. Later on, an appressorium was formed and full development of the germination tube which started to find possible site of penetration. The present observations demonstrate the following sequence of events in the infection of the mite *V. destructor* as a host by *M. ansopliae*: (I) attachment to the host, (II) conidium germination and formation of germ tube, (III) mycelium development on the host, (IV) penetration and growth of the pathogen on and in the host, and (V) sporulation on the surface of the host's body.

Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) studies were carried out to describe the internal development of *M. anisopliae* and *B. bassiana* inside the body-cavity of the mite *V. destructor*. TEM sections of the mite individuals infected with *M. anisopliae* are shown in Fig. 2. It is well known that the infection mainly takes place through the cuticle due to body contamination with the conidia of the entomopathogenic fungus. Therefore, the first step of infection is to adhere to the cuticle to find the more suitable exopenetration sites. After the exopenetration, cylindrical conidia spread through the body fluid and started to produce a toxin (s) that weakness the host's immune system and supply the fungus conidia with the favorable conditions to start the division process. An electron micrograph of a full structure of *M. anosopliae* conidium showed its main components (cell wall, mitochondria, vacuole and nucleus). The nucleus of a mother conidium starts to divide into two nuclei, forming two sister conidia. The division of the conidia of the fungus *M. anisopliae* inside the body-cavity of the mite is a mitosis one with the haploid nuclei. Conidium division is apparently required. The resulting increase in number of conidia makes it possible to excrete more toxin (s) to suppress the host's immunity system and complete the life-cycle within the host which eventually lead to host's mortality. Death of the host might be due to the effect on hemolymph cells and hemolymph characters. Also, conidia germinate in the hemolymph and penetrate muscles, nervous system, malpighian tubules, . . . etc.

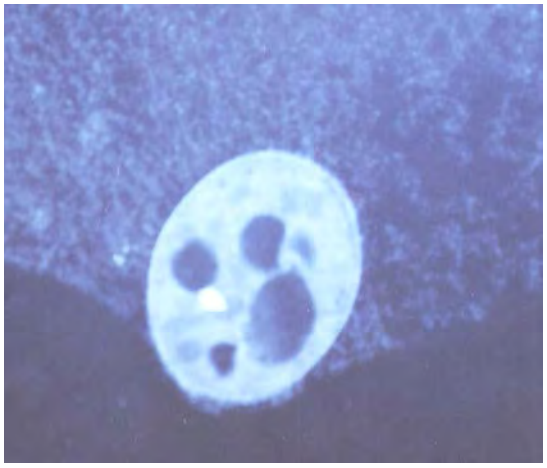

Fig. 2.1. The infection mainly takes place through the cuticle

Fig. 2.2. The nucleus of a mother conidium starts to divide into two nuclei,

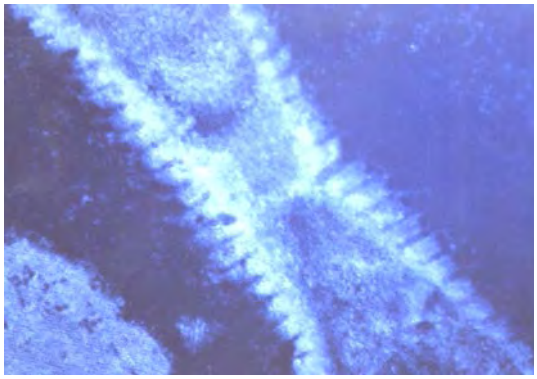

Fig. 2.3. The nucleus of a mother conidium divide, forming two sister conidia.

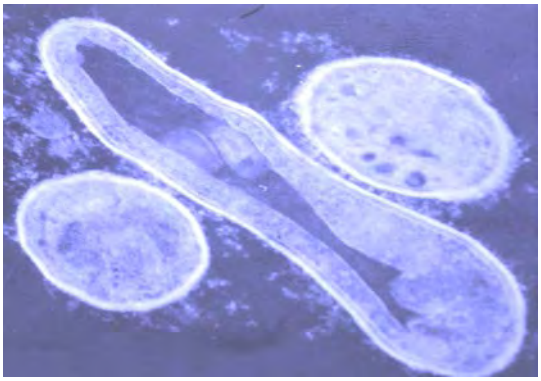

Fig. 2.4. Death of the host might be due to the effect on hemolymph cells and hemolymph characters.

Discussion

Efficacy of the two biopesticides

Mite infestation level varied ,significantly, between treatments 7 and 14 days post-treatments. The biopesticide Bioranza which is dependent upon the entomopathogenic fungi, *M. anisopliae* was the most efficacious fungal formulation tested because it caused the highest reduction in mite counts in the hives. Observations in this investigation indicated that it was critical to have fungal spores with good germination, pathogenicity and virulence. In the fungal treatments, mite infestations in the hives were significantly lower than those recorded in the control at day 14 after treatment. The overall differences between all treatments and the controls were statistically significant at the end of the experiments. All treatments caused significant change in mite infestations on adult bees over time and that the relationships between the treated and control hives varied over time. It was determined that fungal formulated spores provided successful control of mite populations in established honeybee colonies at 10 g of conidia per hive applied two times (day 0 and day 7). Microbial control of Varroa mite with *M. anisopliae* is feasible and could be a useful component of an integrated pest management program for the honeybee industry. In addition, organic beekeepers, homeowner, hobby beekeepers should benefit from a novel, user-friendly and chemical-free strategy to manage the destructive pest of honeybees. Overall, this user-friendly delivery method for the fungus, *M. anisopliae* could provide beekeepers with effective, environmentally sound and sustainable control option for varroa mite populations in honeybee colonies. *B. bassiana* appeared to be harmless to honeybee workers (Martin 2001, James and Hayes 2006, James *et al*., 2006 and Meikle *et al.*, 2006). The high correlation between mite mortality and fungal infection is an indication that the fungus was the major mortality factor of the varroa mite population in the observation hive experiments. However, the fungus proved of good persistence as the infecting mites were recorded 14 days after the application. Also, the low infection rates found in the control hives may be an indication that bees carrying fungus-treated mites drifted the fungus between hives. The current and future target of the present research is to develop a more efficient application technology to reduce the time required per application and to make the treatments more economical for beekeepers. Biological control could provide a key component in the development of a sustainable integrated pest management strategy for *V. destructor*.

Electron Microscopy Examination

The aforementioned explanations agree with Moino *et al*. (2002) who concluded that the infection processes varies in different entomopathogenic fungi. He reported that the initiation of *B. bassiana* conidial germination happened between 12 to 48 hrs after the inoculation of the subterranean termite *Heteroterms tenuis* (Order: Isoptera). These observations coincide with the commonly described sequence of events characterizing other entomopathogenic fungal infections (Chanley, 1989; Askary and Yarmand, 2007). Therefore, the use of entomopathogenic fungi such as *B. bassiana*, alone or associated to safe chemicals is an efficient and environmentally favorable method for controlling the mite *V. destructor* attacking honeybee. In this respect, Fargues *et al*. (1994) stated that penetration, colonization and sporulation occurs faster in the fungus *M. anisopliae* than in *B. bassiana* resulting in the earlier death of hosts infected with the former fungus.

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Antimicrobial activity of *Artemisia judaica* L. essential oil from Egypt

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Abstract

Artemisia judaica is a widely distributed plant in the Middle East (Sinai Peninsula, Jordan and Saudi Arabia). The essential oil derived via hydro-distillation method from aerial parts of *A. judaica* L., were evaluated for antimicrobial activities. Two Gram-positive bacteria (*Bacillus subtilis* and *Streptomyces* spp.); one Gram-negative bacterium, *Escherichia coli* O157; five genera of fungi strains (*Fusarium oxysporum, Rhizopus stolonifer, Rhizoctonia solani, Macrophomina phaseolina* and *Aspergillus niger*) and three yeasts (*Candida albicans, Candida tropicalis* and *Saccharomyces cerevisiae*) were used for evaluation of antimicrobial activities of *A. judaica* essential oil. The agar gel diffusion method was used to assay for the antimicrobial properties on the test isolates. The activity was more pronounced against Gram-positive bacteria and candidal than against fungal organisms except *R. stolonifer*. The chemical composition of the essential oil was analyzed by GC-MS and the resulting oil contained piperitone (32.4%), camphor (20.6%) and *E*-ethyl cinnnamate (8.2%) as major components, which may provide the antimicrobial properties of the essential oil against tested organisms. There is a relationship between the tolerances to *A. judaica* EO and the alterations in protein profile of the tested organisms. To estimate the level of the stress response, the increase and decrease of protein expressions can be analyses The principle aim of the present work was to identify the phytochemicals present in the essential oil of *A. judaica* which are responsible for antimicrobial activity.

Key words: *Artemisia judaica*, essential oil, antimicrobial activity, GC/MS analysis, chemical composition.

Introduction

Several plant and herb species used traditionally have potential antimicrobial and antiviral properties (Sumathi *et al*., 2011) and this has raised the optimism of scientists about the future of phyto-antimicrobial agents (Das *et al.,* 1999). In the last decades, application of synthetic toxins for control of weeds, pests and plant disease caused serious environmental problems (Razavi, 2011). Resistance of some bacteria has created an urgent need to develop alternative antimicrobial drugs from herbs that are safe, cheap and may overcome the resistance of the pathogens (Geidam *et al*., 2007 and Bonjar *et al*., 2004). The investigation of plants for bioactive secondary metabolites is an area which most plant scientists have recently focused with the aim of discovering new clinically useful and commercially important plant products was reported by (Dewick, 1997). Approximately 20% of the plant found in the world have been submitted to pharmacological or biological tests (Suffredini *et al.,* 2004).

Artemisia judaica L. (Asteraceae) is a perennial fragrant small shrub with pubescent leaves, which grows widely in Egypt (desert and coast). It is one of the common species of the genus *Artemisia* (El-Sharabasy, 2010). *A. judaica* is commonly called ''Shih Balady'' which is a medicinal herb in Egypt. It has been used to treat gastro intestinal disorders, poor eyesight, cardiovascular disease, skin disorders and weak immune systems as well as to decrease risk of atherosclerosis, cancer and arthritis. A number of volatile chemical constituents from the aerial parts of *A. judaica* have been identified. Phytochemical analysis showed it to be a rich source of flavonoids including apigenin, cirsimaritin and various novel compounds (Liu *et al.,* 2003, 2004). Biological and pharmacological activities of phytochemical compounds take into account different parameters and factors such as species, ecological factors and environmental conditions. Phenological age of the plant, percent humidity of the harvested material and method of extraction are possible sources of variation for the chemical composition, toxicity and bioactivity of the extracts (Rajakaruna *et al.,* 2002). There is a wide variation in the susceptibility of organisms to toxic compounds Probably a large number of plants with biological activities remain untested.

The principle aim of the present work was to study the antimicrobial activity of the *A. judaica* essential oil (EO). Also, chemical composition of essential oil extracted that could be responsible for antimicrobial activity was identified by using GC/MS analysis from the *A. judaica.*

Materials and methods

Plant material

The aerial parts of *A. judaica* (L.) were collected from El-Arish Region, Sinai Peninsula, Egypt in May, 2009 and shaded for 7-9 days at room temperature (25±2°C) until being crisp. The plant material was identified by botanists in the Department of Agriculture Botany, Menoufiya University, Egypt.

Isolation of essential oil (EO)

Air-dried aerial parts from the sample (200 g), were subjected to hydrodistillation in Clevenger type apparatus for 3 h as described by Negahban *et al.*, (2006). The EO was dried over anhydrous sodium sulphate and stored in a refrigerator at 5°C until required for experiments.

Antimicrobial activity

Microbial strains

For the determination of antibacterial activity of *A. judaica* essential oil (EO), two Gram-positive bacteria; *Bacillus subtilis* and *Streptomyces* spp.; one Gram-negative bacterium *Escherichia coli* O157; five fungi strains (*Fusarium oxysporum, Rhizopus stolonifer, Rhizoctonia solani, Macrophomina phaseolina* and *Aspergillus niger*) and three yeasts (*Candida albicans, Candida tropicalis* and *Saccharomyces cerevisiae*) were used. The selected microorganisms were obtained from the culture collection of the Department of Agriculture Botany, Menoufiya University, Egypt.

Screening for antibacterial and anti-yeast activities

Antimicrobial activity was tested by disc-diffusion inhibition on agar method as described by Musyimi *et al*. (2008). Culture suspension (200 µl) of the tested bacteria (2 x 10^6 CFU/ml) was spread on the media. Circular paper discs of 6.0 mm diameter were cut out from Whatman No. 1 filter paper using a paper punch. Each sterile filter disc was dipped in a known concentration of the essential oil for about 2 min, then gently transferred to the inoculated agar media. Antimicrobial activity was determined by measuring the zone of growth inhibition around the discs after 48 h of incubation at 30°C.

Screening for antifungal activity

For screening the antifungal activity of EO, the agar-disc diffusion method was used. The discs of mycelial felt (6 mm diameter) of the pathogenic fungi were transferred aseptically to the centre of Petri- dishes. Tolcofos-methyl was used as a reference fungicide. The treatments were incubated at 25°C for 72 h. The antimicrobial activity was determined by measuring the diameter (mm) of the growth inhibition zones including the 6 mm disk. The measurements of inhibition zones were measured for three sample replications and values were the average of three replicates.

Antibiotic and Minimum inhibitory concentrations (MIC) assay

The standard discs (6 mm diameter) of some antibiotics were served as positive antibacterial control and compared to the control disc to nullify the effect of the solvent on the growth of the tested organisms. The antibiotics used were amoxycillin (AML10) (10µg), Imipenem (IPM10) (10µg), Cefoxitin (FOX30) (30µg), Ampicillin (SAM 20) (20µg), and Sulphamethoxazole trimethoprim (SXT25) (25µg). The antibiotic disks were placed on the surface of the media; incubation period was 48 or 72 hours at 30 or 25°C according to the microbial organism used. The inhibition zones were measured with a millimeter ruler including the diameter of the disc. The MIC was determined after 48 h for the bacteria and yeasts and after 72 h for fungal strains. Serial dilutions of the EO were made in a concentration range from 0.63 to 80 mg/ml. Each concentration was tested in triplicate. The MIC was determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate.

GC/MS analysis

The oil compounds were isolated, identified and quantified on a Shimadzu GC-17A gas chromatograph (Shimadzu Corp., Kyoto, Japan), coupled with a Shimadzu mass spectrometer detector (GC-MS QP-5050A). The GC-MS system was equipped with a TRACSIL Meta X5 column (Teknokroma S. Coop. C. Ltd., Barcelona, Spain; 30 m × 0.25 mm i.d., 0.25 µm film thickness). Analysis was carried out using helium as carrier gas at a flow rate of 1.0 ml/min at a split ratio of 1:10 and the following temperature program: 40°C for 5 min; rising at 3.0°C/min to 200°C and held for 1min; rising at 15°C/min to 280°C and held for 10 min. The injector and detector were held at 250 and 300°C, respectively. Diluted samples (1:10 pentane, v/v) of 0.2 µL of the extracts were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of *m/z* 45-450. The identification of individual compounds of essential oil was accomplished using two different analytical methods: (a) KI, Kovats indices in reference to *n*-alkanes (C_8-C_{32}) by National Institute of Standards and Technology (NIST) 2009; and (b) mass spectra (authentic chemicals and Wiley spectral library collection). Identification was considered to be tentative when it was based on mass spectral data only. The relative concentration of each compound in the essential oil was quantified according to the peak area integrated by the analysis program.

Protein extraction and electrophoresis

Tested organisms were grown on different liquid media supplemented with 40 mg/ml of *A. judaica* EO. Cells were harvested by centrifugation and resuspended in 150 ml of treatment buffer (Hames, 1981). Samples were then sonicated and boiled for 5 min at 95°C. Lysates were centrifuged to remove cell debris and the supernatant was collected. Proteins were separated by SDS-PAGE, carried out in 12.5 and 18% acrylamide slab gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad) and screened in a Densitometer apparatus (Bio-Rad– Model GS 710). The molecular weight

was determined comparing with a protein standard (Broad Range Prestained Standard, Bio-Rad) and relative amount of proteins corresponding to each band were calculated using Quantity One Program Software (Bio-Rad).

Statistical analysis

Data of all measurements were then subjected to one-way analysis of variance (ANOVA) appropriate for a randomized complete block design (CoStat system for Windows, version 6.311, CoHort Software (2006), Berkeley,CA, USA).

Results

Antimicrobial activity

The antimicrobial activity of *A. judaica* EO was evaluated against three bacterial genera, three yeasts and five fungal genera. According to results in Table 1, the EO had variable antimicrobial activity against all tested microorganisms. Gram-positive bacteria were shown to be more sensitive to the *A. judaica* EO than Gram-negative bacteria. The most susceptible bacterium for *A. judaica* EO was *B. subtilis* (18 mm). The growth of Gram-negative bacteria *E. coli* O157 was not inhibited. The growth inhibitions of tested microorganisms ranged from 10 mg/ml (W/V) to 80 mg/ml (W/V) with the lower MIC value against *B. subtilis* and *Streptomyces* spp. at 10 mg/ml in both strains. It means that the EO had most activity against Gram-negative ones. Moreover, most of the studies investigating the action of essential oils against food spoilage organisms agreed that, essential oils are slightly more active against Gram-positive than Gram-negative bacteria (Lambert *et al.,* 2001). Furthermore, the most susceptible yeasts for *A. judaica* EO was *C. albicans* (13 mm), it had great anticandidal activity against *C. albicans* and *C. tropicalis* but the EO was ineffective against *S. cerevisiae*. Under the same experimental conditions, 40 mg/ml of *A. judaica* EO had a low inhibitory effect on the growth of *R. stolonifer* and had no effect on the growth on the other studied fungi. *R. stolonifer* was the most sensitive fungal strain (8.3 mm) at 40 mg/ml of *A. judaica* EO.

GC/MS analysis

The hydrodistillaton of the dried aerial parts of *A. judaica* L. gave light yellowish oil with yield of 0.6 % (W/W). Fourty-seven components were identified in the oil, representing 94.8% of the total composition (Table 2).

Table 2. Chemical composition of essential oil isolated by hydrodistillation from aerial parts of *Artemisia judaica* analyzed by GC-MS

Number	RI [a]	Compound [b]	Peak Area (%) [c]
1	923	α-Pinene	0.3
2	937	Camphene	0.3
3	956	β-Pinene	0.2
4	971	Myrcene	0.3
5	989	α-Phellandrene	1.2
6	1012	α-Terpinene	0.3
7	1021	1,8-Cineole	0.4
8	1037	Artemisia ketone	1.4
9	1062	Artemisia alcohol	0.3
10	1073	Terpinolene	0.2
11	1079	Fenchone	0.6
12	1089	Linalol	0.3
13	1097	β -Thujone	0.6
14	1105	Chrysanthenone	3.9
15	1114	Camphor	20.6
16	1131	*iso*-Borneol	0.2
17	1141	Terpinene-4-ol	4.6
18	1152	Lavandulol	0.5
19	1164	Borneol	2.2
20	1179	α-Terpineol	0.3
21	1196	Verbenone	0.3
22	1202	Carveol	0.4
23	1230	Piperitone	32.4
24	1240	Geraniol	0.8
25	1248	Perilla aldehyde	0.2
26	1257	Geranial	0.5
27	1265	Bornyl acetate	3.0
28	1296	Carvacrol	0.2
29	1334	Citronellyl acetate	0.7
30	1355	(*E*)-Ethyl cinnamate	8.2
31	1374	α-Ylangene	0.2
32	1385	β-Elemene	0.2
33	1414	α-Cedrene	0.3
34	1429	β -Caryophyllene	0.3
35	1435	(*E*)-β -Farnesene	0.3
36	1442	*allo*-Aromadendrene	0.4
37	1481	Valencene	1.3
38	1487	β -Bisabolene	0.2
39	1498	γ -Cadinene	0.7
40	1541	Spathulenol	0.3
41	1548	Caryophyllene oxide	1.1
42	1560	Davanone	0.3
43	1597	1-*epi*-Cubenol	1.4
44	1610	Humulene oxide II	1.2
45	1616	T-Cadinol	0.4
46	1622	β -Eudesmol	0.2
47	1649	Cadalene	0.6
		Total	94.8

Table 1. Antimicrobial activity of *Artemisia judaica* essential oil (EO) against some microorganisms tested by disc diffusion inhibition on agar method

	Microbial strains	Diameter of inhibition zone (mm) *										MIC**	MIC for Tolcofos-methyl (µg /ml)
		Essential oil (mg/ml)				Antibiotics (µg)					Control		
		10	20	40	80	AML 10	IPM 10	FOX 30	SAM 20	SXT 25			
Bacteria	*Streptomyces* sp.	7.3±0.6^{f}	9.3±0.6^{e}	13.0±1.0^{d}	15.3±0.6^{c}	16.0±1.0^{c}	19.0±1.0^{b}	24.6±1.2^{a}	NE	16.7±0.3^{c}	NE	10	n.d.
	B. subtilis	8.0±0.0^{e}	10.3±0.6^{d}	16.3±1.2^{bc}	18.0±1.0^{b}	15.0±1.0^{c}	21.0±1.0^{a}	22±0.4^{a}	10.3±0.6^{d}	14±0.0^{c}	NE	10	n.d.
	E. coli O157	NE	NE	NE	NE	9.6±0.6^{b}	23.7±1.0^{a}	21.4±0.8^{a}	8.0±0.0^{b}	NE	NE	NE	n.d.
Yeasts	*C. albicans*	NE	7.6±0.6^{c}	11±0.0^{b}	13.3±0.6^{b}	NE	NE	18.7±1.0^{a}	NE	8.7±0.7^{c}	NE	20	n.d.
	C. tropicalis	NE	7.3±0.6^{d}	8.0±0.0^{cd}	12.3±0.6^{b}	NE	NE	17.7±1.4^{a}	NE	9.3±0.9^{c}	NE	20	n.d.
	S. cerevisiae	NE	NE	NE	NE	NE	8.2±0.6^{b}	15.6±0.3^{a}	NE	NE	NE	NE	n.d.
Fungi	*F. oxysporum*	NE	NE	NE	7.3±0.6	NE	NE	NE	NE	NE	NE	80	64
	R. stolonifer	NE	NE	8.3±0.6^{b}	10.6±0.6^{a}	NE	NE	NE	n.d.	n.d.	NE	40	40
	R. solani	NE	NE	NE	8.3±0.6	NE	NE	NE	NE	NE	NE	80	8
	M. phaseolina	NE	NE	NE	NE	NE	NE	NE	NE	n.d.	NE	NE	96
	A. niger	NE	NE	NE	NE	NE	NE	NE	n.d.	NE	NE	NE	120

Means followed by the same letter(s) within each horizontal column are not significantly different at P = 0.05.
* Diameter of inhibition zone including disc diameter of 6 (mm). Values are means of three replication ±SD.
**MIC, minimum inhibitory concentration as (mg/ml).
NE, no inhibition zone observed.
n.d., not determine
[a] RI, retention index on a TRACSIL Meta X5 column
[b] Compounds are listed into order of their elution from a TRACSIL Meta X5 column
[c] Compound percentage

The major components of the essential oil were piperitone (32.4%), camphor (20.6%), *(E)*-ethyl cinnamate (8.2%) and terpinene-4-ol (4.6%). The essential oil of *A. judaica* L. was rich in monoterpenoids and ester of cinnamic acid.

Protein alterations

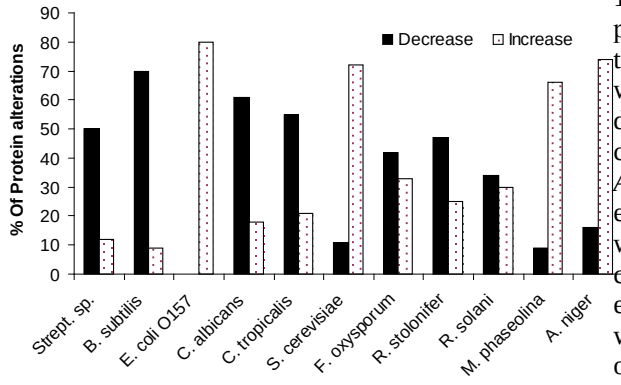

Fig. 1. The effect of *A. judaica* EO on protein profile of tested organisms

Discussion

The use of natural products can be considered as an important alternative for antimicrobial activity. The results from this study indicated that the essential oil of *A. judaica* exhibited antimicrobial activity. wich may be related to its components. The chemical composition of the essential oil from *A. judaica* could be changed according to geographical distribution and might be an effective factor on its antimicrobial activity. The principal components of *A. judaica* essential oil are piperitone and *trans*-ethyl cinnamate, which has been found to act as a key factor against some plant pathogenic fungi (Abdelgaleil *et al.*, 2008). On contrast in other studies Artemisia 1,8-cineole were the major constituents of other species such as *A. siberi* (Amiri, 2007) *A. pedemontana* (Perez-alonso *et al*., 2003), *A. annua* (Jutteau *et al.*, 2002), and *A. parviflora* (Orva *et al.*, 2006). The antimicrobial activity of *A. judaica* EO would be related to its monoterpene components. Indeed, in essential oils, it was shown that monoterpenes in essential oils are able to destroy cellular integrity resulting in respiration inhibition and permeability alteration (Cox *et al.*, 2000). Some oils of basil types showed strong antibacterial activity against *E. coli* (Nour *et al*., 2009).

In general, the toxic activity of essential oils is mostly due to the presence of phenols, aldehydes and alcohols (Sacchetti *et al.*, 2005). However, it is difficult to attribute the activity of a complex mixture to a single or particular constituent. Major or trace compounds might give rise to the antimicrobial activity exhibited. In the oil, the possible compounds synergistic and antagonistic effects would play an important role in microbial inhibition and should also be taken into consideration. It is also possible that various minor components may be involved in some type of synergism with other active components (Yu *et al.*, 2004).

Stress usually results in adaptive responses in many organisms that lead to changes in their regular metabolic process, which are then relfected in the alteration of their protein proifles (Saxen□ □*et al.*, 1996). It is generally assumed that the increases of protein expression relfect a positive attempt of cel□□ to adjust to the new environmental conditions, whereas the decreases of expression is indicative of disruption of cellular metabolism. In the present case of the tested organisms, under the treatments of *A. judaica* EO increases/decreases of polypeptides expression were observed (Figure 1). *B. subtilis* were the less tolerant and most of protein alterations corresponded to decreases, suggesting a deleterious effect on basic cell metabolism. In *E. coli* O157, it was the highest tolerant and showed high increases of protein expression, which leads to conclude that, these isolates expressed mechanisms, that allowed them to enhance resistance to the Oil. A higher protein expression was usually related to tolerance mechanisms according to Saxena *et al.*,(1996). These ifndi□ □□ led to the conclusion that the analysis of protein alterations seems to be a good indicator to estimate the level of stress. The results of the present study suggest the possible use of *A. judaica* essential oil in research for selecting new natural antimicrobial compounds,

Conclusion

Antimicrobial properties of the essential oils and various extracts from many plants have recently been of great interest, because their possible use as natural additives emerged from a growing tendency to replace synthetic compounds with natural ones. Extracted volatile oils from the aerial parts of *A. judaica* with stream distillation were found to have antibacterial and anticandidal activity.

The EO was effective against two gram positive bacteria, *B.subtilis* and *Streptomyces* spp. While no antibacterial activity occurred against *E.coli.* These results may partly justify the traditional use of *Artemisia* species. Antifungal activity was observed only for *R. stolonifer* at 40 mg/ml of the EO. All these results indicated that EO is a source of biologically active compounds. However, further studies are needed. One of these is to obtain more information regarding the practical effectiveness of the extracts in animal models.

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Antifeedant and toxic activity of some plant extracts, chemical and bio insecticides against the cotton leafworm, *Spodoptera littoralis* (Boisd.)

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Abstract

The effect of three different compounds, namely protecto (bioinsecticide based on *Bacillus thuringienses*), coumarin (plant extract of Chicory flower) and Neemix (plant extract of *Azadirachta indica*) were assayed on the 4th larval instar of cotton leafworm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). All tested compounds exhibited a significant antifeedant activity, coumarin recorded the highest mean of antifeedant activity reached (89.19) followed by azadirachtin (81.7) at 100 x 10^3 µg/ml and protecto (32.01) at 200 x 10^3 µg/ml. The overall larval mortality after treatment with lannate, protecto, coumarin and azadirachtin ranged between (48.3 – 70.0%), (20.0 – 50.0%), (25.0 – 60.0%) and (21.6 – 55.0%), respectively. Coumarin and azadirachtin concentrations exhibited longer larval duration, while protecto gave a slight difference in the total larval period. All tested concentrations of all compounds led to significant prolongation in pupal stage duration. Percentage of adult emergence increased with decreasing the tested concentrations of the tested compounds, the percentage ranged between (73.3 – 89.5%), (52.4 – 94.8%) and (66.6 – 93.1%) after treatment with protecto, coumarin and azadirachtin, respectively. The average number of males was higher than that of females after larval treatments with protecto, coumarin and azadirachtin. Adult and pupal deformation percentage increased with increasing concentration of tested compounds. Number of eggs/female and hatchability % decreased with increasing concentrations of tested compounds. Female sterility percentage increased by increasing concentrations of tested compounds.

Key words: Antifeedant, toxic activity, plant extracts, chemical, bio insecticides against, cotton leafworm, *Spodoptera littoralis* (Boisd.)

Introduction

Cotton leaf worm control programs were based mainly on use of insecticides, which created some problems such as insecticides- resistance, environmental pollution and hazard to natural enemies and beneficial insects (Toscano *et al.*, 1974 and Abbas *et al.*, 1996). Hence, the recent approaches now focus upon the use of environmentally safe compounds as pest control agents. One of the latest- approaches for pest control is the use of entomopathogenic bacteria, *Bacillus thuringiensis* representing a good example as a biological control method. The use of *B. thuringiensis* represents a highly active control method against insect pests, especially the cotton leafworm, *S. littorals* (Gaaboub *et. al*. 2005 and Gaaboub, 2004) , *S. exigua* and the black cutworm *Agrotis ipsilon* (Zaki, 1993). Bioinsecticide proved its efficacy against the 2nd and 4th larval instars of *S. littorals* using dipping technique in laboratory, it prevented small larvae from becoming larger and preventsd also metamorphosis of the full- grown larvae that showed failure of pupation, deformed pre pupae and pupae. High mortality and malformations occurred. The apparently normal moth was small in size giving few numbers of small egg masses and even the tiny egg masses were sterile (Gaaboub *et. al*. 2005 and Gomaa, 2005). Also recently, plant extracts, that are non toxic to man and animals, have more attention in controlling many pests, possess distinct toxicity and also lead to antifeeding activity and inhibition of growth of some pests (Sharaby and Ammar, 1997; Badr *et al*., 2000, D'Andrea *et al*; 2001 ; Halawa *et al*, 2007 and Adel and Abd El-Hakim, 2007).

The aim of the present investigation is to evaluate the antifeedant activities, toxicity and biological effect of tested chemical insecticide (methomyl), bio-insecticide (protecto) and plant extracts namely (coumarin & azadirachtin) against 4th instar larvae of *S. littoralis*.

Material and methods

Tested compounds

Plant extracts

1. Coumarin: Isolated from the flower of *Chichorium intybus* (chicory)
2. Azadirachtin (Neemix): from the neem tree, *Azadirachta indica* (Meliaceae)

6 (2, 4-dinitrophenylamino hexanyl) - 22, 23-dihydroazadirachtin

Bio insecticide (protecto);

W.P. based on *Bacillus thuringiensis subsp. kurstaki* (32x10^3 I. U/mg). Active ingredient 9.4%, inert ingredient (Carrier) 90.6%.

Insecticide (methomyl)

Lannate 90% W.P. (a synthetic carbamate compound) Carbamate oxime

Chemical name: S. methyl- N-[(methyl carbomoyl) oxy] thioecetimidate.

Insect maintenance

The stock culture of susceptible Egyptian cotton leaf worm, *S. littoralis* strain was reared on castor bean leaves (*Ricinus communish*) for several generations at laboratory conditions 27 ± 1°C and 70 ± 5% RH. Egg masses were placed on castor bean leaves in cylindrical glass jars. The jars were covered with muslin cloth and fastened with rubber bands. First instar larvae hatched within 2-3 days, the newly hatched larvae were transferred into rearing jars bottomed with sheets of towel paper to absorb excess humidity. Castor bean leaves were provided daily to the larvae in sufficient amounts, the accumulated faeces and debris were cleaned out daily. After pupation, pupae were collected and placed in wide clean jars until adults' emergence. The emerged adults were supplied with pieces of cotton wetted with 5-10% sugar solution for nutrition and branches of (*Nerium oleander*) as a suitable site for oviposition. Deposited egg masses were collected daily and transferred into the rearing jars.

Bioassay tests

Tests for feeding deterrence were carried out using fourth instar larvae of a laboratory culture of the Egyptian cotton leafworm, *S. littoralis* (Boisd.). Test larvae were starved for about 4 hours before treatment and divided into 6 replicates (5 larvae for each replicate). Each larva was kept in a Petri-dish. Discs of 37.5 cm^2 area of castor bean leaves were dipped in each concentration for each compound under investigation and allowed to dry. Only one disc was offered to each tested larva. Untreated discs were introduced to larvae as blank control. The eaten areas were estimated after 24 hours by a Planimeter. The reduction percentage of feeding over control was the factor used for determining the presence of feeding deterrent effect. The anti-feeding activity was evaluated on the basis of the feeding ratio of the treated to the untreated leaf discs. The anti-feeding activity was calculated by using the formula of Saleh *et al.* (1986) as follows:

$$\text{Antifeeding activity} = \left(1 - \frac{\%\text{ of eaten area in treatment}}{\%\text{ of eaten area in control}}\right) X100$$

Effect of tested compounds on certain biological aspects of *S. littoralis* (Boisd.)

Fourth instar larvae of *S. littoralis* were starved for a period 4 hrs before being fed on treated leaves and left to feed for 24 hrs on leaf discs of castor bean leaves treated with different concentrations for each compound. Castor bean leaves were dipped in various concentrations for seconds, then the leaves were left to dry in air. Three replicates were used for each concentration (20 larvae each). Another group of larvae were fed on untreated leaves kept as a check group. After feeding on the treated discs for 24 hrs, the larvae were kept in clean glass jars provided with fresh untreated leaf discs for comparison. Survivors were repeatedly transferred to new jars supplied with fresh leaves every couple of days until pupation. Daily records were taken for the percentages of larval mortality, pupation percentage, larval period, pupal duration and pupal malformation. Emergence and malformations of moths were also recorded. Pairs of moths were kept every pair in a glass jars with a strip of paper for egg deposition. Pieces of cotton soaked in 10% honey solution were supplied for moths feeding. Records were taken for the fecundity of moths. Eggs deposited were incubated at 28 ± 2ºC and the percentage of eggs' hatching was recorded. The overall latent effect on reproduction was expressed as percent sterility and calculated according to Toppozada *et al.* (1966) as follows:

$$\%\text{ sterility} = 1 - \frac{a \times b}{A \times B} \times 100$$

Where a = number of eggs/female in treatment
b= hatching % in treatment
A = number of eggs/female in control
B = hatching % in control

$$\%\text{ Hatchability} = \frac{\text{No. of hatched eggs}}{\text{Total no. of eggs}}) X100$$

Toxicological studies

Castor bean leaves were dipped for 30 seconds in each concentration for each compound and left for natural dryness before being administered to the tested fourth instars larvae. Three replicates of ten larvae each were used for each concentration. Also similar replicates of the same number of larvae were fed on untreated castor bean leaves to serve as check. Mortality counts were recorded after 1, 2, 3, 5 and 7 days and mortality percentages were corrected for natural mortality according to Abbott's formula (1925). To estimate the LC_{50} values, the corrected mortality percentages were subjected to probit analysis according to the method of Finney (1971).

$$\text{Corrected mortality \%} = \frac{\text{Observed mortality \% - Control mortality \%}}{\text{100 - Control mortality \%}}) X100$$

Results and discussion

Effect of different tested compounds as antifeedants on 4th instar larvae of *S. littoralis*

In this trial only 4th instar larvae of *S. littoralis* were used to establish the presence of antifeeding properties in these tested compounds. Data in Table 1 indicated that coumarin gave the lowest consumed

leaf area (cm^2) treated 24 hrs for all tested concentrations followed by azadrachtin extract therefore, the two plant extracts were the best for lowering the consumed area of leaves. All tested compounds gave significantly lower consumed area than control. Also, the same trend was obtained for percentages; the mean percentages of eaten area were 6.06, 8.23 and 15.79 obtained by coumarin, azadirachtin and protecto, respectively. In addition, data in Table 1 clearly indicate the important role of tested compounds used in determining antifeeding activities. Coumarin showed the highest antifeeding activity, followed by azadirachtin. On the other hand protecto gave the lowest antifeeding activity. It could be observed the increasing of consumed area with decreasing the concentration of each tested compounds. The highest used concentration (100 x 10^3 µg/ml) of coumarin and azadirachtin gave the highest antifeeding activity (89.19 and 81.7, respectively). On the other hand, the lowest concentration used of protecto (1.25 x 10^3 µg/ml) gave the least antifeeding activity value (2.25).

Table 1. Effect of different concentrations of coumarin, azadirachtin and protecto as antifeedants on 4th instar larvae of *S. littoralis* (Boisd.).

Compound	Concentration (µg/ml)	Consumed area in $(cm)^2$ treated 24 hrs	% of eaten area	Antifeedant activity
Protecto	20 x 10^3	5.03±0.17	13.34	32.01
	10 x 10^3	5.48±0.33	14.67	25.23
	5 x 10^3	5.73±0.11	15.2	22.53
	2.5 x 10^3	6.25±0.66	16.58	15.49
	1.25 x 10^3	7.23±0.08	19.18	2.25
Coumarin	100 x 10^3	0.80±0.04	2.12	89.19
	50 x 10^3	0.98±0.13	2.60	86.75
	25 x 10^3	1.61±0.24	4.27	78.24
	12.5 x 10^3	2.85±0.22	7.56	61.47
	6.25 x 10^3	5.20±0.16	13.79	29.72
Azadirachtin	100 x 10^3	1.40±0.14	3.71	81.7
	50 x 10^3	1.68±0.12	4.46	77.27
	25 x 10^3	3.13±0.19	8.30	57.69
	12.5 x 10^3	3.64±0.16	9.66	50.77
	6.25 x 10^3	5.66±0.08	15.02	23.45
Control		7.30±0.04	19.62	-

Data presented in Table 2 show the lethal values of different tested compounds against 4th instar larvae of *S. littoralis*. Depending upon the LC_{50} values which are frequently used to measure the tested compounds toxicity, the bioinsecticide, protecto is higher toxic against the fourth instar larvae than plant extracts, coumarin and azadirachtin. However, all tested compounds are lower toxic compared to methomyl. Significant positive correlation coefficient values between concentrations and mortality % were obtained for all tested compounds, indicating that the increasing concentrations increased the mortality percentages.

The toxicity and sublethal effects of profenofos, emamectinbenzoate, spinosad and azadirachtin were studied on 2nd and 4th instar larvae of *S. littoralis* under laboratory conditions by El-Aw (2003). Based on the LC_{50} values, profenofos was most toxic, the toxic action extended for 2 days, whereas the toxicity of emamectinbenzoate, spinosad, hexaflumuron and azadirachtin persisted for 5 days based on LC_{50} values. A negative relationship was found between the time elapsed after treatment (24, 48, 72, 96 and 120 h) and the LC_{50} values of the insecticides.

Table 2. Lethal concentrations of the tested compounds against the fourth instar larvae of *S. littoralis* after 10 days from treatment compared with methomyl after 24 hrs

Compounds	Lethal values and their 95% confidence limits %				
(µg/ml)	LC_{50}	LC_{90}	LC_{95}	Slope ±SD	R
Protecto	3910	68730	154910	1.029±0.256	0.966
Azadirachtin	18540	229390	468020	1.173±0.261	0.959
Coumarin	8430	141880	315890	1.045±0.268	0.961
Lannate	13.729	217.527	476.045	1.068±.0.275	0.933

SD: Standard deviation of mortality regression line
R: Correlation coefficient of regression line

Effect of protecto on some biological parameters of *S. littoralis* (Boisd.), when 4th instar larvae were fed for 24 hrs on treated leaves

Data presented in Table 3 indicated that increasing protecto concentration increased mortality percentage of larvae from minimum 20.0 % at 1.25 x 10^3 µg/ml to maximum of 50.0 % at 20 x 10^3 µg/ml. The highest tested concentration of protecto exhibited significantly high mortality percentage compared by untreated larvae. All protecto concentrations exhibited longer larval duration when compared with untreated to reach maximum of 11.0 days at the highest concentration 20 x 10^3 µg/ml compared with untreated larvae (8.6) days. Also, significant increases in the pupal duration was observed after exposure of *S. littoralis* larvae to protecto concentrations compared with untreated larvae. There was a reduction in pupation percentage compared with the pupation percentage resulted from untreated larvae. The pupation percentage decreased with the increase of concentration that reached (50.0 %) at 20 x 10^3 µg/ml and (80.0%) at lowest concentration 1.25 x 10^3 µg/ml, while it was (95.0 %) among the control pupae. All concentrations of protecto showed significant effect on emergence rates of adults, longevity and sex ratio. On the other hand, results in the same table indicated significant decrease in number of eggs/female with increasing concentrations of portecto (1.25 x 10^3 to 20 x 10^3 µg/ml). The highest number of eggs /female was obtained from control female (1611.0 eggs). The minimum number of eggs/female (774.0eggs) was recorded at the tested protecto concentration (10 x 10^3 µg/ml). Significant increase in pupal deformation percentage and this rate increased with increasing protecto concentrations. Also, significant decrease in eggs hatchability was recorded at various protecto concentrations. Adult female sterility increased with raising protecto concentrations. The sterility percentages ranged from 100.0 % at 20 x 10^3 µg/ml to 31.48 at 1.25 x 10^3 µg/ml of protecto. Koja *et al.* (2006) studied two *B. thuringiensis* commercial formulations, Dipel-2X and protecto against eggs and 1st instar larvae of *E. insulana* (Lepidoptera: Noctuidae). These treatments caused 73.3% larval mortality after 10 days. Larval period was prolonged to 21.3 and 19.1 days when Dipel-2X or protecto was used, compared with 17.5 days in the control. Pupal stage duration was not significantly affected; however, the weight of pupae was significantly reduced when larvae were treated with either *B.t* products, longevity of adults was shortened in treated insects. Several morphological malformations were observed in pupae and also emerged moths.

Effect of coumarin on some biological parameters of *S. littoralis* (Boisd.), when 4th instar larvae were fed for 24 hrs on treated leaves

Data in Table 4 indicated that increasing coumarin concentration increased larval mortality percentage from 25.0 at 6.25 x 10^3 µg/ml to 60.0% at 100 x 10^3 µg/ml concentration. The concentrations of coumarin caused elongations in the larval period to reach maximum of (17.2) days at the highest concentration of (100 x 10^3 µg/ml) compared with untreated larvae (10.9) days. The pupal duration from treated larvae seemed to be relatively longer at than the cheek group and the average were (10.7) and (12.7) days of the lower and the higher concentrations (6.25 x 10^3 µg/ml and 100 x 10^3 µg/ml, respectively). While, it was (9.7) days at the check groups. There was a reduction in pupation and emergence percentages with all used concentrations, compared with the pupation and emergence percentage resulted from untreated larvae. The pupation percentage decreased with the increase of concentration that reached 40.0 %) at 100 x 10^3 µg/ml and (75.0 %) at lower concentration 6.25 x 10^3 µg/ml while it was (96.6 %) at the control. The emergence percentage recorded (52.4 %) at the highest concentration 100 x 10^3 µg/ml and (82.2 %) at the lowest concentration 6.25 x 10^3 µg/ml, while with untreated larvae reached (94.8 %). Results showed increased deformations among pupae and adults and the rate increased with increasing coumarin concentrations. The highest malformation percentage was obtained at 100 x 10^3 µg/ml that recorded (12.5 %) at pupae and (18.1 %) among adults followed by 25 x 10^3 µg/ml that recorded (5.8 %) at pupae and (8.0 %) in adults. On the other side, the lowest value was obtained by 12.5 x 10^3 µg/ml that recorded (7.5 %) at pupae and (6.6 %) in adults. No malformation was recorded in case of the control pupae and adults. In addition the lowest concentration (6.25 x 10^3 µg/ml) did not cause

any malformation, being similar to control. The results indicated that longevity of adult males was shortened with coumarin treatments at concentrations from 6.25 x 10^3 to 100 x 10^3 µg/ml. Also, all concentrations of coumarin gave the higher male : female sex- ratio. Also, data showed decreased number of eggs/female and hatchability % with increasing concentrations of coumarin (6.25 x 10^3 to 100 x 10^3 µg/ml). The highest number of eggs / female and hatchability percentage were obtained by control. Also, female sterility percentage increased by increasing coumarin concentration. The phagodepression activity of five coumarins (=2H-1-benzopyran-2-ones), 6-hydroxy-7-isoprenyloxy-coumarin (1), 6-methoxy-7-isoprenyloxy coumarin (2), 6, 7-methyl lenedioxycoumarin (3), 5-methoxy-6, 7methylenedioxy coumarin (4) and 6-methoxy-7-(2-hydroxy ethoxy) coumarin (5), from the Argentine native herb *Pterocaulon polystachyum*, was tested against *Spodoptera frugiperda* larvae. The compounds were added to an artifical diet at doses ranging from 50 to 200 g per g of diet. The results indicated that 50 micro g/g of compound 1 and 3 (non-methoxylated coumarins) incorporated to the larval diet caused 80 and 50% pupal mortality, respectively, while, 100 micro g /g dose of compounds 2, 4, 6 and 7 produced 60, 50, 10 and 80% pupal mortality, respectively. Larval growing rate during the early larval instars was significantly reduced by treatments with the methylenedioxy coumarins 3 and 4 coincidentally, the larval period duration was significantly increased by the latter compounds. (Vera *et al.,* 2006).

Effect of azadirachtin on some biological parameters of *S. lilloralis* (Boisd.), when 4th instar larvae were fed for 24 hrs on treated leaves

Data in Table 5 indicated that increasing of azadirachtin concentrations increased mortality percentage from 21.6 at 6.25 x 10^3 µg/ml to 55.0 at 100 x 10^3 µg/ml. All azadirachtin concentrations exhibited high larval mortality percentages and longer larval durations compared with control. At 100 x 10^3 µg/ml, the longest larval duration was recorded (15.2 days), followed by 50 x 10^3 µg/ml (14.0 days) when compared with untreated larvae (10.3 days). Also, insignificant effects of azadirachtin on pupal duration and it did not differ significantly from untreated pupae. Higher rates of malformed pupae at various azadirachtin treatment were recorded, the higher malformation percentage was obtained at 100 x 10^3 µg/ml followed by 12.5 x 10^3 µg/ml. Also, results showed higher malformed adult rates at various azadirachtin concentrations. Highest rate of malformed adults occurred at 50 x 10^3 µg/ml, followed by 25 x 10^3 µg/ml and then by 12.5 x 10^3 µg/ml. There was a reduction in pupation percentage than among those resulted from untreated larvae. The pupation percentage decreased with the increase of concentration that reached (45.0 %) at 100 x 10^3 µg/ml and (78.3) at lower concentration 6.25 x 10^3 µg/ml, while it was (96.6 %) from the control larvae. Significant decrease in adults' emergence percentage occurred with increasing concentrations of azadirachtin. The lowest value of emergence percentage was detected at 100 x 10^3 µg/ml followed by 50 x 10^3 µg/ml and then by 25 x 10^3 µg/ml compared with control adult emergence that recorded (93.1 %). The results indicated that longevity of female adults increased with raising azadirachtin concentration from (6.25 x 10^3 to 50 x 10^3 µg/ml) while in higher concentration (100 x 10^3 µg/ml), the longevity of adult female was shortened. Also, all concentrations of azadirachtin gave the higher male : female sex-ratio. Results indicated significant reductions in number of eggs and hatchability% of eggs laid per female. The deposited eggs were 2000 eggs/♀ in control females and it was found to be reduced to 852, 1128, 1150, 12000 and 1227 eggs /female at 100 x 10^3, 50 x 10^3, 25 x 10^3 , 12.5 x 10^3 and 6.25 x 10^3 µg/ml of azadirachtin, respectively. Also, higher concentrations of azadirachtin gave the highest sterility percentage (78.66 %), while, sterility percentage decreased by decreasing azadirachtin concentration. Abd El-Rady and Osman (2005) found that (Neemix 4.5% azadirachtin) neem and (Nat –1 96%) jojoba oils on 4th instar *A. ipsilon* larvae caused an increase in larval and pupal durations and decrease in pupal weight. Also, Neemix was more effective than Nat-1 in decreasing pupation, pupal weight, adult emergence, fecundity and fertility. Malformed pupae were increased with the Neemix treatments, while Nat-1 was high in malformed adults. In summary, the results presented in this investigation indicated that bio-insecticide, protecto caused high mortality percentage and the plant extracts, coumarine and azadirachtin revealed strong antifeedant activity. Furthermore, compound coumarine exhibited potent inhibition of hatching, malformation and emergency.

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Table 3. Biological effects of protecto after treatment of *S. littoralis* 4th instar larvae by feeding for 24 hrs. on treated leaves.

Conc-entrat-ion µg/ml	Larval stage		Pupal stage				Adult stage							
							Sex ratio		Longevity		Egg/ female		% Hatching	% Sterility
	% Mortality	Duration day	Duration day	% Malformed	% Pupation	% Emergence	% Malformed	M	F	M	F	Deposit	Hatch	
20 x 10^3	50.0	11.0	10.7	13.3	50.0	73.3	0.0	2.4	1.7	8.3	8.1	0	0	0	100.0
10 x 10^3	40.0	10.8	10.3	8.2	60.0	80.5	0.0	1.9	2.1	7.7	7.3	774.0	515.0	66.5	60.39
5 x 10^3	30.0	10.3	10.2	7.1	70.0	83.3	5.7	2.3	1.7	8.0	7.0	899.0	603.0	67.1	53.59
2.5 x 10^3	25.0	9.8	9.3	6.6	75.0	86.6	0.0	1.6	2.2	8.0	7.3	1018.0	760.0	74.7	41.51
1.25	20.0	9.4	8.0	4.2	80.0	89.5	4.6	3.0	1.7	7.7	7.7	1209.0	890.0	73.6	31.48
Control	0.0	8.6	7.9	0.0	95.0	94.7	0.0	2.0	2.0	7.7	8.0	1611.0	1300.0	80.6	-
L.S.D (5%)	7.39	0.92	0.87	4.21	8.91	6.01	0.79	1.02	0.28	0.83	0.59	113.0	80.6	7.14	-

Table 4. Biological effects of coumarin on 4th instar larvae of *S. littoralis* (Boisd.) fed for 24 hrs. on treated leaves.

Conc. µg/ml	Larval stage		Pupal stage				Adult stage							
							Sex ratio		Longevity		Egg/ female		% Hatching	% Sterility
	% Mortality	Duration day	Duration day	% Malformation	% Pupation	% Emergence	% Malformation	M	F	M	F	Deposit	Hatch	
100 x 10^3	60.0	17.2	12.7	12.5	40.0	52.4	18.1	2.7	1.4	8.7	9.0	0	0	0	100.0
50 x 10^3	51.6	17.0	12.5	0.0	48.3	68.9	0.0	1.5	2.8	8.3	9.7	390.0	150	38.5	81.91
25 x 10^3	43.3	16.3	12.2	5.8	56.6	78.1	8.0	2.5	1.6	6.0	7.3	566.0	250	44.1	70.0
12.5 x 10^3	33.3	16.1	11.9	7.5	66.6	81.0	6.6	2.0	2.0	8.0	7.6	686.0	343	50.0	58.68
6.25 x 10^3	25.0	15.0	10.7	0.0	75.0	82.2	0.0	2.4	1.7	7.0	10.0	750.0	470	62.6	43.43
Control	0.0	10.9	9.7	0.0	96.6	94.8	0.0	2.0	1.9	6.9	6.5	914.0	830	90.8	-
L.S.D (5%)	10.35	1.61	0.87	3.46	15.26	21.58	4.96	0.73	0.95	1.41	1.76	134.0	96.8	5.93	-

Table 5. Biological effects of azadirachtin on 4th instar larvae of *S. littoralis* (Boisd.) fed for 24 hrs. on treated leaves .

Conc. µg/ml	Larvae stage		Pupae stage				Adult stage							
							Sex ratio		Longevity		Egg/ female		% Hatching	% Sterility
	% Mortality	Duration day	Duration day	% Malformed	% pupation	% Emergence	% Malformed	M	F	M	F	Deposited	Hatch	
100 x 10^3	55.0	15.2	10.7	11.1	45.0	66.6	0.0	3.6	1.3	7.0	5.3	852.0	385.0	45.1	78.66
50 x 10^3	43.3	14.0	10.5	0.0	56.6	73.5	12.0	1.3	4.2	8.7	10.7	1128.0	600	53.1	66.73
25 x 10^3	35.0	13.5	10.0	7.6	65.0	79.4	9.6	2.2	1.8	11.0	8.0	1150.0	693.0	60.2	61.54
12.5 x10^3	28.3	12.3	9.8	9.3	71.6	83.7	5.5	3.3	1.4	7.0	6.0	1200.0	800	66.6	55.60
6.25 x10^3	21.6	11.7	9.3	6.3	78.3	85.1	0.0	2.0	2.0	7.2	5.8	1227.0	900	73.3	50.01
Control	0.0	10.3	9.0	0.0	96.6	93.1	0.0	2.1	1.8	6.0	6.3	2000.0	1800	90.0	--
L.S.D(5%)	12.30	0.86	0.74	2.21	16.4	18.7	2.63	1.12	0.93	1.23	2.13	136.0	107.0	13.39	-

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Biochemical and histological effect of some plant extracts, insecticide (methomyl) and bio insecticide (protecto) against cotton leafworm, *Spodoptera littoralis* (Boisd.)

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Abstract

The toxic effect of four different compounds, namely protecto (bioinsecticide based on *Bacillus thuringienses*), coumarin (plant extract of Chicory flower), Neemix (plant extract of *Azadirachta indica*) and lannate (chemical insecticide) against the fourth instars larvae of cotton leafworm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) by LC_{50} concentrations were evaluated under laboratory conditions. Methomyl led to basement and peritrophic membrane detachment and destruction, appearance of numerous vacuoles, destruction of epithelial cells that emptied their cytoplasmic contents in the lumen, while protecto caused detachment and destruction of the basement and peritrophic membrane, vacuolization and destruction of the epithelial cells. Plant extracts (coumarin and azadirachtin) caused basement membrane detachment followed by destruction, destruction of epithelial cells which showed thickness in some points, in some cases the epithelial cells appeared deformed and become elongate in size than those of the control. The tested compounds caused significant decrease in the total soluble protein content of the 4th instar larvae. All compounds (except methomyl) caused significant increase in the activity of acetylcholinesterase enzyme and caused significant increase in the activity of acid phosphatase and amylase enzyme (except protecto and methomyl decrease the activity of amylase enzyme), on contrary, tested compounds showed significant decrease in the activity of alkaline phosphatase enzyme, coumarin and azadirachtin significantly increased α-esterase, while the other compounds caused significant decrease in the activity of this enzyme and β-esterase enzyme. Also tested compounds expect methomyl caused significant inhibition in the activity of GOT, on contrary, all tested compounds except coumarin caused significant decrease in the activity of GPT and invertase activity. Trehalase showed the same decreasing effects (except coumarin), while azadirachtin similar to control and had no effect.

Key word: Biochemical, histological, plant extracts, methomyl, bio-insecticide, cotton leafworm, *Spodoptera littoralis* (Boisd.)

Introduction

The cotton leaf worm, *S. littorals* (Boisd.) is considered as one of the most severe destructive cotton pests in Egypt and many other parts of the world infesting over 112 plant species belonging to 44 families. The larval stage of *S. littoralis* is known as a notoriously leaf eater accepting almost all herbaceous plant (Hill., 1975). Cotton leaf worm control program was based mainly on use of insecticides, which created some problems such as insecticides- resistance, environmental pollution and hazard to natural enemies and beneficial insects (Toscano *et al.*, 1974 and Abbas *et al*., 1996**).** Hence, it recent approaches now focus upon the use of environmentally safe compounds as pest control agents, the entomopathogenic bacteria, *B. thuringensis* and plant extracts as pesticide alternatives possess distinct toxicity and also lead to antifeeding activity and inhibition growth of some pests (Sharaby and Ammar, 1997; Badr *et al*., 2000 and D'Andrea *et al*; 2001). Latent effect of larval exposure to various antifeedants was reported to disturb growth and development in the subsequent pupal and adult stage, i.e., *S. littoralis* (Meisner *et al*., 1982); *Ostrinia nubilalis* (Arnason *et al*.,1987), *S. littoralis* Adel and Abd El-Hakim (2007) and *Heliothis virescens* (Barnaby and Klocke, 1987) The histological studies are very important to do on the mid gut of the treated larvae because it show and explain the changes resulting from treating larvae with different tested compounds**.** The changes in the biochemical content especially the transaminase enzymes activities such as GOT, GPT, trehalase, invertase, amylase and soluble protein content have an important role in biological and physiological activities of insects. (Mead-Hala, 2000 and Khedr, 2002). Esterase in insects have been implicated in reproductive behavior, pheromone and hormone metabolism, digestion, neurotransmission, the action of end resistance to insecticide, particularly organophosphates. Esterases may contribute to resistance by hydrolyzing the pesticide (Parkes, *et. al*. 1993, Gaaboub, 2004 Hanafy, *et. al.* 2005).

The aim of the present work to evaluate histological effects of tested compounds against the mid gut of 4th instar larvae of *S. littoralis* and biochemical study was conducted on the treated larvae, i.e. total protein and enzyme activities, (acid phosphatase, alkaline phosphatase, alpha and beta esterases, GOT and GPT transaminase enzymes, trehalase, amylase and invertase carbohydrate hydrolyzing enzymes and acetyl cholinesterase enzymes).

Material and methods

The tested compounds

Plant extracts

Coumarin: - Isolated from the flower of *chichorium intybus* (chicory), Azadirachtin (Neemix): from the neem tree, *A. indica* (Meliaceae), 6 (2, 4-dinitrophenylamino hexanyl) - 22, 23-dihydroazadirachtin

Bio insecticide (protecto)

W.P. based on *B. thuringiensis subsp. Kurstaki* ($32x10^3$ I. U/mg). Active ingredient 9.4%

Insecticides (methomyl):

Lannate 90% W.P. (a synthetic carbamate compound) Carbamate oxime

Chemical name: S. methyl- N-[(methyl carbomoyl) oxy] thioecetimidate.

Insect maintenance

The stock culture of susceptible Egyptian cotton leaf worm, *S. littoralis* was reared on castor leaves (*Ricinus communish*) for several generations at laboratory conditions 27 ± 1°C and 70 ± 5% RH. Egg masses were placed on castor oil leaves in cylindrical glass jars. The jars were covered with muslin cloth and fastened with rubber band; first instar larvae hatched within 2-3 days, the newly hatched larvae were transferred into reading jars bottomed with sheeted of towel paper to absorb excess humidity. Castor bean leaves were provided daily to the larvae in sufficient amounts, the accumulated faces and debris were cleaned out daily. After pupation, pupae were collected and placed in wide clean jars until adult emergence. Then, the emerged adults were supplied with a piece of cotton wetted with 5-10% sugar solution and branches of tafla (*Nerium oleander*) as a suitable site for oviposition. Newly laid egg masses were collected daily and transferred into the rearing jars.

Histopathological studies

All tested compounds at their LC_{50} concentration were applied to the fourth instar larvae using the leaf dipping technique method at the LC_{50} concentration of each compound. The larvae were collected after 1, 2, 3, 4 and 5 days post treatment then, transferred into Bouin's solution. It was used as a fixative, for dehydration and removal of the yellow colour of Bouin's solution the larvae were rinsed in a series of ethanol solutions. They were transferred first into 50% ethyl alcohol for 2 hrs. (Two changes) then left for 24 hours. Then the larvae passed through a series of alcoholic treatment each for two hours at room temperature starting with 80% followed by 90%, 96% and ending with 100% alcohol. After dehydration the larvae were placed in solution of amylacetate solution and soft paraffin wax and leaving them for 24 hours at 50°C. The larvae were replaced by soft paraffin wax three times at 24 hours intervals at 50°C. A mixture of one part of hard paraffin wax was added to the larvae. The larvae were imbedded in wax mixture used in the last step. Serial sections at 6 microns were made by microtome and mounted on clean slides using Mayer's albumin. Sections were mounted on glass slides and stained with haematoxylene and counterstained in alcoholic solution and prepared for examination and photo microscopy.

Biochemical studies

Samples preparation

Larval samples used for biochemical assays were collected at 1, 2, 3, 4, 5 and 7 days post treatment of the 4^{th} instar larvae with the LC_{50} concentration for each compound. Untreated larvae were used as control. Samples were homogenized in distilled water using a Teflon homogenizer. The homogenates were centrifuged at 500 r.p.m for 10 minutes at 5°C. The supernatants were immediately assayed to determine the total soluble protein, the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and the carbohydrate hydrolyzing enzymes (Trehalase, invertase, amylase),α-β esterases, phosphatesas (acid and alkalin).

Determination of total soluble protein

Colorimetric determination of total soluble protein in total homogenate *S. littoralis* larvae was carried based out as described by (Gornall *et al.*, 1949). A volume of 0.2 ml of larval homogenate was added to 5 ml of Biuret reagent and incubated for 30 min at 20-25°C. The absorbance of the sample against a blank Biuret reagent was measured at wave length of 546 nm.

Determination of enzymes activities

Transminase enzymes (GOT and GPT)

Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) enzyme activities were determined colorimetrically according to the method of Reitman and Frankle (1957). GOT transfer the amino group from L-aspartate to α-ketyo acid (α-ketoglutaric acid) producing a new amino acid (L-glutamate) and a new keto acid (oxaloacetic acid). GPT transfer the amino group from D,L alanine to α-keto acid (α-keto glutaric acid), resulting in a new amino acid (L-glutamate) and a new keto acid (pyruvic acid). Oxaloacetate or pyruvate reacts with 2, 4-dinitrophyenylhedrazine forming oxaloacetate or pyruvate hydrazone which in alkaline medium form a brown colour which can measure spectrophotometerically. The reaction mixture consisted of 1ml of a mixture of phosphate buffer (pH 7.4) 0.2 mM α-ketoglutaric and 200 mM D-L alanine or L-aspartate, 0.2 ml of larval homogenate was then added to the reaction mixture. The mixture was incubated for 30 min. then after, 10 ml of 0.4 N

NaOH was added. The optical density of the produced brown color is measured after 5 min using spectrophotometer at 520 nm. The enzyme activity is expressed as M Pyruvate/gm body weight/min.

Carbohydrate hydrolyzing enzymes

The methods used to determine the digestion of trehalose, starch and sucrose by trehalase, amylase and invesrtase enzymes respectively, were similar to those described by Ishaaya and Swiriski (1976). The free aldehydic group of glucose were determined using 3, 5 dinitrosalicylic acid reagent. The optical density (OD) of the produced colour is measured at 550 nm using spectrophotometer. The enzymatic activity was expressed as mg glucose released/gm body weight/min.

Determination of non-specific esterases activity

Alpha- and Beta- esterases (α-E, β-E) were determined according to the method of Van Asperen (1962). Using α-naphthyl acetate and β- naphthyl acetate as substrates, respectively. Napthol produced as a result of substrate hydrolysis can be measured by the addition of diazoblue sodium lauryl sulphate solution which produces a strong blue colour in case of α-naphthol or strong red colour in the case of β-naphthol. The colour was measured spectrophotometrically. The developed colour was read at 600 and 555nm for α- and β-naphthol, respectively. The activity was expressed as mg α- or β- naphthol released/min/ larva.

Acid and alkaline phosphatases enzymes (AcP & AlkP)

Acid phosphatase (AcP) and alkaline phosphatase (AlkP) were determined according to the method described by Powell and Smith (1954).

Acetylcholine esterase (AChE):

Acetylcholine esterase (AChE) was measured according to the method described by Simpson *et al.,* (1964), measured at 515 nm.

Results and discussion

Histological changes in the mid-gut of the 4th instar larvae of *S. littoralis* as affected by the tested compounds

Across section in the mid-gut of untreated larvae of *S. littoralis* appeared the epithelium of mid-gut surrounded by the basement membrane. They possess oval, conspicuous nuclei nearly central in position. Scattered between them are small goblet cells with reduced granular cytoplasm and spherical nuclei. The epithelial shows a striated (brush border) which is in the fact, the microvilli of columnar cell extending from their free ends. The wall of the gut contains two distinct layers of muscle fibers longitudinal muscles fibers to the outside and circular muscles fibers to the inside. The spaces between the different gut wall layers are almost filled with connective tissue.

Effect of methomyl

The present histological study on the effect of methomyl on the mid-gut of treated larvae of *S. littoralis* after 1, 2 and 3 days of treatments revealed certain changes appeared within Plates (1) that showed epithelium cells detached from the basement membrane in many areas and thickness of epithelial cells in the mid-gut larvae and some cells were broken and emptied their cytoplasmic contents in the space between the epithelial and peritrophic membrane. However, after 4 and 5 days of treatments complete degeneration of the mid-gut epithelial cells becomes more deformed and losses the columnar structure while the peritrophic membrane still intact in many areas.

Effect of protecto

Plates (2) indicated that, the epithelial cells lost their close association with the basement membrane and with each other. The epithelial cells were destroyed and lose their columnar structure in some point and caused disorganization of peritrophic membrane and in some cases disappeared after 1, 2 and 3 days of treatments. While after 4 and 5days of treatments the mid-gut epithelial cells were filled with scattered vacuoles and the basement membrane appear and still intact and the epithelium cells destruction in some point.

Effect of coumarin

Plates (3) indicated that, the effect of coumarin on the mid-gut tissues of 4th larvae of *S. littoralis* were most pronounced and extensive after 1 and 2 days of the treatments, where some cells become thickness and deformed also, emptied their cytoplasmic contents in the space between the epithelial and peritrophic membrane. After 3 and 4 days of treatment, the epithelium cells are destroyed, broken and separation completely from the broken basement membrane. The cellular debris from degenerating cells filled the gut lumen. While after 5 days of treatments microscopic examination showed no differences between the larvae which treated by coumarin and the control in concern of epithelium and basement membrane also, peritrophic membrane not affected and still intact.

Effect of azadirachtin

Plates (4) indicated that, there is no effect of azadirachtin extract on the 4th larvae instar of *S. littoralis* after 1 and 2 days of treatments, while after 3 and 4 days of treatments many abnormalities appeared in internal components of the mid-gut compared with the untreated larvae, the epithelial cells appeared deformed, destroyed in some points and in some cases the epithelial become elongate in size than control, detachment of the epithelium cells from the basement membrane in the same areas, but it was still intact in other. Also, azadirachtin caused

disintegration of peritrophic membrane compared with control. while after 5 days of treatment the epithelial cells become more apparent and the microvili of some columnar cells are still intact, also peritrophic membrane and basement membrane not affected.

Mohamed (2002) found that plant seed oil extracts, i.e., sunflowers, soybean, castor and cotton caused abnormalities in the tissue of mid-gut larvae of pink bollworms.

Gamil (2004) studded that histological changes were observed in *S. littroalis* mid-gut. Although, damage in mid-gut tissue by the two tested bacteria was relatively similar, Ahmed *et. al.* (2007) studied the effect of different neem products on the mid gut tissues of the black cut worm, *Agrotis ipsilon*. Its occurrence was much more evident and severe when *S. marcescens* was tested compared to the effect of HD 129 and protecto. Heba (2005) found that *B. thuringiensis* var. kurstaki caused detachment and destruction of the basement and peritrophic membranes, vacuolization and destruction of the epithelial cells.

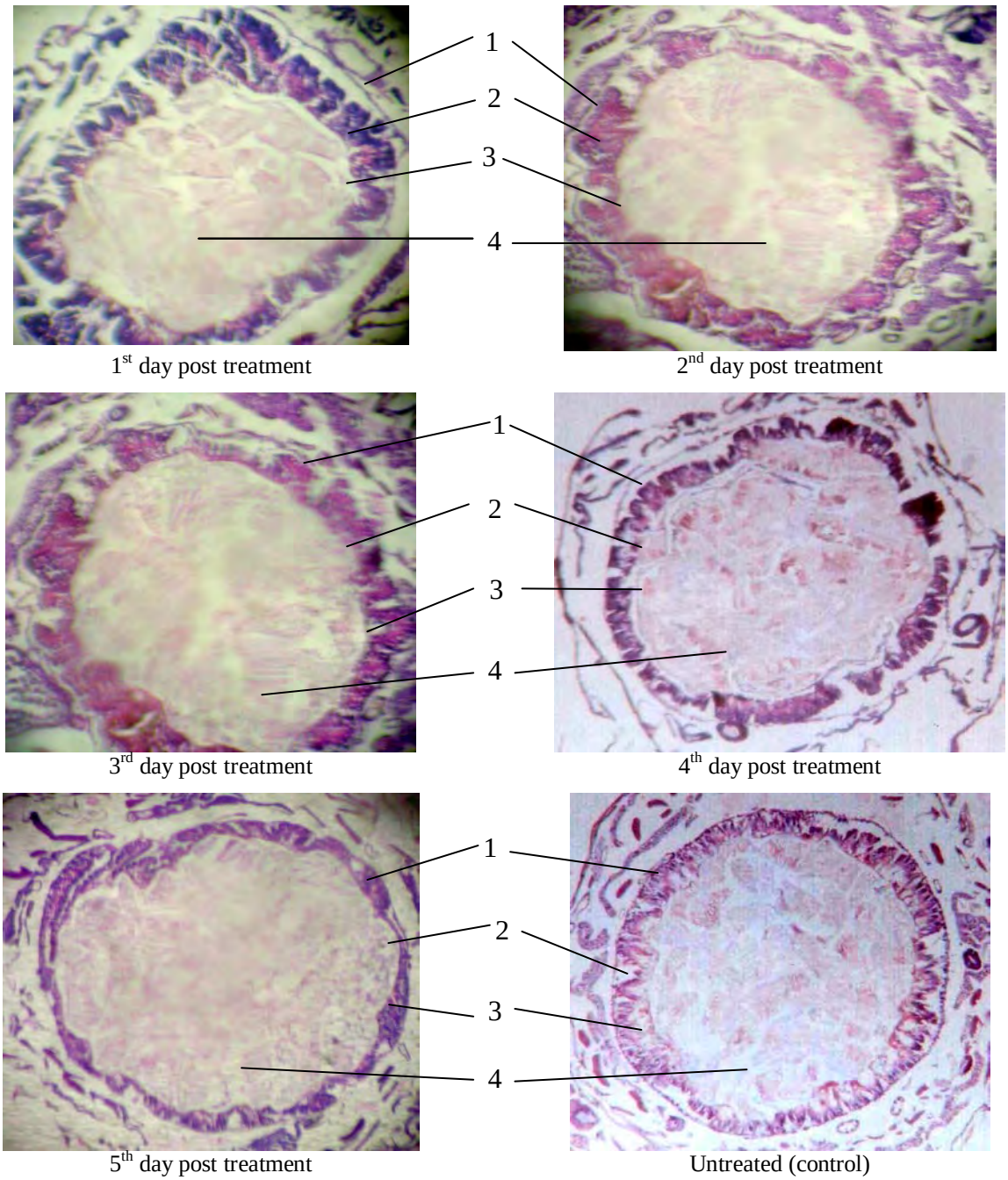


Plate 1. Effect of lannate on the mid gut of the 4th instar larvae of *S. littoralis*.
(1) Basement membrane (2) Epithelium layer
(3) Peritrophic membrane (4) Lumen

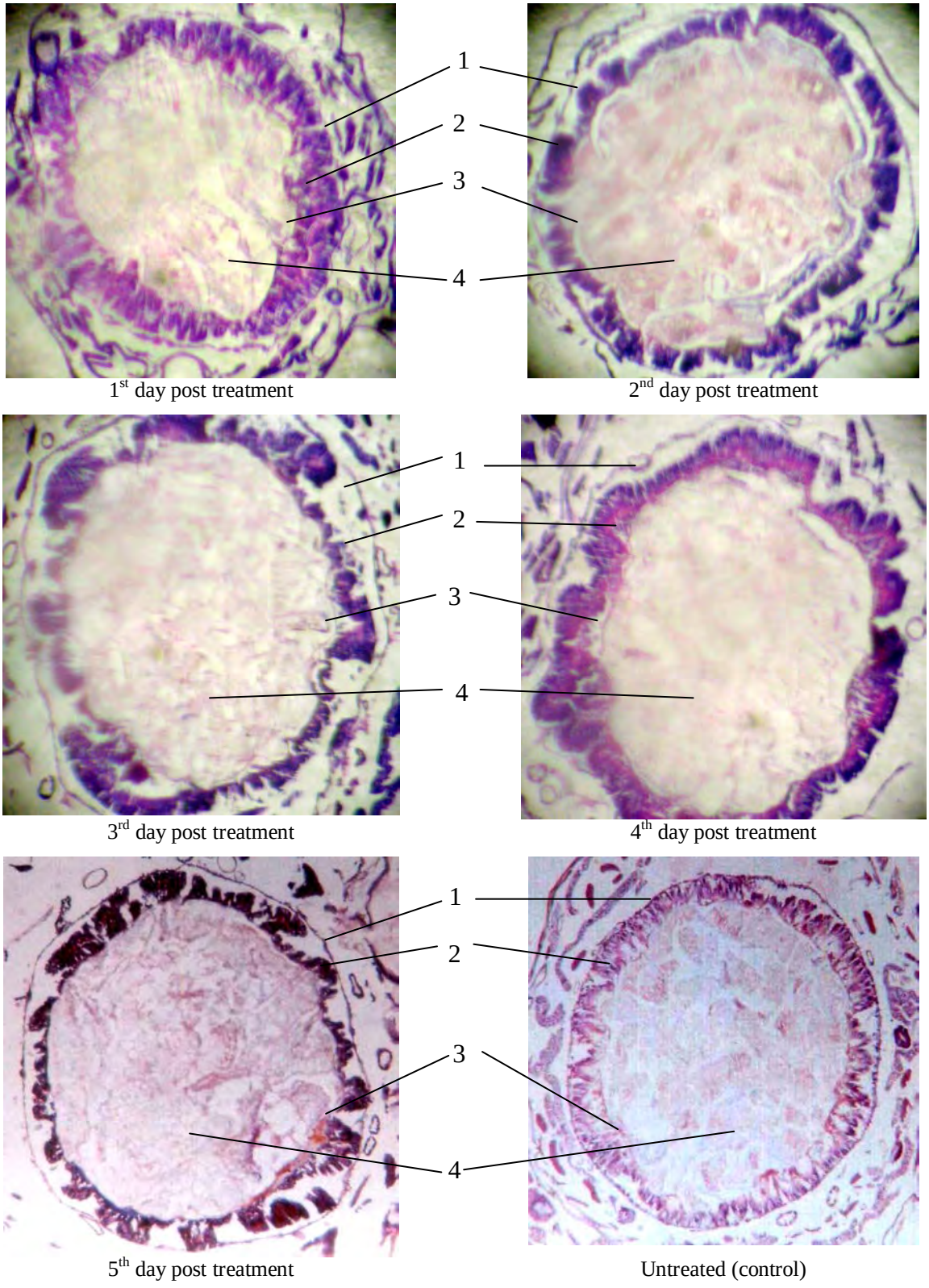
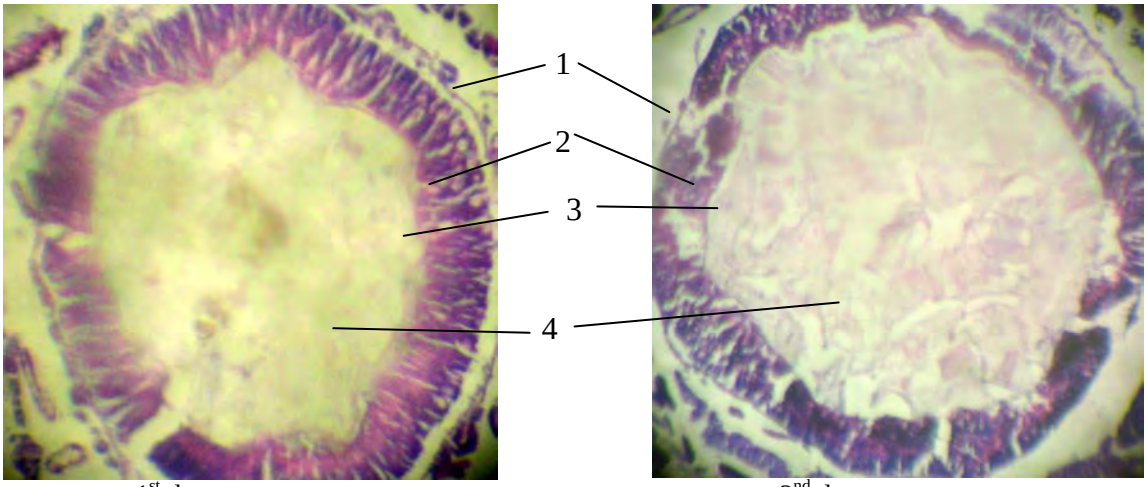


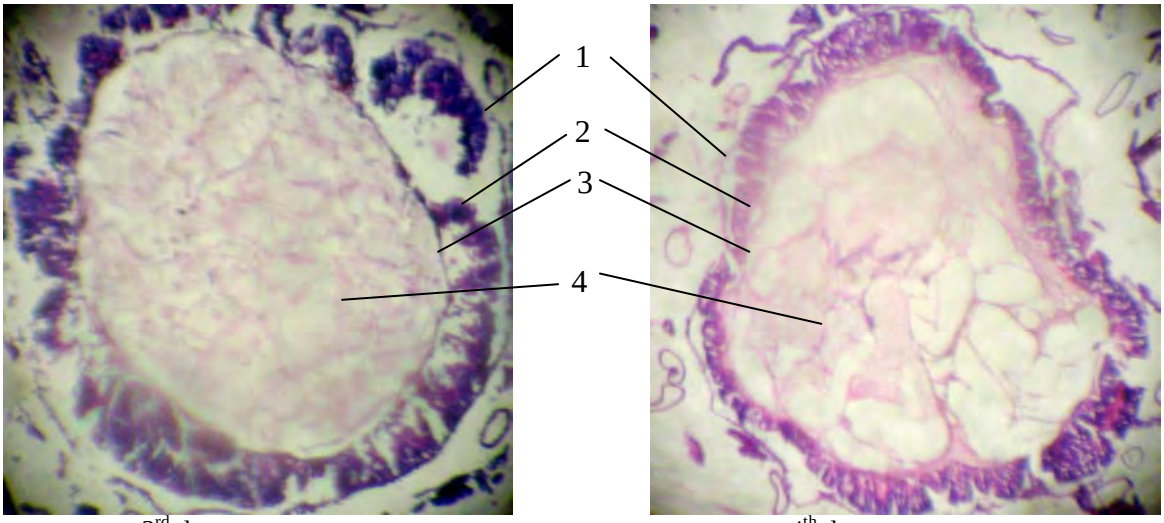
Plate 2. Effect of protecto on the mid gut of the 4th instar larvae of *S. littoralis.*

(1) Basement membrane (2) Epithelium layer
(3) Peritrophic membrane (4) Lumen



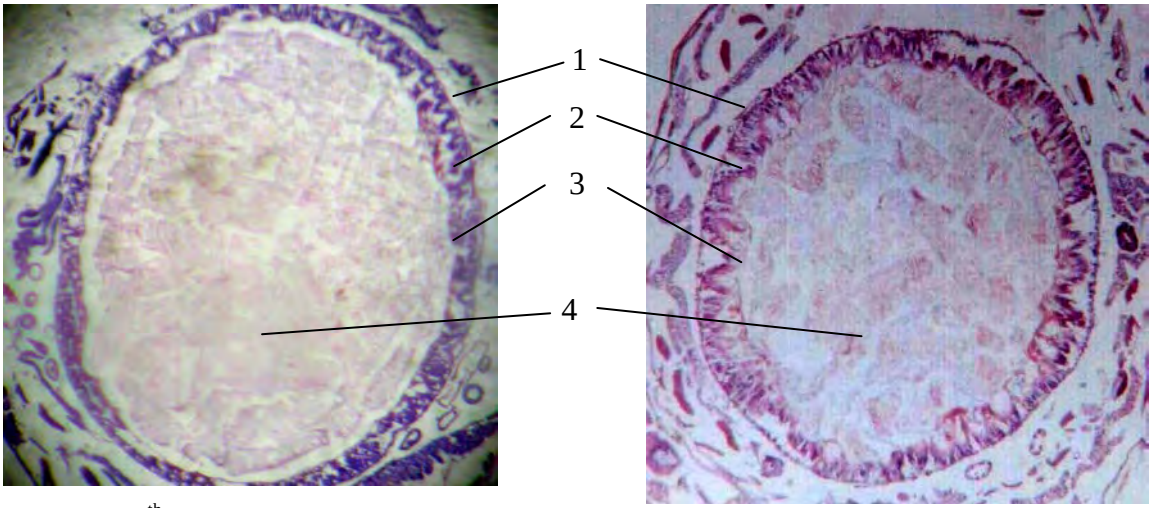
1st day post treatment

2nd day post treatment



3rd day post treatment

4th day post treatment



5th day post treatment

Untreated (control)

Plate 3 Effect of coumarin on the mid gut of the 4th instar larvae of *S. littoralis*.
(1) Basement membrane (2) Epithelium layer
(3) peritrophic membrane (4) Lumen

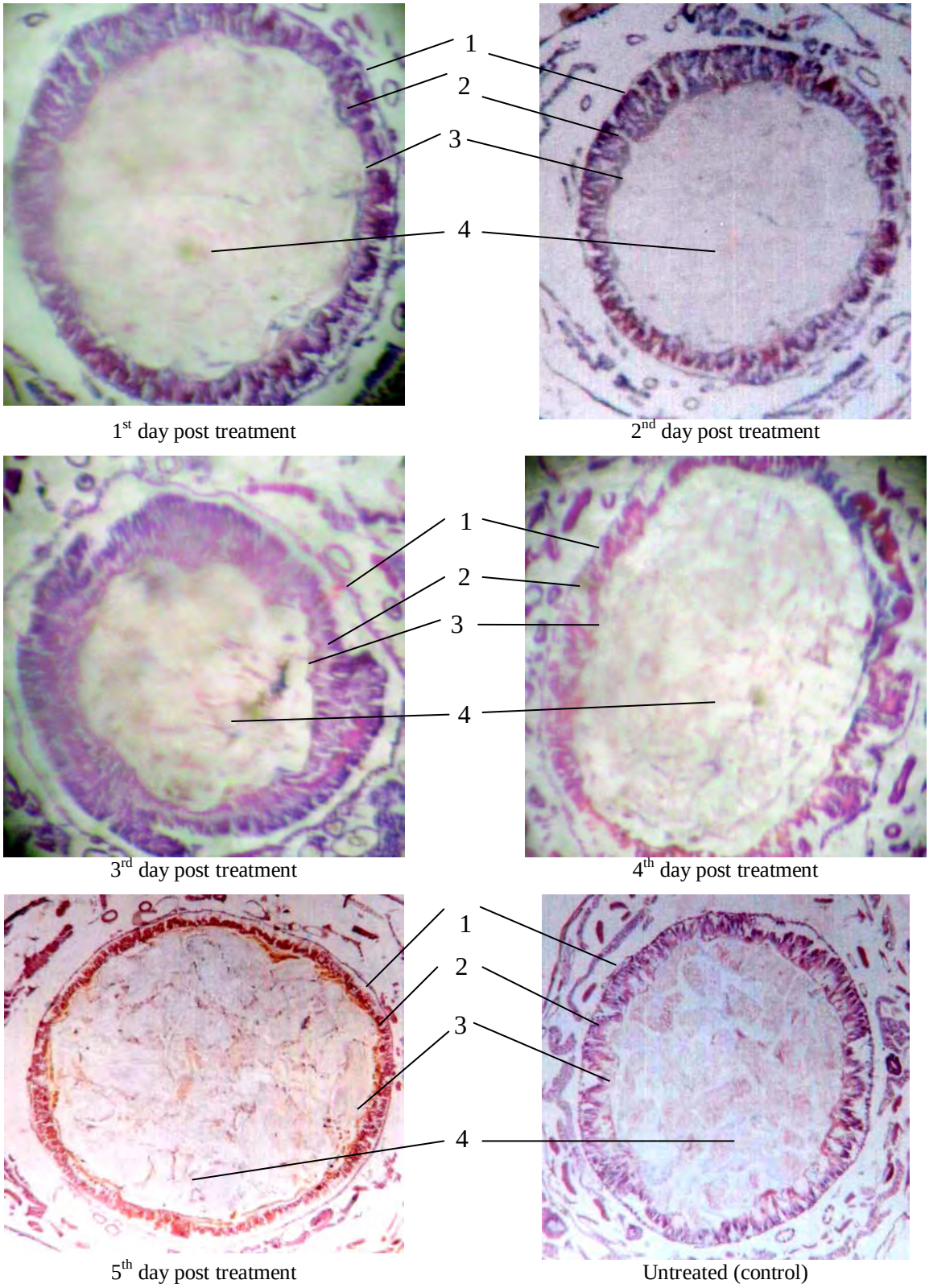


Plate 4. Effect of azadirachtin on the mid gut of the 4th instar larvae of *S. littoralis*.

(1) Basement membrane (2) Epithelium layer
(3) peritrophic membrane (4) Lumen

Effect of the tested compounds on some biochemical aspects of the cotton leafworm larvae in the laboratory

Fourth instar larvae of *S. littoralis* treating with the LC_{50} concentration for each compound was used for biochemical assay to evaluate total soluble protein, the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and the carbohydrate hydrolyzing enzymes (trehalase, invertase, amylase), α-β esterases, phosphatesas (acid and alkaline) and acetylcholinesterase (AChE).

Concentration of total soluble protein (T.S.P)

Data in Table (1) showed that, all tested compounds significantly reduce the total protein except protecto which significantly increase in the concentration of total protein. The other tested compounds caused significant reduce of total protein. The values of total protein in the supernatant of the homogenate larvae reached to 3.120, 1.291, 0.635 and 0.475 mg/g b.w. when larvae treated with LC_{50} of the tested protecto, methomyl, coumarin and azadirachtin, respectively, compared with 2.296 mg/g b.w. of the control. The percent differences between treatments in the concentration of total protein reached 135.88 % more than the check in case of protecto Mohamed-Sondos, 2002 and Abo-El-Ftooh (2004). Mohamady (2000) investigated the effect of treatment of the 4th instar larvae of *S. littoralis* with the LC_{25} and LC_{50} of fenvalerate on the total protein at different time intervals (24, 48 and 72 hrs). The results indicated that there was high reduction in the level of total protein due to the treatment.

Table (1): Effect of the tested compounds on the concentration of total soluble protein of the 4th instar larvae of *S. littoralis*

Treatments	**Total soluble protein (mg/g body weight) Days after treatment**					
	1st	**3rd**	**5th**	**7th**	**Mean**	**%**
Control	2.844	1.265	3.312	1.763	2.296	100.0
Protecto	3.673	3.475	2.796	2.626	3.120	135.88
Methomyl	2.203	1.026	1.330	0.604	1.291	56.22
Coumarin	0.587	0.548	0.687	0.718	0.635	27.66
Azadirachtin	0.662	0.387	0.288	0.561	0.475	20.68

L.S.D. at (0.05) for treatment = 0.32

Carbohydrate hydrolyzing enzymes:

Invertase

Data in Table 2 indicated that, the tested compounds showed reduction in the activity of invertase of the treated 4th instar larval of *S. littoralis*. The mean values of invertase activities in the supernatant of the homogenate larvae reached to 1.128, 1.131 and 1.502 mg/g b.w when larvae treated with (LC_{50}) by protecto, methomyl and azadirachtin compared with 1.636 mg/g. while, there were no effects in the activity of invertase when larvae treated by coumarin (1.636 mg/g). The percent differences between treatments in the activity of this enzyme irrespective to the time after treatment reached 68.94, 69.13 and 91.80 % less than the check in case of protecto, methomyl and azadirachtin, respectively. In general, protecto and methomyl were high effective on invertase activity 68.94 and 69.13, respectively.

Table (2). Effect of the tested compounds on the invertase, trehalase and amylase activty of the 4th instar larvae of *S. littoralis*

Treatments	Invertase activity (µg Glu/g body weight/min) Days after treatment					
	1st	3rd	5th	7th	Mean	%
Control	1.816	1.153	2.418	1.156	1.636	100.0
Protecto	0.353	1.076	1.808	1.278	1.128	68.94
Methomyl	1.274	0.899	1.416	0.935	1.131	69.13
Coumarin	1.827	1.046	1.542	2.077	1.636	100.0
Azadirachtin	1.284	1.710	1.623	1.393	1.502	91.80
L.S.D. at (0.05) for treatment = 0.34						
	Trehalase activity (µg Glu/g body weight/min) Days after treatment					
Control	0.530	0.600	1.150	0.506	0.697	100.0
Protecto	0.353	0.704	0.775	0.455	0.572	82.06
Methomyl	0.574	0.697	0.466	0.419	0.539	77.33
Coumarin	0.639	0.540	0.702	1.097	0.744	106.74
Azadirachtin	0.771	0.500	0.772	0.747	0.697	100.0
L.S.D. at (0.05) for treatment = 0.13						
	Amylase activity (µg Glu/g body weight/min) Days after treatment					
Control	0.143	0.265	0.344	0.291	0.261	100.0
Protecto	0.353	0.100	0.133	0.169	0.189	72.41
Methomyl	0.131	0.329	0.100	0.265	0.206	78.92
Coumarin	0.230	0.292	0.347	0.442	0.327	125.28
Azadirachtin	0.113	0.215	0.364	0.692	0.346	132.56
L.S.D. at (0.05) for treatment = 0.08						

Trehalase

Data in Table 2 indicated that coumarin and azadirachtin are almost the same with control for the the activity of trehalase. The mean values of trehalase activities in the supernatant of the homogenated larvae reached 0.572, 0.539, 0.744 and 0.697 mg /g b.w. when larvae treated (at LC_{50}) by protecto, methomyl, coumarin and azadirachtin, respectively. Compared with 0.697 mg/g. for the control on the contrary, protecto and lannate showed reduction in enzyme activity, reaching 82.06 and 77.33 % less than the check. About 22.77 and 17.94 % inhibition were obtained by methomyl and protecto, respectively.

Amylase

Data in Table 2 indicated that coumarin and azadirachtin caused significant increase while protecto and methomyl caused significant decrease in the activity of amylase of 4th instar larvae of *S. littoralis* compared to the untreated ones and other tested compounds. The mean values of amylase activities in the supernatant of the homogenated larvae reached to 0.189, 0.206, 0.327 and 0.346 mg/g. when larvae were treated at LC_{50} by protecto, methomyl, coumarin and azadirachtin, respectively, compared with 0.261 mg/g for the untreated control. The other products variously decreased this enzyme activity, i.e. 72.41 and 78.92 % less than the

untreated control in case of protecto and methomyl. Khedr (2002) found an increase in the activity of trehalase enzyme of *S. littoralis* (2nd & 4th instar larvae) after treatment with Biorepel.

Transaminase enzymes:

Glutamic pyruvic transaminase (GPT)

Data in Table 3 showed that the tested compounds caused significant decreased in activity of GPT of the treated 4th instar larvae of *S. littoralis* than the untreated control. While, protecto showed no significantly increased the activity of GPT. The mean values of GPT enzyme activities in the supernatant of the homogenated larvae reached to 1.07, 0.58, 0.93 and 1.02 mg/g. When larvae were treated at LC_{50} by protecto, lannate, coumarin and azadirachtin, respectively, compared with 1.09 mg/g b. w for the untreated larvae. The tested compounds decreased this enzyme activity, i.e. 98.17, 53.21, 85.32 and 93.57 % less than the untreated check in case of protecto, methomyl, coumarin and azadirachtin. It was found that the most inhibitor compound was methomyl followed by coumarine this result agree the studied by Vera *et. al.* (2006).

Glutamic oxaloacetic transaminase (GOT)

Data in Table 3 revealed the significant increasing effect of protecto, coumarin and azadirachtin on GOT activity of the treated 4th instar larvae of the cotton leafworm compared with the untreated larvae. The enzyme activity changed from 7.32 mg/g of larvae (normal) to 7.49, 8.40 and 9.97 mg/g , respectively. On the contrary, methomyl gave (5.94 mg/g b.w.) showed 81.15 decreased in GOT activity than the check. In general, the percent differences between treatments in the activity of this enzyme irrespective of the time after treatment reached136.20, 114.75 and 102.32 % more than the check in case of azadirachtin, coumarin and protecto respectively. Mohamady (2000) investigated the effect of treatment of the 4th instar larvae of *S. littoralis* with the LC_{25} and LC_{50} of fenvalerate on the activity of GOT at different time intervals (24, 48 and 72 hrs). Data showed that, there was irregular effect on GOT activity at the different time intervals where it fluctuated between increase and decrease throughout the 72 hrs period of the experiment.

Alpha and beta esterases (α-E and β-E)

Alpha esterase (α-E)

Data in Table 4 were caused indicated that, the tested compounds coumarin and azadirachtin significant increase in the activity of α–esterase of the treated 4th instar larvae of *S. littoralis* than the untreated larvae. The mean values of α–esterase enzyme activities in the supernatant of the homogenate larvae reached to 1.643, 1.890, 3.047 and 2.665 mg/g b.w when larvae were treated at LC_{50} by the tested protecto, lannate, coumarin and azadirachtin, respectively, compared with 2.510 mg/g b.w the untreated control.

Table 3. Effect of the tested compounds on the activities of GPT and GOT enzymes of the 4th instar larvae of *S. littoralis*

Treatments	GPT activity (mg/g pyrovate/ body weight/min) Days after treatment					
	1st	3rd	5th	7th	Mean	%
Control	1.39	0.96	1.63	0.39	1.09	100.0
Protecto	1.08	0.83	1.90	0.48	1.07	98.17
Methom-yl	0.46	0.70	0.45	0.73	0.58	53.21
Coumari-n	0.63	0.94	0.88	1.28	0.93	85.32
Azadirac-htin	0.44	1.95	1.13	0.58	1.02	93.57
L.S.D. at (0.05) for treatment = 0.13						
	GOT activity (mg/g pyrovate/ body weight/min) Days after treatment					
Control	11.18	9.37	4.28	4.44	7.32	100.0
Protecto	9.68	6.47	9.78	4.02	7.49	102.32
Methomyl	3.49	7.23	5.43	7.59	5.94	81.15
Coumarin	6.29	8.32	9.36	9.64	8.40	114.75
Azadirachtin	12.14	11.16	12.09	4.49	9.97	136.20
L.S.D. at (0.05) for treatment = 1.11						

The differences in the activity of this enzyme between treatments reached 121.39 and 106.18 % more than the check in case of coumarin and azadirachtin, respectively. Protecto and methomyl showed slight decrease in α – esterase activity, reached to 65.45 and 75.29 % less than the control, respectively. Mohamed and Azab (2002) found that pyrethroids caused a remarkable increase in alpha-esterase of pink bollworm compared to that recorded in untreated check larvae.

Beta esterase (β -E)

Data in Table 4 revealed that, 4th instar larvae of the cotton leafworm showed significant decreased in β -esterase enzymes activities in the supernatant of the homogenated larvae which treated at LC_{50} by protecto (1.855 mg/g b.w.), methomyl (2.248 mg/g b.w.), coumarin (2.591 mg/g b.w.) and azadirachtin (1.882 mg/g b.w.), respectively. Compared with 2.699 mg/g b.w. of the control. The tested compounds showed decreased in the activity of this enzyme after treatment reached 68.72, 83.29, 95.99 and 69.73 % less than the check in case of protecto, lannate, coumarin and azadirachtin, respectively.

Acetyl cholinesterase (ACHE)

Data in Table 4 indicated that, the tested compounds increased the activity of (AChE) of the 4th instar larvae of *S. littoralis* compared with the untreated larvae. The mean values of (AChE) in the supernatant of the homogenate larvae reached to 0.175, 0.265, 0.266 and 0.249 mg/g b.w when larvae were treated with LC_{50} of the tested protecto,

lannate, coumarin and azadirachtin compared with the check (0.148 mg/g b.w). The percent differences between treatments in the activity of this enzyme reached 118.24, 179.05, 179.72 and 168.24 % more than the check in case of protecto, lannate, coumarin and azadirachtin, respectively.

Alkaline and Acid phosphatase

Alkaline phosphatase

Data in Table 4 revealed that, the tested compounds inhibited the alkaline phosphatase of the treated 4th instar larvae of *S. littoralis* than the untreated ones. The mean values of alkaline phosphatase enzymes activities in the supernatant of the homogenate larvae reached to 0.058, 0.077, 0.054 and 0.073 mg/g b.w. when larvae were treated with the LC_{50} by the tested protecto, lannate, coumarin and azadirachtin, respectively, compared with 0.085 mg/g b.w. of the untreated control. The tested compounds caused decrease in Alkaline phosphatase activity reached to 68.24, 90.58, 63.52 and 85.88 % less than control. In case of protecto, lannate, coumarin and azadirachtin, respectively.

Table 4. Effect of the tested compounds on the activities of α - esterase, β - esterase, acetyl-cholinesterase, alkaline phosphates, acid-phosphates enzymes of the 4th instar larvae of *S. littoralis*

Treatments	α - esterase activity (µg a-naphth/g body weight/min) Days after treatment					
	1st	3rd	5th	7th	Mean	%
Control	1.989	2.581	4.242	1.260	2.510	100.0
Protecto	2.090	1.148	1.429	1.905	1.643	65.45
Methomyl	2.272	2.701	1.144	1.437	1.890	75.29
Coumarin	3.004	3.401	2.632	3.153	3.047	121.39
Azadirachtin	2.244	2.295	2.922	3.200	2.665	106.18
L.S.D. at (0.05) for treatment = 0.23						
	β - esterase activity (µg a-naphth/g body weight/min) Days after treatment					
Control	3.236	1.552	3.625	2.386	2.699	100.0
Protecto	1.418	2.361	2.880	0.760	1.855	68.72
Methomyl	3.706	2.213	1.578	1.496	2.248	83.29
Coumarin	2.423	2.815	1.437	3.687	2.591	95.99
Azadirach-tin	0.931	2.077	2.073	2.447	1.882	69.73
L.S.D. at (0.05) for treatment = 0.21						
	Acetyl cholinesterase activity (µg AchBr/g body weight/min) Days after treatment					
Control	0.071	0.099	0.302	0.120	0.148	100.0
Protecto	0.401	0.105	0.090	0.104	0.175	118.24
Methomyl	0.03	0.045	0.094	0.1	0.0672	29.05
Coumarin	0.532	0.051	0.192	0.288	0.266	179.72
Azadirach-tin	0.327	0.060	0.255	0.356	0.249	168.24
L.S.D. at (0.05) for treatment = 0.56						
	Alkaline phosphates activity (µg phenol/g body weight/min) Days after treatment					
Control	0.092	0.103	0.082	0.061	0.085	100.0
Protecto	0.119	0.041	0.031	0.042	0.058	68.24
Methomyl	0.057	0.075	0.122	0.055	0.077	90.58
Coumarin	0.035	0.059	0.052	0.070	0.054	63.52
Azadirach-tin	0.049	0.090	0.067	0.087	0.073	85.88

Table 4. cont.

L.S.D. at (0.05) for treatment = 0.02						
	Acid-phosphates activity (µg phenol/g body weight/min) Days after treatment					
Control	0.045	0.049	0.031	0.013	0.034	100.0
Protecto	0.085	0.025	0.017	0.020	0.037	108.82
Methomyl	0.042	0.046	0.035	0.018	0.035	102.94
Coumarin	0.053	0.107	0.011	0.026	0.049	144.11
Azadirachtin	0.045	0.052	0.020	0.024	0.035	102.94
L.S.D. at (0.05) for treatment = 0.002						

Acid phosphatase

Data in Table 4 indicated that, the tested compounds were increased the activity of acid phosphatase enzymes compared to untreated larvae. The mean values of acid phosphatase enzymes activities in the supernatant of the homogenate larvae reached to 0.037, 0.035, 0.049 and 0.035 mg/g b.w. when larvae were treated with LC_{50} by protecto, lannate, coumarin and azadirachtin, respectively compared with 0.034 mg/g b.w. of the untreated control. The percent differences between treatments 108.82, 102.94, 144.11 and 102.94 % more than control when larvae treated by protecto, lannate, coumarin and azadirachtin, respectively. Abdel-Hafez *et al.* (1993**)** studied the efficacy of two OP insecticides, two IGR's and their combined mixtures on laboratory of strain *S. littoralis*. Data showed that the larvae with the LC_{50} of the tested compounds caused variable reduction (much lower than control) in alkaline phosphatase.

In general, all tested compounds decreased the total soluble protein of the 4th larvae cotton leaf worm except the bio-insecticide, protecto. The carbohydrate enzymes were inhibited by protecto and methomyl. All tested compound reduced the activity of GPT, α - esterase and β - esterase**.** Alkaline phosphate was inhibited by all tested compounds while the activity of acid phosphates was increased. It was interested that the effect of tested compounds on the different enzymes was agreement with their effect on the mid gut membrane. The disturbances of the enzyme activities in the treated larvae due to the damage of mid gut membrane, particularly epithelial tissues.

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Biochemical and histopathological effects of *Bacillus thuringeinsis kurstaki* and methomyl insecticide on larval stage of the greater sugarcane borer, *Sesamia cretica* (Lederer)

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Abstract

The present study indicates the direct influence of *Bacillus thuringeinsis, kurstaki (Bt)* and methomyl on some physiological parameters of *Sesamia cretica* (Lederer). The activity of protease decreased in case of *Bt* compared with methomyl and control. Significant difference between *Bt*, methomyl and control treatment; values were 65, 105.33 and 106.67 O.D unit x 103, respectively. Amylase activity decreased in *Bt* and methomyl treatment compared to control, values were 401, 396 and 513 µg Glucose/g/min. *Bt* and methomyl caused significant decreases in invertase activity in treated larvae of *S. cretica* than control; values were 417.9, 402.5 and 590.9 Glucose/g/min. Trehalase activity decreased at *Bt*, methomyl and control; values were 418.13, 409.23 and 448.63 Glucose/g/min. α–Esterase and β–Esterase activity decreased in *Bt* and methomyl treatments compare to control with α–Esterase. The values were 52.37, 66.62 and 64.1 α-naphthol/g/min. and 10.26, 22.63 and 11.6 β- aphthol/g/min., respectively. Methomyl caused significant increase in the total proteins of *S. cretica* compared with the control, recording 3.07 and 1.8 mg/g and *Bt* caused 1.57 mg/g, respectively. Also, methomyl caused highly significant increase in the total carbohydrate level than control and *Bt*, the values were 3.18, 2.5 and 1.41 (mg/g) for methomyl, control and *Bt*, respectively. Methomyl caused increase in the total lipids; values were 1.6, 0.9 and 0.78 (mg/g) for methomyl, control and *Bt* of *S. cretica*, respectively.

The histopathological observations obtained from histological sections of midgut of *S. cretica* larvae treated as 2nd instar showed a progressive loss of epithelial cell definitions and considerable disorganization. In addition, the epithelial cells sloughed off into the midgut lumen, appearing as vesicle-like structures. Extensive cellular disintegration was also observed.

Key words: Biochemical, histopathological, *Sesamia cretica*, *Bacillus thuringeinsis kurstaki* and methomyl.

Introduction

There is a great demand to increase agricultural production in order to cover the increase in the human requirements. This leads to the increase in the use of chemical pesticides in order to decrease loses caused by different pests. These chemicals are considered as a main source of environmental pollution and cause danger to all organisms including humans. This massive and unwise use of chemical pesticides can also lead to development of resistant strains. It is importance to use alternative methods for the control of crop pests. The use of biocontrol agents, have the advantages of being pest selective, environment friendly, and selectively easily applicable. The bacterial infection induces several changes in the haemolymph contents of insects included *S. cretica*. Various investigations were made to study the histological and pathological changes, which occur in the insect tissues generally, and insect midgut in particular**.** Two bacterial pesticides (Dipel 2X and Xentari) on *S. cretica* are effective on total protein and haemolymph of *S. cretica* (Abd El-Kareem, 2007). The aim of this part of investigation is to conjugate the obtained data with the observed biological events of *S. cretica*. From the physiological parameter; the observation of the biological aspects can be well reasoning**.** The toxicants used were the entomopathogenic bacteria, *Bacillus thuringiensis* (Dipel 2X 6.4 % WP**)** and methomyl (Lannate 90 % SP).

Materials and methods

The present study was carried out to study the effect of *Bacillus thuringiensis* and methomyl insecticide against the greater sugarcane borer; *Sesamia cretica* (Lederer).

Tested compounds

Dipel 2x, 6.4% W.P.; a microbial bioinsecticide derived from the entomopathogenic bacteria *Bacillus thuringeinsis* subsp. *kurstaki*.
Methomyl (Lannate 90 % SP).

Biochemical Studies

The proteolytic activity was determined by the casein digestion method described by Ishaaya *et al.* (1971). The reaction mixture consisted of 0.2 ml (0.2 *M*) glycine buffer (pH 10), 0.4 ml 1.5% casein solution, and 0.2 ml enzyme solution. Enzymatic activity was terminated after 60 min. incubation at

37°C and adding 1.2 ml of 5 % trichloroacetic acid solution. The reaction mixture was centrifuged at 6000 rpm for 15 min. and the supernatant was taken for enzymatic activity evaluation. The proteolytic activity was determined as O.D. unit x 10^3 at an absorbancy of 280 nm.

The method was based on the digestion of trehalose and starch by trehalase and amylase, respectively, according to the method described by Ishaaya and Swiriski (1976). The free aldhydic group of glucose formed after trehalose, starch and/or sucrose digestion was determined using 3, 5 dinitrosalicylic acid reagent. The trehalase reaction mixture consisted of 0.2 ml 3% trehalose (substrate), 0.18 ml (0.2 *M*) acetate buffer (pH 5.4) and 20 µl of larval homogenate. The amylase reaction mixture consisted of 0.2 ml 2% starch (substrate), 0.16 ml (0.2 *M)* phosphate buffer (pH 6), and 20 µl of larval homogenate.

The invertase reaction mixture consisted of o.2 ml 4% sucrose (substrate), 0.18 ml (0.2 *M)* acetate buffer (pH 5.4) and 20 µl of larval homogenate. The dinitrosalicylic acid reagent was prepared by dissolving one gram of 3,5-dinitrosalicylic acid in 20 ml of 2*N* NaOH and 50 ml of distilled water with the aid of a magnetic stirrer. Potassium sodium tartarate (30 g) was added, and magnetic stirring was continued until a clear solution was obtained. The final volume to l00 ml was completed with distilled water.

All test tubes were incubated at 37 °C for exactly 60 minutes, and then 0.8 ml of 3, 5 dinitrosalicylic acid reagents was added. The reaction mixture was heated for 5 minutes at 100°C in a boiling water bath followed by immediate cooling in an ice bath. The optical density (OD) of the produced colour was measured at 550 nm using spectrophotometer. The enzymatic activity was expressed as µg glucose released/gram body weight/minute. Serial concentrations of glucose solution containing 50, 100, 150, 200 and 250 µg glucose /0.4 ml distilled water were pipetted into test tubes. 0.8 ml of dinitrosalicylic acid solution was added to each tube. The mixture was heated for 5 minutes at 100°C in boiling water bath, and then cooled immediately in an ice bath. The resulting colour was measured spectrophotometrically at 550 nm. The optical densities is plotted against concentrations, thus a curve could be constructed. The standard curve was blotted by O.D. (Optical Density) against concentration.

α- and β-esterases were determined according to the method of Van Asperen (1962) using α- naphthyl acetate and β- naphthyl acetate as substrates, respectively. Naphthol produced as a result of substrate hydrolysis could be measured by the addition of diazoblue sodium lauryl sulphate solution which produces a strong blue colour in case of naphthol or strong red colour in the case of α-naphthol or strong red colour in the case of β-naphthol. The colour was measured spectrophotometrically (by Milton Roy Spectronic model 1201 spectrophotometer). The reaction mixture consisted of 5 ml. substrate solution ($3x10^{-4}$ *M* α- or β- naphthyl acetate, 1 % acetone and 0.04 *M* phosphate buffer pH 7) and 20 µl of larval homogenate. The mixture was incubated for exactly 15 minutes at 27 °C then 1 ml of diazoblue color reagent (prepared by mixing 2 parts of 1% diazoblue B and 5 part of 5% sodium lauryl sulphate) was added. The developed color was read at 600 and 555 nm for α- and β- naphthol, respectively. The activity was expressed as µg α- or β- naphthol released /min./larva. Stock solutions were prepared by dissolving 20 mg α- or β-naphthol in 100 ml of 0.04 *M* phosphate buffer (pH 7). Ten milliliter of stock solution was diluted up to 100 ml by the phosphate buffer. Aliquots containing of 2.5, 5, 10, 20 and 40 µg of α- naphthol or 2.5, 5, 7.5, 10 and 20 µg β-naphthol were pippetted into test tubes and completed to 5 ml by phosphate buffer.One milliliter of diazoblue reagent was added and the developed color was measured as mentioned before. The standard curves of both α-E and β-E were blotted by O.D. against concentration.

Total proteins were determined by the method of Bradford (1976). Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95 % ethanol. To this solution, 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Sample solutions 50 µl were pipetted into a test tube and the volume was adjusted to 0.1 ml with phosphate buffer (pH 6.6). 5 ml of protein reagent were added to the test tube and the contents were mixed (inversion or vortexing). The absorbance at 595 nm was measured after 2 min. and before 1 hr against blank prepared from 0.1 ml of phosphate buffer (pH 6.6) and 5 ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in standard curve used to determine the protein in unknown samples.

For preparation of standard curve, serial concentrations of Bovine serum albumin solutions containing 10 to 100 µg of protein were pipetted into test tubes and the volume was adjusted to 0.1 ml with phosphate buffer (pH 6.6). Five ml of protein reagent were added and the resulting colour was measured spectrophotometrically at 595 nm. The optical densities is plotted against concentrations, thus a curve could be constructed. The standard curve was blotted by O.D. against concentration.

Total carbohydrates were determined according to Singh and Sinha (1977). Anthron reagent was prepared by adding 28 ml of H_2O to 72.0 ml concentrated H_2SO_4. While this mixture was still warm, 50 mg of anthron was added with vigorous shaking. Sample solution of 100µl was diluted to one ml with H_2O, then 5 ml anthron reagent. A blank containing 1.1ml of H_2O and 5 ml of anthron reagent

was placed. All tubes were placed in a boiling water bath for 10 min., then left to cool for 15 min. at room temperature. The absorbance was recorded at 620 nm.

For standard curve, from stock solution (250 mg of glucose in 100 ml of distilled water), serial concentrations of glucose containing 50, 100, 150, 200 and 250 µg glucose per ml water were prepared. To each, 5 ml of anthron reagent was placed. All tubes were placed in a boiling water bath for 10 min. Tubes were left for 15 min. to cool at room temperature, then O.D. reading were made at 620 nm**.**

Total lipids were estimated according to Knight *et al*. (1972) using phosphovanillin reagent. Pure vanillin (0.6 gm) was dissolved in 10 ml ethanol and completed to 100 ml with distilled water. 400 ml of concentrated phosphoric acid were added, and the solution was stored in dark glass bottle at room temperature. Sample solution of 250 µl was added to concentrated sulfuric acid (5 ml) in a test tube and heated in a boiling water bath for 10 min. After cooling to room temperature, the digest (500 µl) was added to phosphovanillin reagent (6.0 ml). After 45 min. incubation in dark, the developed colour was measured at (525 nm) against reagent blank prepared from 500 µl distilled water and 6.0 ml phosphovanillin reagent. The result is expressed as mg lipid / insect.

For standard curve, serial concentrations of oleic acid and palmitic acid mixture (7:3) from 1 to 5 mg/ml were prepared in absolute ethanol were used and treated in the same manner as the unknown. The standard curve was blotted by O.D. (Optical Density) against concentration**.**

Histopathological studies

Histopathological changes which occurred in larvae treated with the tested bacteria and insecticide were documented through histological preparation following treatment of *S. cretica* larvae with LC_{50} value of the tested bacterial compound and insecticide. This investigation was carried out on 2nd instar larvae after 2 and 7 days post treatment with *B. t* and after one day and 7 days post treatment with methomyl. Similarly, investigation on healthy untreated larvae of the same age was carried out and considered as a control. Larvae not responding to needle stimulus were selected. The insects were dissected in Ringer' s solution and the midgut was rapidly removed and submerged in the fixative. Alcoholic Boiun's solution was used as a fixative. After approximately 36 hours, the larval samples were removed from the fixative and washed in several changes of 70% ethyl alcohol until the disappearance of the fixative's yellow color. The samples were then dehydrated in a graded series of ascending ethyl alcohol and finally in two changes of absolute alcohol for nearly 3-4 hours. Samples were, then, transferred to an equal mixture of absolute alcohol and xylene for a couple of hours before being cleared with xylene overnight. Paraffin wax was gradually impregnated in the xylene and finally samples were removed and placed in melted paraffin in an oven set at 55-60ºC for 4 hours (the paraffin was changed twice). Midgut samples were then embedded in paraffin and cross section of the midgut was carried out by a microtome at 5µ thickness. Sections were mounted on glass slides and stained with Haematoxylene and counter stained in alcoholic Eosin and prepared for observation and photo microscopy.

Results and Discussion

Biochemical Studies

Carbohydrates, proteins, and lipids are very efficiently utilized by insects and most species derive the main part of their nourishment from these nutrients. The utilization of these nutrients depends on the digestive enzymes; protease, amylase, trehalase and invertase (Abdel-Khalek, 2007). The aim of this part of investigation was to conjugate obtained data with the observed biological events of *S. cretica*. From the physiological parameter; the observation of the biological aspects can be well reasoning.

Effect of *Bt* and methomyl on the biochemical parameter of *S. cretica*

The effect of *Bt* and methomyl on proteases activity of *S. cretica* was presented in Table (1). Data in this table revealed that the activity of protease values were 65.0, 105.3, 106.7 O.D unit x 103 at LC_{50} of *Bt*, LC_{50} of methomyl and control, respectively. Statistically, there was significant difference between *Bt* and control, while the difference between methomyl and control was nonsignificant.

Carbohydrates hydrolyzing enzymes

Amylase

The changes in amylase activity of *S. cretica* in the normal state and after treatment with LC_{50} of *B. t.* and LC_{50} of methomyl were given in Table (1). Amylase activity decreased in *Bt* and methomyl treatment compared with control, values were 401, 396, 513 µg Glucose/g/min**.** The differences between values of the two treatments and the untreated control were significant.

Invertase

The tested *Bt.* and methomyl caused a significant decrease in invertase activity in treated *S. cretica* larvae than the untreated ones; recording 417.9, 402.5 and 590.9 glucose/g/min. at LC_{50} of *Bt*, LC_{50} of methomyl and control respectively. Statistical analysis showed that there were significant differences between the two treatments and the untreated control Table (1).

Trehalase

Trehalase is a carbohydrate hydrolyzing enzyme in which hydrolyzes trehalase a non reducing disaccharide which is present in high concentration as the main sugar in insect's haemolymph. *S. cretica* trehalase activity values were 418.13, 409.23 and 448.63 Glucose/g/min. at LC_{50} of *Bt*, LC_{50} of methomyl and control respectively. Statistical analysis indicated significant differences between the two treatments and the untreated control Table (1).

Table 1. Effect of *Bt* and methomyl LC_{50}s on activity of Protease and Carbohydrates hydrolyzing enzymes in the haemolymph of *S. cretica* 2nd instar larvae.

Treatment	Protease units*	Amylase units**	Invertase units**	Trehalase units**
Bt	65 ± 2.31^{b}	401.17 ± 5.32^{b}	417.9 ± 1.36^{b}	418.13 ± 1.01^{b}
Methomyl	105.33 ± 4.10^{a}	396.10 ± 2.57^{b}	402.47 ± 0.50^{c}	409.23 ± 0.78^{c}
Control	106.67 ± 3.53^{a}	513.1 ± 4.59^{a}	590.9 ± 1.17^{a}	448.63 ± 1.57^{a}

Values with the same letter vertically are non-significantly different.
* 1 unit: (O.D unit x 10^3), ** 1 unit: (µg Glucose/g/min.), Non-specific esterases, ** 1 unit: (β - naphthol/g/min)
* 1 unit: (α - naphthol/g/min)

Esterase

The determined values of α – Esterase activity of *S. cretica* in the normal state and after LC_{50} of *Bt* and LC_{50} of methomyl treatments were given in Table (2). α – Esterase activity decreased in *Bt* and methomyl treatments while increased in control; values giving 52.37, 66.62, 64.1 (α - naphthol/g/min.). Statistical analysis showed significant differences between the two treatments and the untreated control.

Esterase

The tested *Bt* and methomyl caused significant decreases in β – Esterase activity in treated larvae of *S. cretica* than the untreated ones and their values were 10.26, 22.63 and 11.6 (β -naphthol /g/min.) at LC_{50} of *Bt*, LC_{50} of methomyl and control, respectively. Statistical analysis showed significant differences between treatments and the untreated control Table (2).

Table 2. Effect of *Bt* and methomyl LC_{50}s on activity of β - Esterase and α - Esterase in the haemolymph of *S. cretica* 2nd instar larvae.

Treatment	α – Esterase units*	β – Esterase units**
Bt	52.37 ± 0.25 c	10.26 ± 0.17 c
Methomyl	66.62 ± 0.31 a	22.63 ± 0.16 a
Control	64.1 ± 0.11 b	11.60 ± 0.17 b

Values with the same letter vertically are non-significantly different.

The main metabolic components

The main metabolites (total proteins, total carbohydrates and total lipids) are major biochemical components necessary for an organism to develop, grow and perform its vital activities (Abdel-khalek, 2007). Thus the present work was carried out to study the changes in the main metabolites after the treatment of *Bt* and methomyl.

Total proteins

Data in Table 3 show the changes in the total soluble proteins of *S. cretica* in the normal state and post treatment of *Bt* and methomyl. The values were 1.57, 3.07 and 1.8 (mg/g) in B.T., methomyl and control treatments, respectively.

Total carbohydrates

Table 3 shows the changes in the total soluble carbohydrates of *S. cretica* in the normal state and post treatment by *Bt* and methomyl; values were 1.41, 3.18 and 2.5 (mg/g).

Total lipids

Changes in the total soluble lipids of *S. cretica* in the normal state and post treatment of *Bt* and methomyl are shown in Table 3 ; values were 0.9, 1.6 and 0.87 (mg/g), respectively.

Table 3. Changes in main haemolymph components of *S. cretica* after treatment with LC_{50} of *Bt* and methomyl.

Treatment	Total soluble protein (mg/g)	Total soluble carbohydrates (mg/g)	Total soluble lipids (mg/g)
Bt	1.57 ± 0.03^{b}	1.41 ± 0.02^{c}	0.9 ± 0.05^{b}
Methomyl	3.07 ± 0.03^{a}	3.18 ± 0.01^{a}	$1.60 \pm .03^{a}$
Control	1.80 ± 0.09^{b}	2.5 ± 0.04^{b}	0.78 ± 0.02^{b}

Values with the same letter vertically are non-significantly different

.

The results showed that *S. cretica* 2nd instar larval treatment with the LC_{50} of Dipel 2X affected some physiological aspects. Obtained results revealed that the activity of protease decreased in case of *B. t.* compared with control, protease values were 65 and 106.67 O.D. units$X10^3$, respectively. Amylase activity decreased, also, in *Bt* treatment compared with control; values were 401 and 513 µg Glucose/g/min, respectively**.**

A reduction, also, appears in Invertase and Trehalase activity in treated larvae of *S. cretica* than the untreated ones. α–Esterase and β –Esterase activity decreased in *Bt* treatment while increased in control; values were 52.37 and 64.1 (α -naphthol/g/min.), respectively. While, β–Esterase activity values were 10.26 and 11.6 (β -naphthol /g/min.). A reduction occurred in total proteins and total carbohydrates in case of *Bt* compared with control. While, total lipids increased in control than *Bt* treatment. Total protein values were 1.57 and 1.8 (mg/g) and total carbohydrates values were 1.41 and 2.5 (mg/g) for *Bt* and control, respectively. While, total lipid values were 0.9 and 0.78 (mg/g) for *B t.* and control, respectively.

The results of *S. cretica* haemolymph enzymes after treatments with *Bt* are in harmony with those obtained by El-Ghar *et al.* (1995) who determined the effect of Abamectin and *B. thuringiensis* (β-exotoxin of *B. thuringiensis*) on the main digestive enzymes and non-specific esterases in larvae of *Spodoptera littoralis*. The results of the main metabolic components agreed with those obtained by Hoffmann (1980) who found that the injection of the live cells of *Bt* (500 cells/insect) into the haemolymph of the last larval instar and young male of *Locusta migratoria*, insignificantly, affected the total haemolymph content of lipids. Kamel *et al*, (2010) studied the effect of commercial formulations of *B. thuringiensis* subspecies *kurstaki* "Agerin, Dipel 2X and Dipel DF" on total protein and lipid contents on *Spodoptera littoralis*. The results showed significant differences in protein contents between control and treated larvae after 48 hrs of exposure to different commercial formulations of *Bt*. While Dipel 2X showed no significant difference in lipid content between control and treated larvae after 48 and 120 hrs of exposure.

The present results disagreed with El-Kattan (1995) who studied the effect of *B. thuringiensis* on the total protein and lipid contents in the haemolymph of *Plodia interpunctella* (Hb.) larvae after injection with LC_{50} concentration. Infection produced a gradual decrease in protein content of infected larvae at 12 and 18 hr intervals following injection. Zidan *et al*., (1996) found also that *B. thuringiensis* caused a significant reduction in protein content of the *Spodoptera littoralis* larvae. Also, Abd El-Aziz (2000) found reduction in the lipid content after treating *Spodoptera littoralis* larvae with Dipel 2X (*B. thuringiensis*). El-Hefny (2006) assayed two bacterial pesticides (Dipel 2X& Xentari) on *Sesamia cretica*. All treatments were effective on total protein in *S. cretica* haemolymph as the lowest total protein was indicator to the highest efficacy among the different treatments which were remarked on Dipel [I] + *Steinernema carporcapsae* LC_{50} (231.51 microgram/ ml) followed by Dipel [I] + *S. riobravae* LC_{50} (239.33 microgram/ml), respectively. Abd-El Aziz *et al.* (2011) studied the effect of commercial formulations of *Bacillus thuringiensis kurstaki* "Agerin, Dipel 2X and Dipel DF" for control of *Spodoptera littoralis* in cotton fields because it was described as cheap and readily available in Egyptian market.

Ezzeldin *et al.* (2009) studied the effect of chemical insecticide (methomyl), Agerin (microbial insecticide of *Bt*) petroleum oil (Sisi 6), and inorganic salt (barium nitrate) on greater sugar cane borer, *Sesamia cretica* L., under field conditions. Their results indicated that methomyl was the most active material followed by Agrein in controlling the pest and resulted increased yield of all maize and sorghum varieties. The results showed also that the infection of *S. cretica,* treated with the LC_{50} of methomyl affected some physiological aspects of larvae treated as 2nd larval instar. Obtained results revealed that there was no significant difference between Methomyl and control. The protease value for methomyl treatment was 105. O.D. unit x 10^3. Amylase activity decreased in methomyl treatment while increased in control; values were 396 and 513 µg Glucose/g/min, respectively**.** A reduction, also, appeared in Invertase and Trehalase activity in treated larvae of *S. cretica* than the untreated ones. α – Esterase and β – Esterase activity increased in methomyl treatment while increased in control, values were 66.62and 64.1 (α -naphthol/g/min.), resp., while β – Esterase activity values were 22.63 and 11.6 (β -naphthol /g/min.).

An incensement occurs in total proteins, total carbohydrates and total lipids in case of methomyl compared with control. Total protein values were 3.07 and 1.8 (mg/g) and total carbohydrate values were 3.18 and 2.5 (mg/g) in methomyl treatment and control, respectively. While, total lipid values were 1.6 and 0.78 (mg/g) for methomyl and control, respectively. The results of *S. cretica* haemolymph enzymes after treatments with methomyl agreed with Naveed *et al.* (2009) who investigated the effect of different insecticides such as endosulfan, monocrotophos, methomyl, phosphomidon and carbaryl on invertase activity at different stages of pentatomid bug *Cyclopelta siccifolia* W. with lethal concentrations of respective insecticides. The data revealed that maximum decrease in Invertase activity was detected with (45%) at 24 hours.

Histological studies

The histological structure of the midgut from untreated *S. cretica* larvae show that it is composed of closely arranged granulated columnar epithelial

cells with a rather broad apex, bearing an apical microvillus border Figure 1.

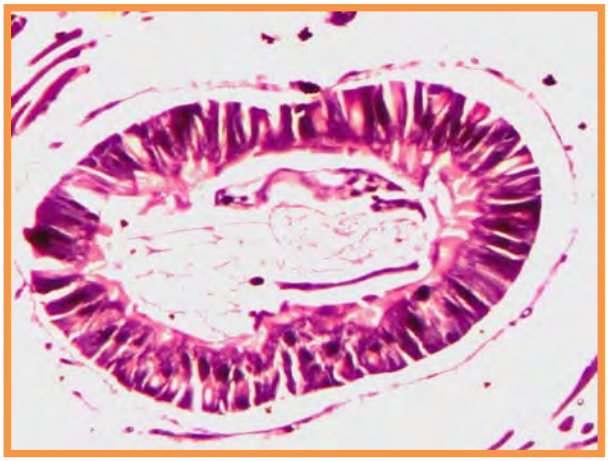

Fig. 1. Transverse section in midgut of untreated 2nd instar larva of S. *cretica* [4 X].

The same figure also, observed small goblet cells and both types of cells rest on a basement membrane. The epithelial cells are shielded from the midgut lumen content by the pertrophic membrane. There is a simple developed muscular layer, encircling the epithelial layer **Figure 2** followed by a peritoneal membrane.

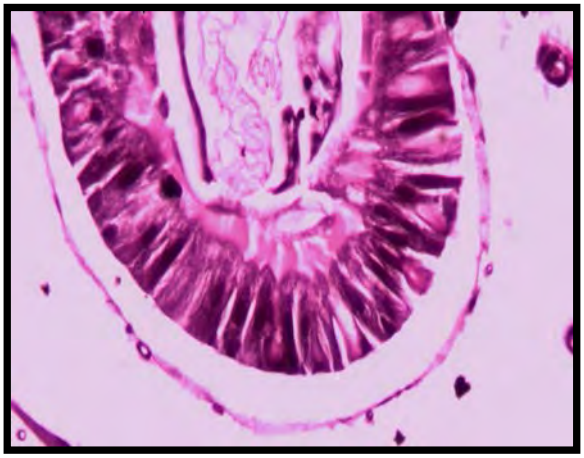

Fig. 2. Part of transverse section in midgut of untreated 2nd instar larva of *S. cretica* [40 X].

Following treatment of larvae with LC_{50} values of methomyl and *B.t*, histological changes were observed. The midgut sections, prepared 2 days post treatment in case *B.t* showed scattered vacuoles within the epithelium cells and some affinity to the dye **(Figure 3).**

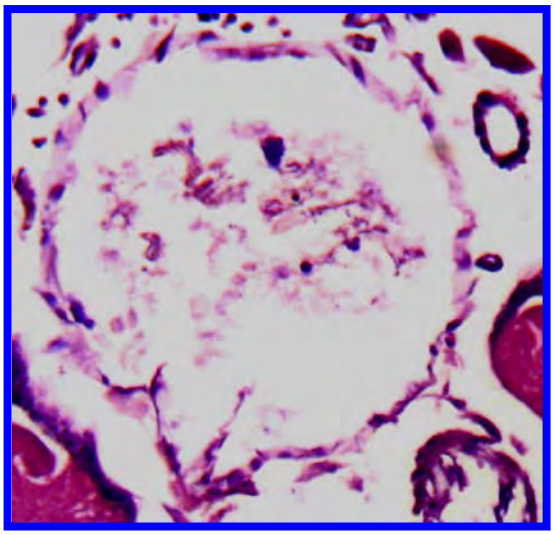

Fig. 3. Transverse section in the midgut of untreated 2nd instar larva of *S. cretica* after 2 days post infection with LC_{50} value of *B.t* [4 X].

The distal ends of some columnar epithelial cells become somewhat distended with disrupted microvilli, while in other areas the cells remain intact**.** The differentiation of the goblet cells was rather difficult. The peritrophic membrane appears impaired and appears stretched or broken down in many places. When histological studies were conducted at a later time, i.e. seven days post infection, degeneration of the midgut epithelial cells becomes more apparent as the cells completely loose their columnar structure (Figure 4).

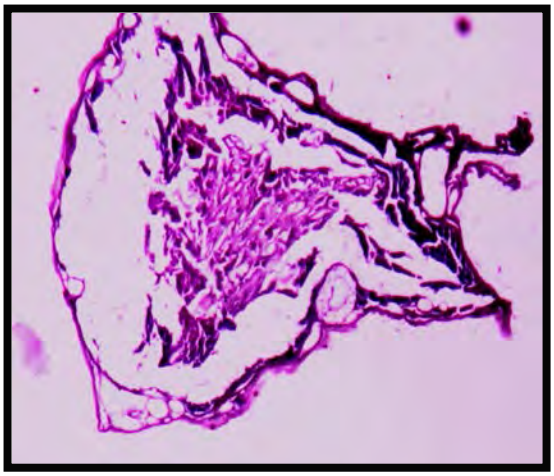

Fig. 4. Transverse section in the midgut of untreated 2nd instar larva of *S. cretica* after seven days post infection with LC_{50} value of *Bt* [4 X].

Only remnants of the peritrophic membrane could sometimes be observed and nearly complete detachment of the epithelial cells from the broken basement membrane occurred.

The histopathological observations obtained from histological sections of midgut of *Sesamia cretica* larvae treated as 2nd instar show a progressive loss of epithelial cell definitions and considerable disorganization (Fig. 5). In addition, the epithelial cells sloughed off into the midgut lumens, appearing as vesicle-like structures. Extensive cellular disintegration was also observed (Fig. 6). These results agree with those obtained when other insects were treated with *Bacillus thuringeinsis* (Abo El-Ghar *et al.*, 1994 ; Zidan *et al.*, 1998 ; Quesada and Santiago 2001; El-Hefny, 2006 and Abd El-Kareem, 2007).

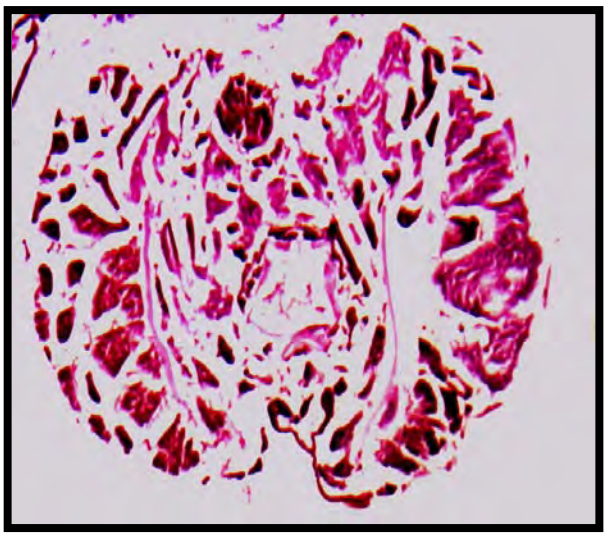

Fig. 5. Transverse section in the midgut of untreated 2nd instar larvae of *S. cretica* 24 hrs. post treatment with LC_{50} value of methomyl [4 X].

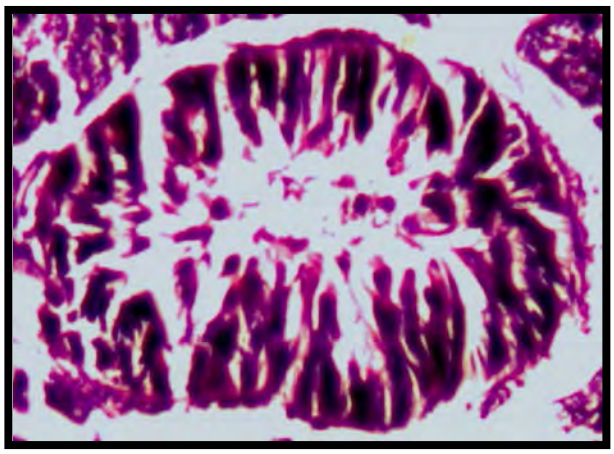

Fig. 6. Transverse section in the midgut of untreated 2nd instar larvae of *S. cretica* 7 days post treatment with LC_{50} value of methomyl [4 X].

Conclusion

Biochemical and histological studies in the present investigation indicated the direct influence of *Bt* and methomyl on larval stage of *Sesamia cretica*. Previous results revealed that using entomopathogenic bacterium could give some promising results. Application of entomopathogenic bacterium proved to have not only direct lethal effect but also a latent effect which appeared in different stages following the larval stage; i.e. pupal and adult stage. This fact can help in the future to minimize *S. cretica* numbers in sugarcane and corn fields.

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Joint action of novel insecticides in binary mixtures with conventional, growth regulator, and biorational ones against *Sopdoptera littoralis* (Boisd.) larvae

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Abstract

In a lab study, the interaction effects between binary mixtures of each of two recently developed insecticides, Pyridalyl and Spinetoram, were studied at different mixing ratios with eight compounds: three conventional insecticides (Chlorpyrifos-E, Methomyl, and Deltamethrin); two insect growth regulators(IGRs) (Hexaflumuron and Pyriproxyfen); and three biorational compounds (Pepronyl butoxide, Sesame oil and Olic acid). This was to investigate the joint actions of these binary mixtures to control cotton (*Gossypium barbadense* L.) leaf worm [S. *littoralis* (bisd.)] 4th instar larvae. Based on the co-toxicity coefficient, both insecticides --pyridalyl and spinetoram-- positively responded when each one was mixed with Chlorpyrifos-E and exhibited remarkable synergistic action at most tested mixing ratios within 24 and 48 hr post applications. On the other hand, slight synergism was only recorded for Pyridalyl-pyriproxyfen mixtures at 9:1 and 4:1, while similar trend was also recorded at 9:1, 4:1 and 1:1 of Pyridalyl-Hexaflumuron mixtures whereas no synergism was almost recorded for Spinetoram-IGRs mixtures. In contrast, the three biorational compounds (pbo, sesame oil and olic acid) exhibited highly considerable synergism at all tested mixing ratios with Pyridalyl, while sesame oil and olic acid evoked remarkable synergism only in their binary mixtures with Spinetoram.

Key words*:* Pyridalyl, Spinetoram, joint action, cotton leaf worm.

Introduction

The Egyptian cotton leaf worm *Spodoptera littoralis* (Boisd.) is one of the most destructive phytophagous insect pests in Egypt, not only to cotton but also to other field crops and vegetables (Kandil *et al.*, 2003). These caterpillars are major polyphagous key pests in Egypt, causing serious and considerable economic losses in both greenhouses and open fields. During the last few decades, Egypt has suffered from the onset of resistance in cotton leaf worm for the most conventional insecticides (Maher, 1975; Yehia *et al.*, 1985; El-Bermawy *et al.*, 1991&92; and Rashwan *et al*,. 1991&92). To overcome development of resistance, several studies have been made for investigating new and non-traditional control agents or/and approaches effective against this pest. One of the new non-traditional control compounds is Spinosyns, a novel family of insecticidal macrocyclic lactones which are active on wide variety of pests, especially lepidopterans and dipterans. Spinosad (Tracer, Success) belongs to this family and possess a mode of action (Salgado *et al.*, 1997) that appears unique with studies suggesting that nicotinic and gamma-aminobutic acid receptor functions are altered in a novel manner (Salgado and Saar, 2004). Another Spinosyns, Spinetoram (Radient) is a recently developed and belong to IRAC mode of action class 5 (IRAC, 2009). Insecticides in this class primarily activate the neonicotinic acetylcholine receptors by acting on a unique site (Salgado et al., 1998; Salgado, 1998). In Egypt, Temerak (2007) used spinosyn products, Spinosad (Spintor 24 % SC) and Spinetoram (Radiant 12 % SC) to combat egg masses of the cotton leaf worm, and he indicated that Radiant 12 % SC was 5 and 7 time stronger than Spintor 24 % SC in field and laboratory, respectively. Another new and non-traditional compound is Pyridalyl, a novel insecticide that has a phenoxy-pyridaloxy derivative structure introduced in 2002 by Sumitomo (Sakamoto *et a*l., 2004). The compound was reported effective generally on the pests of order Lepidoptera and Thysanoptera on cotton and vegetables. The compound was effective in particular against populations of tobacco budworm, *Heliothis virescens*, cotton bollworm *Helicoverpa zea* (Johnson *et al*., 2000) and *Plutella xylostella* (Umeda and Strickland, 1999) which are resistant to various currently used insecticides. Searching for a new approach for use of each of these new compounds to be standing by for the onset of resistance problem in future had led to investigating them in mixtures to improve their performance as effective control measures. Early studies of synergism (Busvine, 1970) indicated that mixing of chemicals of different mode of action can led to more potent use of toxicants which could theoretically prevent or delay the emergence of resistant strains. In this respect, it was recorded with organophosphates/synergizes Pyrethroids against several pests, i.e. *S. littoralis* (Ascher *et al.*, 1986), *Heliothis armigera* (Martin *et al.*, 2003). Also, synergism was obtained by combinations of IGRs and traditional insecticides (El-Guindy *et al.*, 1985; Radwan *et al.*, 1985; Radwan *et al*., 1990; Kandil et al., 2006). Though, the present study aimed to

characterize the interaction between each of Pyridalyl and Spinetoram when combined with three traditional insecticides, two IGRs, and three biorational compounds when tested against the 4th instar larvae of the cotton leaf worm *S. littoralis* (Boisd.).

Materials and methods

Tested insects

The cotton leaf worms, *Spodoptera littoralis* (Boisd.) used in the present study has been reared on cultures in a lab out of contamination with any insecticide for more than two years and was initiated from egg masses supplied from the Central Laboratory of Pesticides, Ministry of Agriculture, Dokki, Giza. All stages of *Spodoptera littoralis* were reared and tested at 27 ± 2 ºC and 70 ± 5 % RH (El-Defrawi *et al.* 1964).

Insecticides

Three groups of formulated insecticides were chosen for investigation both alone and in mixtures. These insecticides include i) Newly developed insecticides, Pyridalyl (2,6-dichloro-4-(3,3-dichloro-2-allyloxy)phenyl-3-[5-trifluoromethyl)-2-pyridyloxy]propyl ether) (S1812 50 % EC), and Spinetoram(hexadecahydro-14-methyl-1H-as-indaceno[3,2-d]oxacyclododecin-7,15-dione) (Radiant 12 % SC); ii) conventional insecticides: Deltamethrin ((*S*)-α-cyano-3-phenoxybenzyl(1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) (Decis 2.5 % EC) and Methomyl (*S*-methyl *N*-(methylcarbamoyloxy)thioacetimidate) (Lannate 90 % SP), andChlorpyrifos-Ethyl(*O*,*O*-diethyl*O*-3,5,6-trichloro pyridin-2-yl phosphorothioate) (Dursban 48% EC)

iii) Insect growth regulators: Hexaflumuron(1-[3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl]-3-(2,6-difluorobenzoyl)urea) (Consult 10%) and Pyriproxyfen (4-phenoxyphenyl(RS)-2-(2-pyridyloxy)propylether 2-[1-(4-phenoxyphenoxy)propan-2-yloxy]pyridine) (Admiral 10%EC); and iv)Bio-Rational:PepronylButoxide (5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole)(Pbo), Sesame oil and Olic acid.

Bioassay

For assessing the toxicity of each insecticide either separately or in binary mixtures, stock solution of each insec

ticide alone or mixture of two components were prepared fresh daily on the basis of active ingredient (W/V). The tested mixing ratios of each mixture were 9:1, 4:1, 1:1, 1:4 and 1:9 on the basis of active ingredient for both components. Stock solutions of either separate insecticide or mixture were serially diluted to 5-7 concentrations, resulting in 10-90 % mortality which were used to calculated LC_{50} values. Castor bean leaves were dipped in either water (control) or in one of the prepared insecticide(s) dilutions for 20 seconds and were air dried under room temperature. Larvae were placed in glass jars (1 1b) and offered a control or treated leaves for 24 h and then were replaced by untreated fresh leaves at the 2nd day post treatment. Five replicates, each of ten 4th instar larvae were used. Percentage mortality was recorded after 24 and 48 h and corrected according to Abbott formula (Abbott, 1925). To calculate LC_{50} values for either mixtures or their components separately, probit analysis (Finney, 1971) was adopted using the corrected mortality percentages. The co-toxicity coefficient based on LC_{50} values was calculated according to the method of Sun and Johnson (1960), using toxicity index (Sun, 1950).

Table 1. The LC_{50} and the co toxicity coefficient (CTC) of different conventional insecticides separately and in binary mixtures with pyridalyl against the 4th instar larvae of *S. littoralis* (Boisd.).

Ratio	Toxicity Indicator					
	Methomyl		Deltamethrin		Chlopyrifos –e	
	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC
			post 24 h			
1:0	275.03	—	275.03	—	275.03	—
9:1	47.65	167.04	337.92	82.13	163.50	121.68
4:1	54.16	85.96	526.08	53.23	38.39	405.82
1:1	153.96	13.45	633.92	45.43	1.68	5620.05
1:4	165.49	8.06	763.55	38.80	95.54	70.89
1:9	65.01	18.33	833.90	35.87	6.82	907.60
0:1	10.77	—	302.09	—	56.99	—
			post 48 h			
1:0	14.91	—	14.91	—	14.91	—
9:1	2.10	576.31	14.30	114.53	14.18	105.84
4:1	15.01	67.84	28.87	62.93	10.10	149.56
1:1	20.93	32.97	19.66	137.35	0.017	90661.44
1:4	29.23	17.86	127.83	41.14	19.70	79.86
1:9	22.39	21.56	131.39	58.57	0.135	11733.18
0:1	4.49	—	143.03	—	15.95	—

Results and discussion

The joint action data (Table 1) illustrate that pyridalyl in combinations with methomyl and deltamethrin for most of the tested mixing ratios generally showed an antagonistic action based on cotoxicity coefficient calculated on the basis of the LC_{50} values at 24 and 48 h post treatment.

However, pyridalyl + methomyl (ratio 9:1) exhibited moderate synergistic action reached 167.04 and 576.31 after 24 and 48 h, respectively and pyridalyl + deltamethrin (9:1and1:1) only after48h.

On the other hand, pyridalyl + chlorpyrifos-ethyl combinations evoked remarkable synergistic action at all mixing ratios at 24 and 48 h post treatment except at mixing ratio of 1:4, recording co-toxicity coefficient ranged between 121.68 and 5620.05, 105.84 and 90661.44 at 24 and 48 h post treatment, respectively. As for combinations of spinetoram with methomyl, deltamethrin and chlorpyrifos-e when tested at the same mixing ratios (Table 2), spinetoram revealed synergistic action at 24 h only when combined with deltamethrin at 1:9 and chlorpyrifos at 4:1, 1:1 and 1:9, resulting in co toxicity coefficient values of 147.42 for deltamethrin and 265.41, 113.32 and 965.56 for chlorpyrifos at the prementioned mixing ratios, respectively. Regarding the degree of synergism at 48 h (Table 2), it was evident that spinetoram revealed moderate synergistic action when combined with deltamethrin and chlorpyrifos-e recording co toxicity coefficient ranged between 101.34-347.93 and 119.87-2156.91 for spinetoram+deltamethrin and spinetoram+chlorpyrifos-e, respectively.

Table 2. The LC_{50} and co toxicity coefficient (CTC) of different conventional insecticides separately and in binary mixtures with spinetoram against the 4th instar larvae of *S. littoralis* (Boisd.).

Ratio	Toxicity					
	Methomyl		Deltamethrin		Chlopyrifos –E	
	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC
			post 24 h			
1:0	344.75	——	344.75	——	344.75	——
9:1	292.17	28.81	525.43	64.69	485.30	47.19
4:1	246.17	19.47	533.49	62.85	64.64	265.41
1:1	20.99	99.52	551.43	58.39	86.31	113.32
1:4	26.41	50.58	2512.71	12.32	349.54	19.57
1:9	100.66	11.85	207.50	147.42	6.44	965.56
0:1	10.77	——	302.09	——	56.99	——
			post 48 h			
1:0	55.20	——	55.02	——	55.02	——
9:1	14.62	177.10	121.66	101.34	36.87	119.87
4:1	52.65	25.80	73.96	146.49	11.82	312.43
1:1	0.905	917.41	50.90	156.11	11.32	218.45
1:4	22.15	24.83	96.51	65.01	11.82	157.27
1:9	34.70	14.25	16.85	347.93	0.796	2156.91
0:1	4.49	——	143.03	——	15.95	——

On the other hand, spinetoram+methomyl exhibited synergistic action only at two mixing ratios 9:1 and 1:1 recording co toxicity coefficient 177.10 and 917.41, respectively. Considering the response of the 4th instar larvae to different tested mixtures of pyridalyl and to insect growth regulators (Table 3), it was obvious that pyridalyl exhibited synergistic action when combined with hexaflumuron at 9:1, 4:1 and 1:1 while when mixed with pyriproxyfen at 9:1 and 4:1, at 24 h post treatment, thid resulted in co toxicity coefficient values of 264.89, 180.77 and 194.74 for the former and 165.66, 119.68 for the later, respectively. Regarding the degree of synergism at 48h it was obvious that pyridalyl revealed synergistic action when combined with hexaflumuronat (9:1 and 1:4) and with pyriproxyfen at (9:1 and 4:1), recording co toxicity coefficient (152.03 and 464.09) and (524.15 and 386.53), respectively. As for combinations of spinetoram with the two insect growth regulators when tested at the same mixing ratios (Table 4), spinetoram exhibited antagonistic action at all mixing ratios at 24 h post treatment except at 1:1 mixing ratio with hexaflumuron which revealed synergistic action. On the contrary, at 48 h spinetoram-hexaflumuron exhibited remarkably considerable synergistic action at all mixing ratios studied and recording co toxicity coefficient ranged between 108.20 to 252.78, while spinetoram-pyriproxyfen exhibited synergistic action at only 9:1, 4:1 and 1:1 mixing ratios, recording co toxicity coefficient of 249.97, 218.21 and 114.81, respectively. The response of the 4th instar larvae when exposed to pyridalyl binary mixtures of Pbo, sesame oil and olic acid are shown

in Table 5. The data of co toxicity coefficient values indicated highly remarkable synergistic action for all tested mixtures of pyridalyl-sesame oil and pyridaly-olic acid recording co toxicity coefficient ranged between 367.63 to 10745.84 and from 598.29 to 1736.73, respectively. Meanwhile, pyridalyl-pbo revealed synergistic action at 99:1and 90:10 mixing ratios recording co toxicity coefficient 338.08 and 650.31 at 24 h post treatment, respectively. The results after 48 h post treatment revealed a remarkable increase in synergistic action for pyridalyl-pbo, pyridalyl-sesame oil and pyridalyl-olic acid at all mixing ratios recording co toxicity ranged between 233.97 to 25010.06, 167.36 to 2258.03 and 1428.46 to 9958.82, respectively. As for combinations of spinetoram with biorational compounds (Pbo, Sesame oil and Olic acid) when tested at the same mixing ratios (Table 6), it was obvious that spinetoram revealed its synergistic action at 24 h when combined with sesame oil and olic acid at all mixing ratios recording co toxicity coefficient ranged between 232.59 to 252.17 and 122.82 to 749.65, respectively. On the other hand, spinetoram-pbo exhibited antagonistic action at 99:1 and 95:5 mixing ratio while slight synergistic was recorded at 90:10 mixing ratio. Following the response of the 4th instar larvae to different tested mixtures at 48 h post treatment, it was clear that high increase in synergistic action was achieved for spinetoram/olic acid at all mixing ratios recording co toxicity coefficient ranged between 505.79 to 36845.81. On the other hand combinations of spinetoram/pbo exhibited synergistic action at 95/5 and 90/10; spinetoram/sesame oil at 99:1 and 90:10 mixing ratios, recording co toxicity coefficient (1405.21 and 107.44) and (153.35 and 118.29), respectively. In conclusion it is obvious that both new compounds pyridalyl and spinetoram when mixed with chlorpyrifos in particular, exhibited considerably high potentials at all tested mixing ratios. Similar findings were achieved by Attique *et al.* (2006) when investigated the synergism between pyrethroids, organophosphates and new insecticide against diamondback moth *Plutella xylostella.* The toxicity of chlorpyrifos in combination with spinosad and emamectin against the field strain increased significantly. In the same study, the toxicity of pyrethroid bifenthrin against the field population increase significantly ($p<0.01$) when combined with spinosad or/and emamectin. Also, our data are in agreement with Abdallah and Kandil (1985) where they found that chlorpyrifos acts as potentiator for several insecticides belonging to different groups including antimoulting compounds (IGRs). As for spinetoram it was obvious that slight synergistic action was achieved when mixed with hexaflumuron at 1:1 mixing ratio after 24 h and that synergistic action increased remarkably at most of mixing ratios of spinetoram-hexaflumuron and spinetoram-pyriproxyfen after 48h, post treatment. This agree with Abdel Rahman and Abou-Taleb (2007) who found that the spinosad or spinetoram at LC_{25}/IGR compounds at LC_{25} mixtures revealed potentiating effect higher than the mixtures of spinosad or spinetoram at LC_{25}/IGR compound at $LC_{10.}$ Although the toxicity of tested IGR compounds appeared after 48 and 72 hrs of exposure, the potentiating effect of these compounds in mixtures with spinosad and spinetoram appeared after 24 hrs. Generally, based on their interaction it is preferred to use high concentrations of spinosad or spinetoram with low concentration of the IGR compounds and not the opposite.

Table 3. The LC_{50} and co toxicity coefficient (CTC) of different insect growth regulators separately and in binary mixtures with pyridalyl against the 4th instar larvae of *S. littoralis* (Boisd.).

Ratio	Toxicity			
	Hexaflumuron		Pyriproxyfen	
	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC
			post 24 h	
1:0	275.03	—	275.03	—
9:1	115.11	264.89	184.36	165.66
4:1	189.23	180.77	286.96	119.63
1:1	276.98	194.74	928.93	58.90
1:4	3154.36	40.41	1775.62	75.82
1:9	24950.83	9.34	3262.28	80.36
0:1	13906.60	—	50523.57	—
			post 48 h	
1:0	14.91	—	14.91	—
9:1	10.88	152.03	3.16	524.15
4:1	52.83	35.16	4.82	386.53
1:1	34.52	85.22	131.34	22.66
1:4	15.24	464.09	363.97	20.35
1:9	165.94	80.12	268.28	54.83
0:1	1107.03	—	9071.99	—

Also, Abdel-Hafez and Mohamed (2009) found that the interaction between spinetoram and chlorfluazuron or fenopropathrin revealed a potential effect in all treatments at different concentration of binary mixtures, except the mixture of spinetoram at (LC_{50}) with fenopropathrin (LC_{10}) which produced an additive effect (+19.1). The observed mortality caused by both agents together was higher than with each agent alone. The most promising synergism occurred when LC_{25} of spinetoram was combined with LC_{25} of fenopropathrin (+118.7), followed by chlorfluazuron (+77.91).

Table 4. The LC_{50} and cotoxicity coefficient (CTC) of different insect growth regulators separately and in binary mixtures with spinetoram against the 4th instar larvae of *S. littoralis* (Boisd.).

Ratio	Toxicity data for spinetoram in mixtures with the indicated insecticide			
	Hexaflumuron		Pyriproxyfen	
	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC
		post 24 h		
1:0	344.75	—	344.75	—
9:1	390.70	97.78	571.51	66.97
4:1	1256.79	34.07	470.16	91.50
1:1	332.41	202.40	941.57	72.73
1:4	8527.56	18.38	20429.00	8.23
1:9	8173.22	34.51	8173.22	39.77
0:1	13906.60	—	50523.57	—
		post 48 h		
1:0	55.02	—	55.02	—
9:1	24.05	252.78	24.44	249.97
4:1	62.79	108.20	31.47	218.21
1:1	77.80	134.73	95.45	114.81
1:4	129.02	177.81	3462.83	7.76
1:9	162.86	233.45	2061.19	25.31
0:1	1107.03	—	9071.99	—

Table 5. The LC_{50} and cotoxicity coefficient (CTC) of different biorational insecticides separately and in binary mixtures with pyridalyl against the 4th instar larvae of *S. littoralis* (Boisd.).

Ratio	Toxicity					
	Pbo		Sesame oil		Olic acid	
	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC
			24 h post treatment			
1:0	5917.07	—	23184.72	—	1571.44	—
99:1	82.13	338.08	75.56	367.63	46.35	598.29
95:5	328.90	87.81	31.13	929.40	25.14	1140.00
90:10	46.75	650.31	2.84	10745.84	17.26	1736.73
0:1	275.03	—	275.03	—	275.03	—
			48 h post treatment			
1:0	41.03	—	34.41	—	98.50	—
99:1	0.06	25010.06	8.96	167.36	0.151	9958.82
95:5	1.94	793.80	4.13	371.53	1.09	1428.46
90:10	7.11	233.97	0.70	2258.03	0.34	4792.14
0:1	14.91	—	14.91	—	14.91	—

Table 6. The LC_{50} and cotoxicity coefficient CTC) of different biorational insecticides separately and in binary mixtures with spinetoram against the 4th instar larvae of *S. littoralis* (Boisd.)

Ratio	Toxicity					
	Pbo		Sesame oil		Olic acid	
	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC	LC_{50} (ug l^{-1})	CTC
				post 24 h		
1:0	5917.07	——	23184.77	——	1571.44	——
99:1	2349.11	14.82	146.58	237.55	46.35	749.65
95:5	1745.45	20.73	155.91	232.59	219.57	163.38
90:10	356.57	106.75	151.65	252.17	304.49	122.82
0:1	344.75	——	344.75	——	344.75	——
				Post 48 h		
1:0	41.03	——	34.41	——	98.50	——
99:1	55.32	99.13	35.67	153.35	0.15	36845.81
95:5	3.85	1405.21	69.72	76.62	2.97	1894.40
90:10	49.52	107.44	43.88	118.29	11.38	505.79
0:1	55.02	——	55.02	——	55.02	——

Laboratory studies suggest that the use of spinetoram in combinations with either chlorfluazuron or fenopropathrin at low doses may reduce the rate of the used insecticides. Generally the present data suggests that the use of pyridalyl or/ and spinetoram in binary mixtures with conventional insecticides particularly chlorpyrifos, sesame oil and olic acid may improve the performance and thus inhibit resistance development in *S. littoralis*, in addition to reduce the rate of used insecticides and consequently reduce the costs as well as reduce environmental pollution.

Conclusion

The bio rational compounds (pbo, sesame oil and olic acid) exhibited highly considerable synergism at all mixing ratios with pyridalyl while sesame oil and olic acid exhibited remarkable synergism only in their binary mixture with spinetoram , whereas both insecticides (spinetoram and pyridalyl) evoked no considerable synergistic action with conventional insecticides and IGRs.

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Prospecting of antagonistic microbe formulations and plant extracts in management of *Meloidogyne incognita* infecting sugar beet, *Beta vulgaris* L.

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Abstract

This experiment was carried out under greenhouse conditions at the laboratories of the City of Scientific Research and Technology Applications, Alexandria, Egypt to evaluate two antagonistic microbe formulations and two plant extracts in the management of root knot nematode, *Meloidogyne incognita* infecting sugar beet, *Beta vulgaris* L. The tested biocontrol agents were: *Serratia marcescens* , *Trichoderma harzianum*, Nemastop and neem oil. Results indicated that the biocontrol agent of the fungus, *T. harzianum* gave the highest increases in the vegetative characters of sugarbeet plants i.e. root diameter, root scale, root length, total weight root, total foliage weight, and total weight plant. Moreover, results indicated that the bacterium biocide, *Serratia marcescens* gave the highest reduction percentages of root gall numbers and egg mass numbers, followed by the fungus biocide, *Trichoderma harzianum*, and neem oil extract.

Key words: Biotechnology, *Meloidogyne incognita*, *Serratia marcescens*, *Trichoderma harzianum*, garlic, neem, sugarbeet.

Introduction

Sugarbeet, *Beta vulgaris* Saccarifera L. is considered one of the most important crops that rank next to sugarcane in importance as sugar crop in Egypt and attribute 28 % of sucrose production. In Egypt, sugarbeet is cultivated in 153.8 thousand faddans with an average production of 20.6 tons per faddan (2001/2002). Recently, reclaimed desert irrigated lands at West Nubaryia and El-Bostan regions has shown that sugarbeet can be successfully grown under sandy soil area condition.

Plant parasitic nematodes, especially root-knot nematodes are known among the most serious pests of sugarbeet in many countries. Of some 50 described species of *Meloidogyne*, only few parasitized sugarbeet, viz, *M. arenaria*, *M. incognita*, *M. javanica*, *M. hapla* and M. naasi are economically important to sugarbeet production (Arnold, 1984). *M. arenaria, M. incognita* and *M. javanica* essentially are hot -weather organisms and most important where beets are grown in regions with long & hot summers and short, mild winter. In Egypt, *M. incognita* and *M. javanica* were reported as major nematode pests of sugarbeet (Oteifa and El-Gindi, 1982; Maareg *et al*., 1988 a&b and 1998 and Ismail *et al*., 1996). The average annual loss in yield of sugarbeet due to *M. incognita* in different states in the U. S. A. was estimated to be as high as 10-50 % and in Italy as 5-15 % (Altman and Thomson, 1971). In Egypt, *Meloidogyne incognita*, due to its frequency of occurrence, high level of infestation and possible interactions with other pathogens, is considered the predominant species attacking sugar beet (Beta vulgaris L.) crop (Ibrahim, 1982; Oteifa and El-Gindi, 1982; Abd El-Massih *et al*., 1986; Maareg *et al*., 1998; El-Nagdi *et al*., 2004, Korayem, 2006). Reduction of crop losses due to nematodes is one way of increasing crop yields. Therefore, some additives such as soil amendments (Maareg *et al*., 1999, Maareg *et al*., 2008) and certain biocontrol agents (Maareg and Badr, 2000; Youssef *et al*., 2008) were tested against root-knot nematode on sugar beet, to minimize environmental pollution and keep management processes more economic.

The aim of this research was to evaluate two biocides (one species of bacterium and other of fungus as antagonistic organisms) in addition to the Nemastop and neem oil as plant extracts in the control of root-knot nematode, *Meloidogyne incognita* infecting sugar beet plants under green field conditions.

Materials and methods

This experiment was carried out under field conditions of Elnubaria province and laboratories of City of Scientific Researches and Technology Applications, Alexandria, Egypt. Sugar beet (*B. vulgaris*) cv. DS 9002 was used as the host plant for *M. incognita*. An area of 1000 square meters was designed as rows (30 cm) at 60 cm intervals, then it divided into 30 plots. Sugar beet seeds were planted at holes (10 cm distance between holes). Treatments were replicated six times and arranged in a completely randomized block design.

The tested materials were incorporated into the soil five weeks after planting. These materials were the fungus, *Trichoderma harzianum* which was mixed with talcum powder (10 g fungus + 90 gram talcum powder) and applied as soil treatment at the rate of 2 g per seedling , and the second was the bacterium, *Serratia marcescens* which was also

mixed with talcum powder at the same weights and applied as soil treatment at the rate of 2 g per seedling. In addition to the commercial bio pesticide Nemastop which consisted of a suspention contains 600 g of ground garlic gloves in one liter of water , the liquid product was applied at the rate of 5 ml per /seedling . Also, neem oil which considered a vegetable oil pressed from the fruits and seeds of the neem (*Azadirachta indica*), was used as a pure commercial product at the rate of one ml per seedling .

Plants in each hole were thinned to one plant after germination, 21 days from sowing. The untreated rows were left as a control. Observations on the plant vegetative characters of different treatments were recorded 4 months after planting date i.e. total leaf weight (g), numbers of leaves/plant, leaf height (cm), total weight of 5 leaf discs 2 cm diam.(g), total leaf area cm 2, root diameter (cm), root scale (cm), root length/(cm), total weight root, total foliage weight (g), and total weight /plant (g).

The number of galls and eggmasses in roots were also counted, in addition to gall area root gall index, egg masses index and reduction percentages % (Barker 1985).

Statistical analysis

Data were analyzed using Fisher's Least Significant Difference (LSD). The means were compared by LSD at the 0.05 levels of significance (Steel and Torrie 1980). Increase or decrease of different means and reduction percentages were calculated according to Abbott's formula (1925).

Results and discussion

Data presented in Table 1 show the effect of the application of some biological agents against root knot nematode, *Meloidogyne* spp on some plant vegetative measurements of sugar beet var. DS9001, under field conditions. Statistical analysis of the results indicate that there were significant differences in the means of leaf characters among the applied treatments. The highest values of total sugar beet leaf area was recorded with the treatment of Nemastop, followed by the treatment of *Trichoderma harzianum, and* Neem oil treatment , while the least values was recorded with the treatment of *Serratia marcescens* .

Table 1**.** Effect of the application of some biological agents against root knot nematode, *Meloidogyne* spp on some plant vegetative measurements of sugar beet Var. DS9001, under field conditions.

Treatments	Average numbers of different characters				
	Ave. leaf weight (g)	Ave. no. of leaves/plant	Average of leaf height (cm)	Ave. weight 5 leaf discs 2 cm diam.(g)	Ave.total leaf area cm ^{2}
			Bacteria		
Serratia marcescens	217.80 c	36.6 c	37.2 ab	0.668 a	661.50 e
			Fungi		
Trichoderma harzianum	345.81 b	56.4 a	39.3 a	0.713 a	880.64 c
			Plant extract		
Nemastop	490.78 a	62.4 a	36.85 ab	0.618 a	1622.17 a
			Plant extract		
Neem oil	259.05 c	45.5 b	33.05 b	0.648 a	773.63 d
			Control		
Control	316.44 b	61.3 a	40.0 a	0.674 a	932.84 b
			LSD 5 %		
LSD 5%	54.5	6.6	3.9	0.152	25.73

Means in each column followed by different letter(s) are significantly different at 5% level.

Regarding to the effect of the application of some biological agents, against root knot nematode, *Meloidogyne* spp, on some root measurements of sugar beet Var. DS9001, under field conditions, statistical analysis of the results in Table 2 indicated that there were significant differences in the means of sugar beet shoot and root characters among the applied treatments. The highest values of total shoot and root weights were recorded with the treatment of *Trichoderma harzianum*, followed by the treatment of Nemastop, and Neem oil treatment , while the least values was recorded with the treatment of *Serratia marcescens*. The highest average numbers of root diameter was recorded with the treatments of *Trichoderma harzianum* and Nemastop without significant differences, while the least one was recorded with the treatment of the bacterium, *Serratia marcescens*.

Data presented in Tables 3 and 4 show the effect of different bioagent treatments on the numbers of root knot nematode, *Meloidogyne incognita* galls and egg masses, and its effect on gall area and gall size in

relation to root gall and egg masses index. Statistical analysis of the results (Table 3) indicated that there were significant differences in the numbers of root galls and egg masses among the applied treatments. The highest numbers of root-knot nematode galls was recorded with the treatment of the plant extract, Nemastop , while the least one was observed with the treatment of the bacterium, *Serratia marcescens*. The highest numbers of root-knot nematode egg masses was recorded with the treatment of the plant extract, Nemastop, while the least one was observed with the treatment of the bacterium, *Serratia marcescens*.

As for the effect application of some biological agents on the reduction percentages of root galls and egg mass numbers of root knot nematode, *Meloidogyne* spp infecting sugar beet Var. DS9001, under field conditions, results in table (4) indicated that the highest reduction percentages in the root knot nematode galls was recorded with the treatment of the bacterium, *Serratia marcescens* 73.4 %, while the least one was recorded with the treatment of the plant extract, Nemastop (31.3%).

The highest reduction percentages in the root knot nematode egg masses was recorded with the treatment of the bacterium, *Serratia marcescens* 47.7 % , while the other treatments did not affect the egg mass numbers where there were numbers much more than at control treatment.

These results are in harmony with those obtained by Ismail, *et al*., (1996), Sharon, *et al*., (2001) Haseeb *et al*. (2005), Abd-Elgawad (2008) and Abd-Elgawad and Kabeil (2010), who controlled in Egypt root knot nematodes successfully with different bio control agents.

Table 2. Effect of the application of some biological agents against root knot nematode, *Meloidogyne* spp on some root measurements of sugar beet Var. DS9001, under field conditions.

Treatments	Ave. foliage weight g	Ave. root weight g	Ave. plant weight g	Ave. root length cm	Ave. root scale cm	Ave. root diameter cm
Serratia marcescens	217.80 d	626.89 d	844.69 e	26.00 a	4.32 b	24.79 b
Trichoderma harzianum	345.81 b	1290.32 a	1636.13 a	29.20 a	5.29 a	33.26 a
Nemastop	490.78 a	987.42 c	1478.20 b	25.55 a	5.16 a	32.44 a
Neem oil	213.67 d	673.88 d	887.55 d	25.55 a	4.36 b	27.41 b
Control	316.45 c	1099.19 b	1415.64 c	26.05 a	5.18 a	32.56 a
LSD 5 %	16.77	82.97	16.94	7.8	0.23	3.25

Means in each column followed by different letter(s) are significantly different at 5% level.

Table 3. Effect of the application of some biological agents on the root galls and egg mass numbers of root knot nematode, *Meloidogyne* spp infecting sugar beet Var. DS9001, under field conditions.

Treatments	Average numbers of character per root					
	root gall numbers	root gall index	gall area	gall size	egg masses numbers	egg masses index
Serratia marcescens	19.6 e	3.4	17.1%	2.45 c	36.9 d	4.1
Trichoderma harzianum	29.3 c	3.9	34.9 %	4.00 ab	76.8 b	4.8
Nemastop	51.3 b	4.2	38.6 %	3.20 bc	86.5 a	4.8
Neem oil	23.7 d	3.6	36.3 %	3.54 abc	69.2 c	4.7
Control	74.7 a	4.7	35.1 %	3.65 a	70.6 c	4.7
LSD 5 %	3.25			1.25	1.63	

Gall or egg mass index: 0 = 0, 1= 1-2 , 2= 3-10, 3= 11-30, 4= 31-100, 5= > 100

Means in each column followed by different letter(s) are significantly different at 5% level.

Table 4. Effect of the application of some biological agents on the reduction percentages of root galls and egg mass numbers of root knot nematode, *Meloidogyne* spp infecting sugar beet Var. DS9001, under field conditions.

Treatments	Root gall numbers	Reduction percentages %	Egg masses numbers	Reduction percentages %
Serratia marcescens	19.6 e	73.4	36.9 d	47.7
Trichoderma harzianum	29.3 c	60.8	76.8 b	0.0
Nemastop	51.3 b	31.3	86.5 a	0.0
Neem oil	23.7 d	68.3	69.2 c	0.0
Control	74.7 a	-	70.6 c	-
LSD 5 %	3.25		1.63	

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Evaluation of *Typhlodromips swirskii* (Athias-Henroit) as a biological control agent of two stored product mites on oyster mushroom (*Pleurotus ostreatus*)

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Abstract

Laboratory studies were conducted to evaluate the effect of three temperature degrees (20, 25 and 30ºC) on the biology and life table parameters of the predator *Typhlodromips* (Amblyseius) *swirskii*, and its efficiency as biological control agent for *Tyrophagus putrescentiae* and *Aleuroglyphus ovatus* on the dried oyster mushroom. The development period of *T. swirskii* was shorter on *T. putrescentiae* than on *A. ovatus*, recording 10.14, 7.90 and 5.20 days on *T. putrescentiae*, and 13.33, 10.31 and 7.71 days on A. ovatus at 20, 25 and 30ºC, respectively. The maximum female longevity of *T. swirskii* was achieved at 20°C on *T. putrescentiae* and *A. ovatus*, recording 30.43 and 21.32 days, respectively. The female longevity was decreased with the increase of the temperature to reach 17.11 and 11.34 days at 30°C. The population doubling time (PDT) and generation time (T) were inversely affected with increasing temperature. In contrast, the intrinsic rate of increase (rm) and finite rate of increase (λ) were improved due to the increase of temperature degrees. The net reproductive rate (R0) of *T. swirskii* was 10.60, 13.89 and 9.71on *T. putrescentiae*, while it was 8.47, 12.00 and 7.12 on *A. ovatus* at 20, 25 and 30ºC, respectively. The increases of rm for *T. swirskii* ranged from 0.079 to 0.190 on *T. putrescentiae* and from 0.061 to 0.135 on *A. ovatus*. The results concluded that the predation effect of *T. swirskii* on *T. putrescentiae* was greater than on *A. ovatus*. The greatest reductions (%) of *T. putrescentiae* and *A. ovatus* by *T. swirskii* were noticed at 25ºC with predator-prey ratio of 1:10, recording 73.90 % and 65.21%, respectively.

Key words: Storage mites, predatory mite, *Typhlodromips swirskii*, life table, biological control.

Introduction

Stored product mites such as Tyrophagus puterscentiae (Schrank) and Aleuroglyphus ovatus (Troupeau,1878) (Acari: Astigmata) can be associated with various kinds of stored products (e.g. grain products, seeds, dried fruits, vegetables, mushrooms, medicinal herbs, spices, dried fish and fruits, seeds and bulbs of ornamental plants, and some traditional cheeses and ham (Hughes, 1976). The infestation of stored food products by T. putrescentiae and A. ovatus can lead to different types of damages. Firstly, storage mites directly affect human health through causing dermatitis, asthma, pulmonary acariasis, and urinary acariasis due to allergenic contamination of food (Li *et al*., 2003). Secondly, they can decrease the food quality due to the accumulation of dead mites, faeces, exuvia and the spread of fungi and thus indirectly contribute to contamination of food and feed with mycotoxins (Hubert *et al.*, 2004). Thirdly, they cause significant grain weight losses and decrease of germinability (Zdarkova and Reska, 1976). The level of damage caused by mites is related to the size of the population, which, in turn, depends on how rapidly the population increases in number (Cunnington, 1976).

Traditionally, the management of storage pest mites relies on chemical and physical measures (Thind and Dunn, 2002). While physical control remains a safe and efficient strategy, the use of acaricides causes problems because of toxic residues in food and rapidly increasing resistance (Szlendak *et al.*, 2000). Currently, biocontrol is almost absent in storage, in contrast to glasshouse, orchard or field environments. It was documented that biological control provides a feasible alternative or complement to pesticides in the food industry and stored grain (Flinn and Hagstrum, 1996). Predacious mites, in particular those belonging to the families Phytoseiidae have been widely used for biological control of pest mites in fruits and other crops worldwide (Arthurs *et al*., 2009; Cakmak *et al.*, 2009). For example, Amblyseius swirskii (Acari: Phytoseiidae) is an omnivorous mite that feeds on many species of small arthropods as well as pollen grains (Nomikou *et al*., 2003). In addition, they mentioned that A. swirskii has been successfully used as biological control agents against phytophagous mites, thrips, white lfies, and other pests on vegetable crops in greenhouses. However, no study documenting the potential role of A. swirskii in the control of stored product mites, especially on the dried mushrooms. Therefore, the objectives of this study were to 1): develop a comprehensive understanding of the

effect of A. swirskii on two stored product mites; 2) estimate the biology and life table of this predator three temperatures (20, 25 and 30°C). These data are necessary to understand and predict the performance of this natural enemy under the stores environments.

Materials and methods

Stored product mites rearing

Stock cultures of *Tyrophagus puterscentiae* and *Aleuroglyphus ovatus* were obtained from Central Science Laboratory, Fujing Province, China. The mites were mass-reared in glass flasks (volume 1000 ml) on a mixture of wheat germ and baker's yeast (ratio 2: 1 W:W).

Oyster mushroom

The dried oyster mushroom (*Pleurotus ostreatus*) samples were collected from Wuhan City, Hubei Province, China. The samples of oyster mushroom were crushed to small pieces before the experiment began.

Source and maintenance of Typhlodromips swirskii

A stock culture of *T. swirskii* used in this study was obtained from the Department of Plant Protection, Huazhong Agricultural University, Wuhan, China. The predatory mite (*T. swirskii*) was maintained on a mixture of *T. puterscentiae* and *A. ovatus* and held in cylindrical plastic cages (30 cm diameter and 15 cm high). The cages were covered with round plastic plates that had a 5 cm diameter hole in the center sealed with filter paper disks for aeration. The rearing cages were kept at 25 ºC, 80 % r.h., and 14:10 (D: L).

Development of T. swirskii

The developmental time of immature stages of *T. swirskii* on the combined stages of *T. puterscentiae* or *A. ovatus* were evaluated. About 10 adult females of *T. swirskii* were mixed with 100 individuals of *T. puterscentiae* or *A. ovatus* and placed on a filter paper carried one gram of the dried oyster mushroom in a Petri dish (6 cm in diameter). The Petri dishes were kept at three constant temperatures (20, 25 and 30 °C). The rearing cells were examined every 12 h to determine the developmental time of immature stages (egg, larva, protonymph and deutonymph) for *T. swirskii* at the studied temperatures.

Reproductive periods and female longevity of T. swirskii

When *T. swirskii* became adults, we transferred pairs of newly emerged males and females to individual Petri dish supplied with *T. puterscentiae* or *A. ovatus* as above. The observations of the reproductive periods at the studied temperatures were continued until the females of *T. swirskii* stopped laying eggs or died. We counted eggs daily and removed them after the observation. The rearing units was examined every 24 h. Additional males were reared at the test temperatures to supply mates if required. The experiment was conducted with ten replicates for each treatment.

Life table of T. putrescentiae, A. ovatus and T. swirskii

Adult survival and reproduction data at each studied temperature were used to calculate the life table parameters. A computer program TWOSEX (Chi, 1997) designed in Visual BASIC for the Windows operating system and available at http://140.120.197.173/Ecology/prod02.htm and http://nhsbig.inhs.uiuc.edu.tw/wes/chi.html. The intrinsic rate of increase (r_m) was estimated by equation recommended by Birch, (1948):

$$\sum e^{-r_m x} l_x m_x = 1$$

where x is the age in days, l_x the age-specific survival rate of females (probability at birth of being alive at age x), and m_x the age-specific oviposition rate (mean number of female offspring produced in a unit of time by a female aged x). The net reproductive rate, R_0, is given by $R_0 = \Sigma\ l_x\ m_x$; the mean generation time, T, in days, is given by $T = \ln R_0/rm$; the finite rate of natural increase, λ, is given by e^{rm} and the population doubling time, PDT, is given by PDT = ln 2/rm.

Biological control of T. putrescentiae and A. ovatus

Twenty grams of oyster mushroom were placed into 250 ml plastic dishes. Two hundred combined stages of prey mite species (*T. putrescentiae* or *A. ovatus*) were added to each dish. Females of *T. swirskii* were transferred to the experimental vessels in four densities (5, 8, 10 and 20 individuals) and giving initial predator-to-prey ratios of 0.025, 0.04, 0.05 and 0.1. The experimental dishes were placed into an incubator machine at three constant temperatures (20, 25 and 30°C), 80 %R.H., and 14:10 (D: L). The reduction percentages of *T. putrescentiae* and *A. ovatus* due to the attack were determined after 21 days.

Statistical analysis

The data of developmental time, duration of preoviposition, oviposition, postoviposition and female longevity was analyzed by one-way ANOVA followed by least significance different tests (LSD) for the separation of significantly different means. The level of significance was $P < 0.05$ in all cases. Each of the above mentioned analyses was conducted

using the statistical package SPSS 10.0 for Windows (SPSS Inc., USA).

Results and Discussion

Developmental time of immature stages of T. swirskii

Data in Tables 1 and 2 show the developmental time of immature stages of *T. swirskii* fed on *T. putrescentiae* and *A. ovatus* on the oyster mushroom. The results indicated that the increase of temperature has negative effects on the development of all immature stages (eggs, larva, protonymph and deutonymph) for *T. swirskii*. The development of *T. swirskii* on *T. putrescentiae* was faster than on *A. ovatus.* Generally, the highest developmental time was recorded for the deutonymph of *T. swirskii*, whereas the lowest was found for the larva. The eggs, larva, protonymph and deutonymph of *T. swirskii* developed after 2.71, 1.59, 2.69 and 3.15 days at 20 °C, after 1.80, 1.27, 2.11 and 2.72 days at 25 °C, and after 1.24, 1.00, 1.39 and 1.57 days at 30 °C, when *T. putrescentiae* was its prey. On the other side, eggs, larva, protonymph and deutonymph of *T. swirskii* on *A. ovatus* durated 3.60, 1.92, 3.56 and 4.25 days at 20 ºC, 2.21, 1.56, 2.74 and 3.30 days at 25 ºC, and 1.89, 1.25, 1.99 and 2.58 days at 30 ºC, respectively. When *T. swirskii* reared on *T. putrescentiae*, the total developmental time (life cycle or egg-adult) was 10.14, 7.90 and 5.20 days at 20, 25 and 30 ºC, respectively.

Furthermore, the total developmental time for *T. swirskii* on *A. ovatus* at 20, 25 and 30 ºC were 13.33, 10.31 and 7.71 days, respectively. Similar results were recorded by Kazak *et al.* (2002). These researchers found that the eggs, larva, protonymph, deutonymph and life cycle of *Neoseiulus umbraticus* the all stages of *Tetranychus cinnabarinns* were 4.40, 1.00, 1.90, 2.20 and 9.70 days at 20 ºC, were 3.00, 0.70, 2.00, 2.10 and 8.00 days at 25 ºC and were 1.80, 0.90, 1.50, 1.50 and 5.90 days at 30 ºC, respectively. Palyvos and Emmanouel (2009) evaluated the development of *Cheyletus malaccensis* on *T. putrescentiae* as a food source at different temperatures. They mentioned that the eggs, larva, protonymph and deutonymph of *Cheyletus malaccensis* were 8.40, 4.30 and 3.00 days at 20 ºC and they increased to 11.00, 6.90 and 5.20 days at 25 ºC and to 9.20, 6.10 and 4.20 days at 30 ºC, respectively. At 20 ºC, 25 ºC and 30 ºC, the life cycle declined from 38.60 days at 20 ºC to 24.80 and 17.00 days at 25 ºC and 30 ºC, respectively. Domingos *et al*.(2010) stated that eggs, larva, protonymph and deutonymph of *Neoseiulus baraki* fed on *T. putrescentiae* developed after 2.10, 1.50, 1.30 and 1.20 days at 27 ºC.

They also observed that the developmental time of eggs, larva, protonymph and deutonymph for *Neoseiulus baraki* on *Aceria guerreonis* decreased from 6.10 to 1.70 days, from 5.50 to 1.00 days, from 7.26 to 1.20 days and from 5.40 to 1.20 days due to the increase of the temperature from 18 ºC to 33 ºC, respectively. The life cycle decreased from 24.60 days at 18 ºC to 5.10 days at 33 ºC. Lee and Gillespie (2010) studied the development of *Amblyseius swirskii* at different temperatures and they found that the increase of the temperature had negative effects on the development of all immature stages of *Amblyseius swirskii*. The eggs, larva, protonymph and deutonymph of *Amblyseius swirskii* on Cattail pollen were 3.10, 1.40, 3.20 and 3.30 days at 20 ºC, 1.70, 1.00, 2.30 and 2.00 days at 25 ºC, and 1.10, 1.00, 2.20 and 1.70 days at 30 ºC, respectively. Additionally, they also showed that the life cycle of *Amblyseius swirskii* was 10.90 days at 20 ºC and it decreased to 7.00 days at 25 ºC and to 6.00 days at 30 ºC. It was noticed by Galvao *et al*. (2010) that eggs, larva, protonymph and deutonymph of *Proctolaelaps bulbosus* durated 1.10, 1.40, 1.20 and 1.20 days on the eggs of *T. putrescentiae* and durated 1.30, 1.40, 1.10 and 1.10 days on combined stages of *T. putrescentiae*, respectively at 25 ºC. The total development time of *Proctolaelaps bulbosus* at 25 ºC were 4.80 and 5.00 on eggs and combined stages of *T. putrescentiae*, respectively.

Table 1. Developmental time of immature stages of *T. swirskii* fed on *T. putrescentiae* on oyster mushroom at different temperatures

Stages	Temperatures (ºC)					
	20		25		30	
Egg	2.71	a	1.80	b	1.24	c
Larva	1.59	a	1.27	b	1.00	c
Protonymph	2.69	a	2.11	b	1.39	c
Deutonymph	3.15	a	2.72	b	1.57	c
Total (Life Cycle)	10.14	a	7.90	b	5.20	c

Means followed by different letters in the same row are significantly different (P< 0.05).

Table 2. Developmental time of immature stages of *T. swirskii* fed on *A. ovatus* on oyster mushroom at different temperatures

Stages	Temperatures (ºC)					
	20		25		30	
Egg	3.60	a	2.71	b	1.89	c
Larva	1.92	a	1.56	b	1.25	c
Protonymph	3.56	a	2.74	b	1.99	c
Deutonymph	4.25	a	3.30	b	2.58	c
Total (Life Cycle)	13.33	a	10.31	b	7.71	c

Means followed by different letters in the same row are significantly different (P< 0.05).

Reproductive periods and female longevity of *T. swirskii*

The preoviposition, oviposition, postoviposition and female longevity of *T. swirskii* are presented in Tables 3 and 4. The results showed that the temperature is an important factor in controlling of the reproductive periods and female longevity of *T. swirskii*. The increase of temperature led to decreases in reproductive periods and female longevity. The oviposition period was the longest stage, whereas the preoviposition period was the lowest one. All adult stages and female longevity for *T. swirskii* durated longer time on *T. putrescentiae* than on *A. ovatus*. Durations of preoviposition, oviposition and postoviposition for *T. swirskii* on *T. putrescentiae* on the oyster mushroom were 2.89, 23.84 and 3.70 days, 2.00, 17.95 and 3.09 days, and 1.40, 13.46 and 2.25 days at 20°C, 25 °C and 30 °C, respectively. On the other hand, 2.05, 16.52, 2.75 days at 20°C, 1.42, 13.28 and 1.60 days at 25 °C, and 1.08, 9.10 and 1.16 days at 30 °C were the needed periods for the development of preoviposition, oviposition and postoviposition of *T. swirskii* on *A. ovatus*. The female longevity of *T. swirskii* was (30.43 days) on *T. putrescentiae* at 20 °C and it decreased to (23.04 days) at 25 °C and to (17.11 days) at 30 °C. Decreases from 21.32 days at 20 °C to 16.30 days at 25 °C, and to 11.34 days at 30°C were observed due to the feed of *T. swirskii* on *A. ovatus*, respectively.

Kazak *et al*. (2002) mentioned that the preoviposition, oviposition, postoviposition and female longevity of *Neoseiulus umbraticus* were 3.00, 34.9, 32.7 and 80.5 days; 3.10, 34.2, 21.6 and 67.0 days; 2.30, 20.2, 19.0 and 57.6 days at 20 C, 25 30 C, respectively on the all stages of *Tetranychus cinnabarinns*. It was observed by Domingos *et al.* (2010) that preoviposition, oviposition, postoviposition and female longevity of *Neoseiulus baraki* on *T. putrescentiae* were 2.90, 14.60, 3.10 and 19.60 days, respectively at 27 °C. Lee and Gillespie (2010) showed that the increase of temperature from 20 to 30 °C led to decreases in the total preoviposition period of *Amblyseius swirskii* on Cattail pollen from 20.30 days to 9.70 days, and from 44.50 days to 21.80 days for the female longevity of this predator mite. Galvao, *et al.* (2010) found that the preoviposition, oviposition, postoviposition and female longevity of *Proctolaelaps bulbosus* fed on the coconut mite (*Aceria guerreronis*) were 0.60, 9.20, 4.70 and 14.50 days, respectively at 25 ºC.

Table 3. Reproductive parameters and female longevity of *T. swirskii* reared on *T. putrescentiae* on oyster mushroom at different temperatures

Periods	Temperatures (ºC)					
	20		25		30	
Preoviposition	2.89	a	2.00	b	1.40	c
Oviposition	23.84	a	17.95	b	13.46	c
Postoviposition	3.70	a	3.09	b	2.25	c
Longevity	30.43	a	23.04	b	17.11	c

Means followed by different letters in the same row are significantly different (P< 0.05).

Table 4. Reproductive parameters and female longevity of *T. swirskii* reared on *A. ovatus* on oyster mushroom at different temperatures

Periods	Temperatures (ºC)					
	20		25		30	
Preoviposition	2.05	a	1.42	b	1.08	c
Oviposition	16.52	a	13.28	b	9.10	c
Postoviposition	2.75	a	1.60	b	1.16	c
Longevity	21.32	a	16.3	b	11.34	c

Means followed by different letters in the same row are significantly different (P< 0.05).

Life tables of *T. swirskii*

Tables 5 and 6 present the life table of *T. swirskii* fed on *T. putrescentiae* and *A. ovatus* on the oyster mushroom at different constant temperatures. The results confirmed that the temperature had different effects on the life table parameters of *T. swirskii*. The net reproductive rate (R_0) rose when the temperature increased from 20 ºC to 25. The increase of temperature to 30 ºC led to decreases in the values of the net reproductive rate (R_0). The net reproductive rates of *T. swirskii* at 30 ºC became lower than those at 20 ºC and 25 ºC. The population doubling time (PDT) and mean generation time (T) were affected negatively by increasing the temperature degrees. In contrast, the intrinsic rate of natural increase (r_m) and, the finite rate of increase (λ) improved due to the temperature increased. The R_0 of *T. swirskii* on *T. putrescentiae* was 10.60, 13.89 and 9.71, while it was 8.47, 12.00 and 7.12 on *A. ovatus* at 20 ºC, 25 ºC and 30 ºC, respectively. The values of r_m of *T. swirskii* ranged from 0.079 to 0.191 on *T. putrescentiae* and from 0.061 to 0.135 on *A. ovatus*. Reductions in the values of PDT from 8.77 days to 3.63 days and from 11.36 days to 5.13 days were noticed due to the feeding of *T. swirskii* on *T. putrescentiae* and *A. ovatus*, respectively.

The use of *A. swirskii* as a predator for *T. putrescentiae* and *A. ovatus* lowered the values of T from 29.88 days to 11.90 days and from 35.03 days to 14.54 days, respectively. In contrast, the finite rate of increase (λ) of *T. swirskii* increased from 1.08 to 1.21 and from 1.06 to 1.14 when *T. putrescentiae* and *A. ovatus* were its food, respectively. Similar results were found by Kazak *et al.*, (2002), who motioned that r_m and T rose due to temperatures increased, whereas, R_0 decreased as temperature increased. The values of r_m, R_0 and T of *Neoseiulus umbraticus* on the all stages of *Tetranychus cinnabarinns* were 0.123, 0.149 and 0.180; 18.8, 25.0 and 23.5; 23.8 days, 21.5 days and 17.5 days at 20 ºC, 25 ºC and 30 ºC, respectively. Domingos et al. (2010) found that R_0, r_m, λ and T of *Neoseiulus baraki* at 27 ºC were 18.67, 0.18, 1.20 and 15.80, respectively, when fed on *T. putrescentiae*. Lee and Gillespie (2010) studied the effect of different temperatures on life table parameters of *Amblyseius swirskii* fed on the Cattail pollen, and they stated that the values of R0 were 9.22, 11.14 and 8.70, at 20, 25 and 30, respectively. In addition, they showed that the values of r_m and λ increased from 0.076 to 0.145 and from 1.08 to 1.16, respectively due to the increase of temperatures degrees from 20 ºC to 30 ºC. In contrast, they observed that T and PDT of *A. swirskii* decreased from 29.20 days to 14.90 days and from 9.51 to 4.80 days as results of temperature increased from 20 ºC to 30 ºC. It was found by Galvao *et al.* (2010) that R_0 and r_m were 17.50 and 0.392, respectively at 25 ºC.

Table 5. Life table parameters of *T. swirskii* fed on *T. putrescentiae* on the oyster mushroom at three constant temperatures

Temperatures	Parameters									
	R_0		r_m		PDT		T		λ	
20	10.60	c	0.079	c	8.77	a	29.88	a	1.08	b
25	13.89	a	0.160	b	4.33	b	16.44	b	1.17	a
30	9.71	b	0.191	a	3.63	c	11.90	c	1.21	a

R_0: net reproductive rate, rm: intrinsic rate of natural increase, PDT: population doubling time (days), T: mean generation time (days), λ: finite rate of increase. Means followed by different letters in the same column are significantly different ($P< 0.05$).

Table 6. Life table parameters of *T. swirskii* fed on *A. ovatus* on the oyster mushroom at three constant temperatures

Temperatures	Parameters									
	R_0		r_m		PDT		T		λ	
20	8.47	b	0.061	c	11.36	a	35.03	a	1.06	b
25	12.00	a	0.112	b	6.19	b	22.19	b	1.11	a
30	7.12	c	0.135	a	5.13	c	14.54	c	1.14	a

R_0: net reproductive rate, rm: intrinsic rate of natural increase, PDT: population doubling time (days), T: mean generation time (days), λ: finite rate of increase.

Means followed by different letters in the same column are significantly different ($P< 0.05$).

Biological control of storage product mites using T. swirskii

The reduction percentages of *T. putrescentiae* and *A. ovatus* as influenced by *T. swirskii* are shown in Tables 7 and 8. The temperature has an important role in the biological control of *T. putrescentiae* and *A. ovatus* by *T. swirskii*. The predation effect of *T. swirskii* on *T. putrescentiae* was higher than

that on *A. ovatus*. With increasing the ratio between *T. swirskii* and *T. putrescentiae* or *A. ovatus*, the efficiency of *T. swirskii* became low. The reduction percentages of *T. putrescentiae* by *T. swirskii* were 61.42 %, 58.81 %, 55.00 % and 53.74 % at 20 ºC; 73.90 %, 69.35 %, 66.43 % and 61.40 % at 25 ºC, and 65.64 %, 60.30 %, 57.12 % and 52.63 % at 30 ºC, when the ratios between *T. swirskii* and *T. putrescentiae* were 1:10, 1:20, 1:25 and 1:40, respectively. The use of *T. swirskii* as a predator at the ratios of 1:10, 1:20, 1:25 and 1: 40 caused decreases in the numbers of *A. ovatus* by 51.49 %, 46.80 %, 43.00 % and 40.76 %, by 65.21 %, 61.70 %, 55.32 % and 51.10 %, and by 58.80 %, 55.19 %, 50.64 % and 46.45 % at 20 ºC, 25 ºC and 30ºC, respectively. The higher reduction percentages of *T. putrescentiae* than *A. ovatus* by *T. swirskii* can be illustrated by the higher population growth (r_m) for *A. swirskii* on *T. putrescentiae* than *A. ovatus* (Figures 5 and 6).

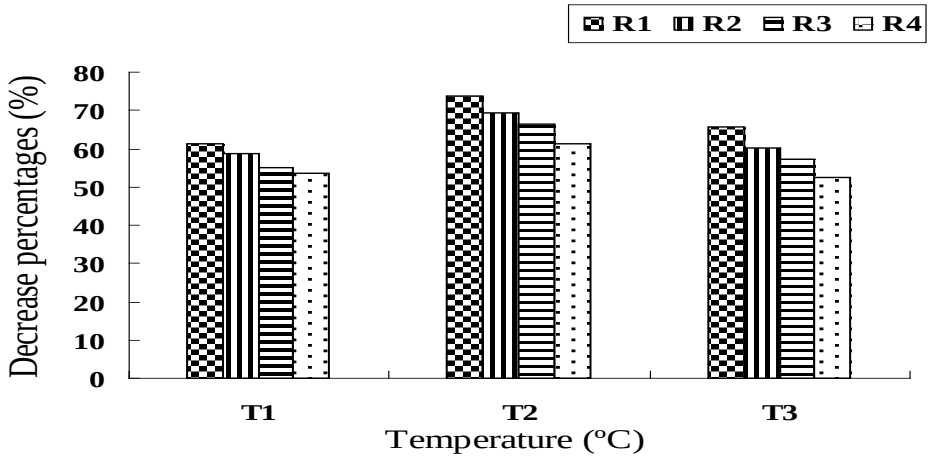

Fig. 1. Decrease percentage (%) of *T. putrescentiae* by *T. swirskii* at different temperature on oyster mushroom

T is Temperature (T1= 20 ºC, T2= 25 ºC and T3= 30 ºC).
R is the ratio between the predator mite and storage mite, R1 = (1:10), R2 = (1:20), R3 = (1:25) and R4 = (1:40).

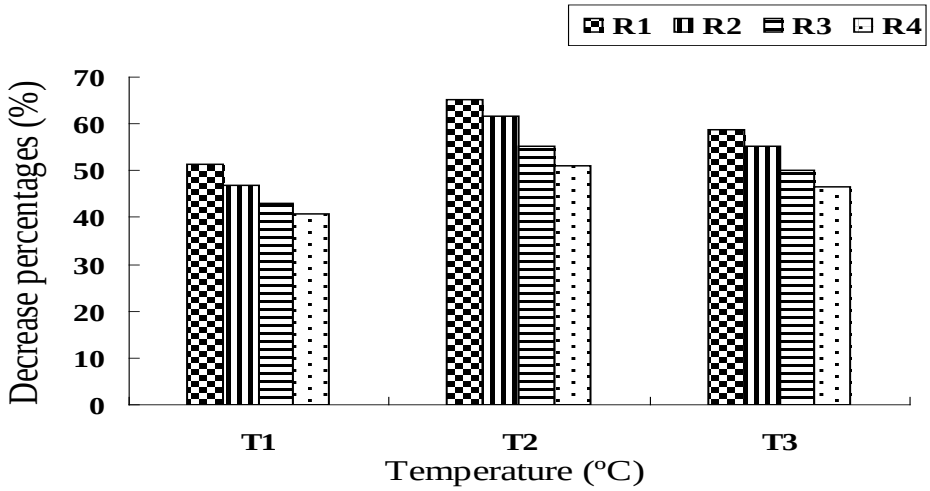

Fig. 2. Decrease percentage (%) of *A. ovatus* (combined stages) on oyster mushroom by *A. swirskii* at different temperature.

T is Temperature (T1= 20 ºC, T2= 25 ºC and T3= 30 ºC).
R is the ratio between the predator mite and storage mite, R1 = (1:10), R2 = (1:20), R3 = (1:25) and R4 = (1:40).

Conclusion

Our data clearly showed that *A. swirskii* is a promising potential biological control agent of *T. puterscentiae* and *A. ovatus* on the dried oyster mushroom at the three studied temperatures. The highest predation effect of *A. swirskii* was noticed at 25 ºC, whereas the lowest was recorded at 20 ºC. Further studies are necessary to evaluate the predation potential of *A. swirskii* on other storage mites under a wide range of temperature and moisture.

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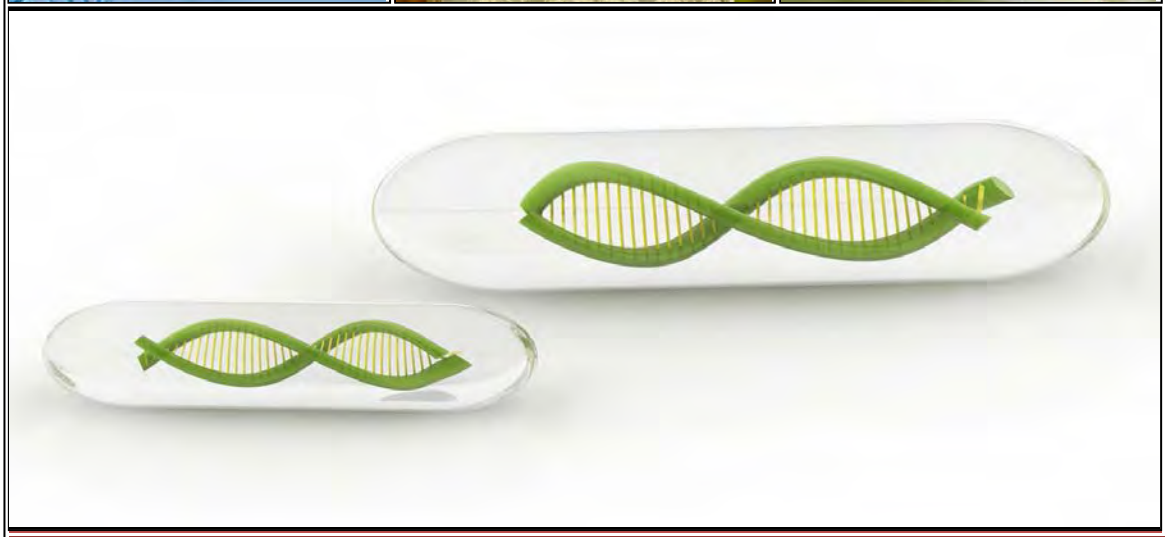


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