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EDITORIAL PREFACE

It is my pleasure to present the eighth volume of the *Jordan Journal of Biological Sciences* (JJBS) to the audience. JJBS is a refereed, peer reviewed quarterly international journal issued by the Jordanian Ministry of Higher Education and Scientific Research Support Fund in cooperation with The Hashemite University, Zarqa, Jordan. This journal publishes papers in Biological Sciences encompassing all the branches at molecular, cellular and organismal levels.

A group of distinguished scholars have agreed to serve on the Editorial Board. Without the service and dedication of these eminent scholars, JJBS would have never existed. Now, the Editorial Board is encouraged by the continuous growth of the journal and its formation into a true multidisciplinary publication. I am also honored to have the privilege of working with all members of the international advisory board served by a team of highly reputable researchers from different countries across the globe. I am also delighted with our team of national and international reviewers who are actively involved in research in different biological sciences and who provide authors with high quality reviews and helpful comments to improve their manuscripts.

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At the end of this preface, I would like to thank our readers and authors for their continuing interest in JJBS, and each member of our editorial and review boards for their continued hard work, support and dedication, which made it possible to bring another new issue of JJBS to the multidisciplinary international audience. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to Jordan Journal of Biological Sciences. I very much appreciate your support as we strive to make JJBS one of the most leading and authoritative journals in the field of Biological Sciences.

June, 2015

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
The Hashemite University, Zarqa, Jordan

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Jordan Journal of Biological Sciences **Review**

Malignant Ovine Theileriosis (*Theileria lestoquardi*): A Review

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Abstract

Malignant Ovine Theileriosis (MOT) is a tick borne disease of sheep and goats, caused by *Theileria lestoquardi* and is considered a major constraint for sheep production in many areas of the world. It has been reported to infect lymphocytes *in vivo* and *in vitro* and the schizonts differentiate into macro-schizonts and micro-schizonts. To date, little is known about the mechanisms involved in the disease pathogenesis, but its high mortality is likely to be linked to the ability of *T. lestoquardi* to stimulate uncontrolled proliferation of the infected leukocyte. Consequently, severe tissue destruction and pulmonary oedema leading to respiratory failure are thought to be the cause of death. Despite an immense amount of small ruminant research, MOT remains an important disease of sheep and goats. Therefore, the present review outlines the current knowledge covering *T. lestoquardi* transmission, distribution, pathogenesis, diagnosis and control. The information may assist in filling the gaps in our knowledge about the economic impact of the disease and new research initiatives. We conclude that the development of a simple, affordable and applicable diagnostic test for an early detection at the field level, and the production of an effective vaccine could have a significant impact on the control of the disease.

Keywords: Malignant Ovine Theileriosis, *Theileria lestoquardi*, Distribution, Pathogenesis, Diagnosis, Control, Economic impact.

1. General Introduction

Malignant Ovine Theileriosis (MOT) or Malignant Small Ruminant Theileriosis (Smith and Sherman, 2011) is a parasitic disease of sheep, caused by *Theileria lestoquardi* and mainly transmitted by *Hyalomma anatolicum*. Sheep are considered a very receptive host for *T. lestoquardi*, as infection usually evolves into sub-acute and acute theileriosis even in indigenous sheep (Tageldin *et al*., 1992; El Hussein *et al*., 1998; Tageldin *et al*, 2005; El Imam *et al*., 2015). Globally, high morbidity and mortality rates have been reported in Iran (Hooshmand-Rad, 1977), Sudan (Salih *et al*., 2003; El Imam *et al*., 2015), and in Sultanate of Oman (Tageldin *et al*., 2005). Sheep from disease-free zones suffer high morbidity when introduced to endemic areas and significant mortality rates are expected (El Imam *et al*., 2015). Consequently, the improvement of livestock production in these zones is severely hampered. Accordingly, the disease is of high economic importance, especially in Sudan where export of sheep and sheep products are a major component of their national economy (El Imam *et al*., 2015). Despite the importance of the disease, there is a considerable lack of knowledge about many aspect of host-parasite relationship and breed susceptibility (Leemans *et al*., 1999 a,b).

2. Taxonomy

Species identification using DNA sequences is the basis for DNA taxonomy. Recently, molecular markers, such as the Major Piroplasma Surface Protein (MPSP), small subunit ribosomal RNA gene (18S), and rRNA internal transcribed spacer region (ITS), have been used in the phylogenetic analysis of *Theileria* spp. (Chae, *et al*., 1999; Gubbels *et al*., 2000; Gou *et al*., 2013). Nonetheless, the exact taxonomic *Theileria* spp. have been difficult to establish and are the subject of a considerable debate (Gubbels *et al*., 2002).

3. Life Cycle

In general, the majority of protozoan parasite life cycles are of a complex and dynamic nature (Mans *et al*., 2015). The parasites have a typical apicomplexan lifecycle involving several differentiation steps, interspersed with phases of proliferation in the mammalian hosts and the vector tick. The detailed *Theileria* life cycle has been reviewed (Shaw, 2003; Uilenberg, 2006; McKeever, 2009; Mans *et al*., 2015). Specific *Theileria* spp. are transmitted by specific tick species; however, the distribution of a particular *Theileria* spp. is directly related to the distribution range of its vector tick(s).

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4. Transmission

T. lestoquardi is transmitted from tick's stage to stage through *Hyalomma anatolicum* (Taha and El Hussein, 2010; Abdigoudarzi, 2013), *H. impeltatum* (El-Azazy *et al.*, 2001), *H. excavatum* (Hashemi-Fesharki, 1997), *H. detritum* (Abdigoudarzi, 2013), *Rhipicephalus sanguineus* (Razmi *et al.*, 2003) *R. turanicus* (Abdigoudarzi, 2013) and through vertical transmission (Zakian *et al.*, 2014).

5. Distribution

Historically, the disease was first described in 1914 by an Egyptian veterinary inspector from two Sudanese sheep exported to Egypt. Later, it was reported in Iraq (Latif *et al.*, 1977), India (Sisodia, 1981), Sudan (El Ghali *et al*., 1995), Turkey (Sayin *et al.*, 1997), Iran (Spitalska *et al.*, 2005), Saudi Arabia (El-Azazy *et al.*, 2001) and in Sultanate of Oman (Shayan *et al.*, 2011; Tageldin *et al.*, 2005). Surprisingly, it has not been reported in Jordan (Sherkov *et al*., 1977) or Israel (Pipano, 1991). Thus, more accurate and precise data are needed on the geographic distribution of the disease.

6. Pathogenesis

The disease is highly pathogenic to sheep (Leemans *et al.*, 1999 a,b; Tageldin *et al.*, 2005) and goats (Taha *et al.*, 2011). Even in indigenous sheep breeds, high morbidity and mortality rates were reported (El Hussein *et al.*, 1998; El Imam *et al.*, 2015). So far, little has been known about the mechanisms involved in the pathogenesis of *T. lestoquardi* infection (Leemans *et al.*, 2001). The pronounced pathology and high mortality are likely to be linked to the ability of *T. lestoquardi* schizonts to stimulate uncontrolled proliferation of the infected leukocyte inducing a phenotype typical of tumor cells (von Schubert *et al.*, 2010). Although these cellular transformation is known to be reversible and dependent on a viable parasite (Dobbelaere and Heussler, 1999) the parasitized cells acquire the capacity to metastasize and multiply in non-lymphoid as well as lymphoid tissues (Dobbelaere and Kuenzi, 2004; Shiels *et al*., 2006; Luder *et al.*, 2009). *T. lestoquardi* appears to transform mainly major histocompatibility complex class II-positive cells (Ahmed *et al.*, 1999; Preston *et al*., 1999) and production of a number of cytokine which may induce fever and play a role in anaemia, muscle wasting and necrosis (Dobbelaere and Heussler, 1999, Ahmed *et al.*, 1999, 2002). The mechanism employed by the *Theileria* parasite to regulate the bovine host cell is studied (Dobbelaere and Kuenzi 2004; Dessauge *et al.*, 2005; Shiels *et al.*, 2006; Dobbelaere and Baumgartner, 2009) but there is still a considerable lack of detailed knowledge regarding the ovine cells.

The hepatization and rubbery texture of the lungs and accumulation of excessive fluids and exudates in the chest cavity were reported (Tageldin *et al.*, 2005); these fluids may impair the host respiration (El Imam, 2010). Serious tissue destruction and pulmonary oedema indicate that emphysema, congestion and collapse (Plate 1) lead to a respiratory failure and provide clinical signs for sheep suffering from acute *T. lestoquardi* infection (Tageldin *et al.*, 2005). The slight distention of the gall bladder together with green bile (Plate 2) may also indicate acute *T. lestoquardi* infection (El imam 2010).

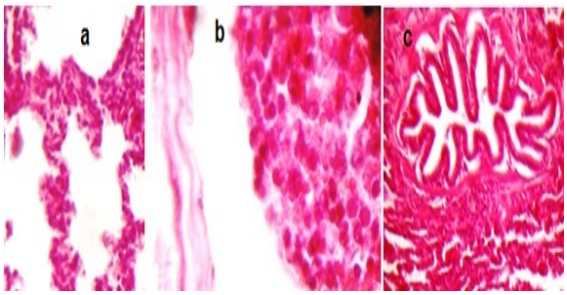

Plate 1. Photomicrograph of lung section showing (a) congestion, (b) emphysema and (d) collapse (H & E stain X100).

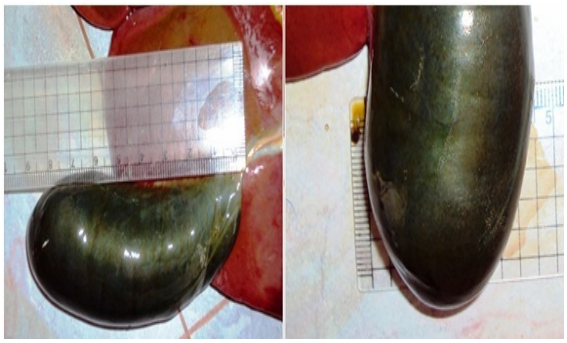

Plate 2. Photograph of distended gall bladder in *T. lestoquardi* infected sheep (a) length, (b) width.

7. Clinical Signs

The most prominent clinical signs of *T. lestoquardi* infections include generalized enlargement of the superficial lymph nodes (Plate 3.), high fever, listlessness, anorexia, emaciation, intermittent diarrhoea or constipation and loss of condition (Leemans *et al.*, 1999a; Tageldin *et al.*, 2005). Initially, infected animals have an apparently normal appetite, but in a few days after the onset of fever they cease eating and later on they become progressively emaciated (El Imam *et al.*, 2015). In fact, elevation of body temperature or fever is associated with many disease states since the hypothalamus is the control center for thermal regulation. Chemical (pyrogens) are released from body tissues and fluids when either or both are injured may influence and alter the hypothalamus function. Fever is the result of either cytokine receptor or Toll-Like Receptor (TLR) triggering; in autoimmune diseases, fever is mostly cytokine mediated whereas both cytokine and TLR account for fever during infection (Dinarello, 2004).

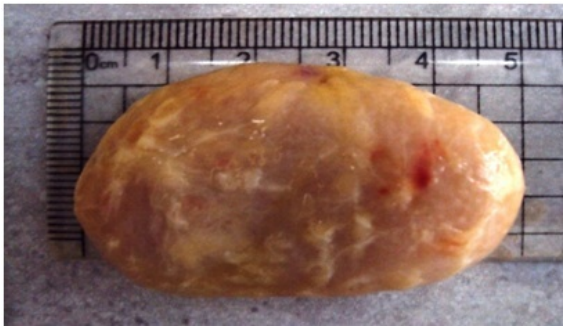

Plate 3. Photograph of enlarged superficial lymph node in *T. lestoquardi* infected sheep. However, the detailed explanation of mechanisms that cause fever in *T. lestoquardi* infections awaits full elucidation.

Sheep infected with *T. lestoquardi* also display anaemia due to erythrocyte destruction (Nazifi *et al*., 2011, 2012; El Imam *et al.,* 2015), but the precise cause of the anaemia is still unknown. Many studies have tried to clarify the mechanisms involved in the development of anaemia (Shiono, *et al.,* 2004; Nazifi *et al.,* 2011). Morphological changes to the surface of the RBC, an increase in osmotic fragility (Yagi *et al.,* 1989), abnormal RBC clearance (Yagi *et al.,* 1991), changes in membrane glycolipid components (Watarai *et al.,* 1995) and oxidative injuries (Shiono *et al*., 2001, 2003; Yagi *et al.,* 2002; Nazifi *et al.,* 2011) take place. In addition, an accelerated destruction of RBC in anaemic sheep could be attributed to the binding of autoantibody (IgG) to parasitized RBC that results in phagocytosis (Shiono *et al*., 2004). A marked fall in WBCs resulting in leukopenia that lasts for several days, and a fall in blood PCV and Hb are often reported (Nazifi *et al.,* 2012, Elsadig *et al.,* 2013, El Imam *et al.,* 2015).

8. Immune Responses

Little is known about the mechanisms involved in the protective immune response against *T. lestoquardi* (Leemans *et al.,* 2001) or the susceptibility of the various ovine breeds (Uilenberg, 1997). *T. lestoquardi* infects the monocytes/macrophages and B cells (Leemans, *et al.,* 2001). It is known that animals that survive infection are resistant to further challenge and indigenous sheep and goats usually acquire immunity at an early age (Hooshmand-Rad, 1985). Comparatively, goats show a greater resistance to the infection than sheep (Brown *et al.,* 1998) despite the fact that indigenous sheep in *T. lestoquardi* endemic areas have a strong natural resistance or tolerance to the disease. The mechanisms of this apparent breed resistance are unknown (El Imam *et al.,* 2015). Experience gained from defining the response to bovine *Theileria* should be useful for addressing this knowledge gap in small ruminants. However, specific studies mapping the small ruminant response against *Theileria* are required and may help to understand the immune responses to other tick borne disease.

9. Diagnosis

Routine field diagnosis of *T. lestoquardi* infection is usually based on a combination of host specificity, transmission mode, adult tick species, epidemiololgical data, clinical signs and pathological findings together with morphological demonstration of the parasite, ideally the macroschizont infected leukocyte. In the last decade, a considerable progress has been achieved in the development of diagnostic tests for tick and tick borne-diseases in general, but the high cost and technological requirements limit the routine field application (Minjauw and McLeod, 2003).

9.1. Microscopic Examinations

The direct method involves identifying the parasite in Giemsa's-stained blood smears (Plate 4) or lymph-node biopsy samples (Plate 5). The method is reliable for diagnosing clinical acute cases, but it is very subjective in pre-immunity and/or long-lasting carrier hosts, where low parasitaemia occur and schizont infected leukocytes cannot be detected. Thus, a level of expertise is required for differentiating mixed *Theileria* spp. infection on the basis of morphology (d'Oliveira *et al.,* 1995; Garcia-Sanmartin *et al.,* 2006). To overcome this problem, a number of serological tests for species-specific detection allowing have been developed.

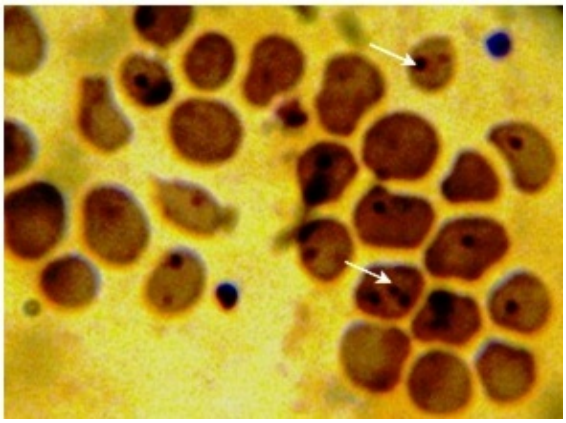

Plate 4. Photomicrograph of peripheral blood smear showing *T. lestoquardi* piroplasms infecting red blood cells (Giemsa's stain X100).

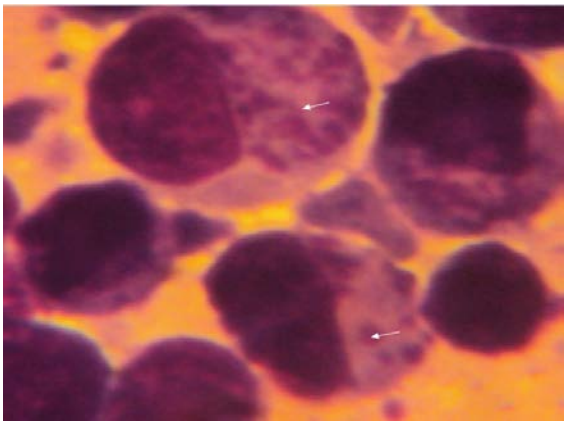

Plate 5. Photomicrograph of lymph node smear showing *T. lestoquardi* schizonts (arrow) infecting lymphocytes (Giemsa's stain X100).

9.2. Serology

Serological diagnostic tools for the major tick-borne protozoan diseases of livestock were reviewed (Bakheit *et al.,* 2007). Antibody detection tests, commonly used in

identification of *T. lestoquardi*, are the Indirect Fluorescent Antibody (IFA) test and the enzyme-linked immunosorbent assay (ELISA). Tests based on antibody detection can be of a little value for the diagnosis of an acute disease since the clinical signs of *T. lestoquardi*, as with other pathogenic *Theileria*, appear before antibodies can be detected. In addition, maternal immunity can produce false positive results. Furthermore, a lack of antibodies in carrier sera may result in long-term infection (Leemans *et al*., 1999a). *T. lestoquardi* and *T. annulata* exhibit astonishing similarities with regard to serology, thus the differential diagnosis between these species is subjective without the use of species specific reagents.

9.2.1. Indirect Fluorescent Antibody Test

The Indirect Fluorescent Antibody Test (IFAT) based on schizont or piroplasms antigen to detect the circulating antibodies against *T. lestoquardi* has been developed (Leemands *et al.,* 1997; Salih *et al.,* 2003; Taha *et al.,* 2003). In Sudan, we subjected lung impression smears during different courses of the disease to IFAT for further demonstration of *T. lestoquardi* schizont infected cell sequestrations in the pulmonary bed. The result of this was that crude antigens derived from in vivo gave clear and bright fluorescence emitted from intracellular schizonts (Plate 6). Our findings could have application significance in diagnosis and developing strategies for therapeutic attack on the parasite (A.H. El Imam and K.M. Taha: unpublished data). However, limitations of IFAT hinder the routine use at large scale epidemiological investigations where a high number of samples required to be screened. These limitations are mainly due to time constrains, the absence of a means of standardization and cross-reactivity with antibodies against other *Theileria* spp. that simultaneously infect sheep (Leemans *et al*., 1997).

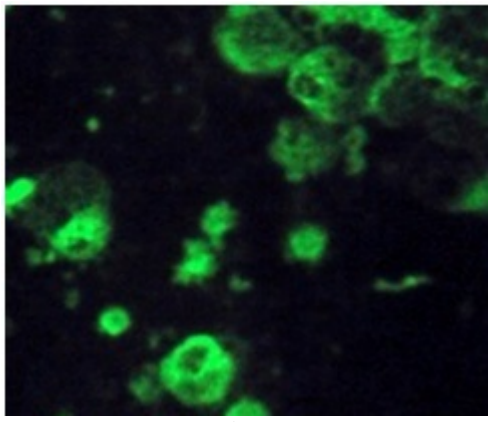

Plate 6. Photomicrograph of a lung impression smear showing massive T. *lestoquardi* schizonts sequestrations in the pulmonary bed (IFA test stain X100).

9.2.2. Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent Assay (ELISA) for serological detection of antibodies against *Theileria* spp. infecting sheep have been documented (Gao *et al*., 2002; Miranda *et al*. 2006; Abdo, 2010). Recently, a newly developed and characterized recombinant protein-based ELISA has been validated to resolve the problems associated with ELISA diagnosis of *T. lestoquardi* (Bakheit *et al*., 2006 a- c). Thus, it may be very useful and applicable for future epidemiological investigation of ovine theileriosis.

9.3. Molecular Based Tests

Advances in molecular biology have enabled identification and classification of several pathogens including *Theileria/Babesia* (Caccio *et al.,* 2000), *Ehrlichia/Anaplasma* (Arens *et al.,* 2003) and Rickettsia group (Christova *et al.,* 2003) at the genotypic level.

9.3.1. Polymerase Chain Reaction

The conventional Polymerase Chain Reaction (PCR) is more sensitive and specific (Figure 1) (A.H. El Imam and K.M. Taha: unpublished data) than other conventional methods (Almeria *et al.,* 2001) and is commonly used to detect ovine theileriosis (Aktas *et al.,* 2005; Altay *et al*., 2008) but it is subjective in mixed infections (Pin *et al*., 2005).

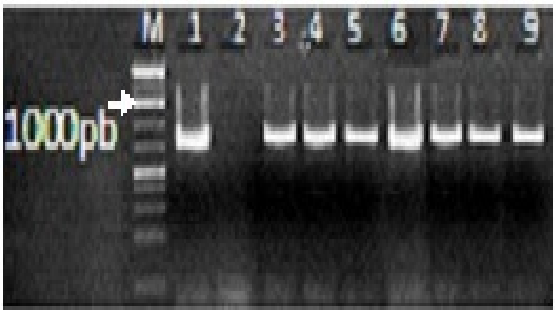

Figure 1. *T. lestoquardi* detected by PCR using *T. lestoquardi* specific primers. Captions: Lane $_{M,}$ standard size marker, L_1 positive control, L_2 negative control, L_{3-9} test samples.

9.3.2. Reverse Line Blot

Reverse Line Blot (RLB) assay was developed for simultaneous specific detection of different piroplasm species (Gubbels *et al.,* 1999). The assay is based on amplification of a fragment of the 18S, 16S ribosomal DNA from virtually all species of *Theileria/Babesia* and of *Ehrlichia* respectively (Schnittger *et al.,* 2004). The advantages of this diagnostic method are its reliability, sensitivity and specificity for the identification of different sheep tick-borne diseases. The RLB assay is a powerful tool and a practical assay since it is able to detect extremely low levels of parasitemia (Gubbels *et al.,* 1999). A possible disadvantage is that it relies on its ability to combine a pair of catch all primers with a region that allows species specific detection via hybridization, and this may not always be achievable for closely related species/gene combinations. It also requires sophisticated laboratory equipment and, due to a complex protocol with a need for controlled hybridization conditions, it may be subject to reproducibility problems in different laboratories. However, the small subunit ribosomal RNA gene (18S RNA gene) sequence data have been successfully used to improve the classification of previously known data and identify several novel *Theileria* and *Babesia* species (Altay *et al.,* 2007; Niu *et al.,* 2009; Oosthuizen *et al*., 2008, 2009; Niu *et al*., 2012; Ranjbar *et al.,* 2012).

9.3.3. Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a novel molecular detection technique that allows target DNA to be amplified with high detection performance under isothermal conditions. The assay is a rapid method with high specificity and efficiency based on a set of four specifically designed primers that can recognize six or eight distinct sequences of the target gene (Notomi *et al.,* 2000; Nagamine *et al.,* 2002). LAMP can be applied using non-denatured template, and DNA extraction may also be neglected, since a drop of blood spotted on to filter paper meets the requirements for the initiation of the reaction (Nagamine *et al.,* 2001a). The test relies on a visual inspection of the reaction product turbidity (Mori *et al.,* 2001; Nagamine *et al.,* 2001b) or a detection of the amplified products through the addition of fluorescent dyes (SYPR Green) and results can be validated using agarose gel electrophoresis (Notomi *et al.,* 2000).

Furthermore, optimal conditions for detection of *T. lestoquardi,* under which the assay exhibited no cross-reaction with other closely related tick-borne diseases, have been established (Liu *et al.,* 2013). The suitability of LAMP for diagnosis of *T. lestoquardi* infection in the field was tested in Sudan, and so was its potential for application in epidemiological surveys (Salih *et al.,* 2012).

9.3.4. Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) of the PCR products allows differentiation between *T. lestoquardi* and *T. annulata* (Spitalska *et al.,* 2004) and between *T. annulata, T. lestoquardi* and *T. ovis* (Zaeemi, *et al.*,2011). It also seems to be useful for the differentiation between *T. separata* and *Theileria* spp. China (Bami *et al.,* 2009). High sensitivity and specificity of PCR–RFLP method have been recently proven and they appeared to be a very powerful tool to detect extremely low parasitemia rates, with discrimination between ovine *Theileria* species in mixed infections (Bami *et al.,* 2009; Zaeemi, *et al.,* 2011).

The development of a simple and applicable diagnostic test suitable for routine diagnosis and useful for detecting mixed infections could have a significant impact on the control of malignant theileriosis of sheep and goats. Thus, there is a pressing need to develop an affordable diagnostic test to detect an early infection at the field level.

10. The relationship between T. lestoquardi and T. annulata

T. lestoquardi and *T. annulata* exhibit a strong serological cross-reactivity (Leemans, *et al.,* 1997), similarities with regard to morphology (Brown, *et al.,* 1998), share the same vector and two immunogenic macroschizont proteins (Namavari *et al.,* 2008) and their geographic distribution tends to overlap (Taha *et al.,* 2013). Both species parasitize the similar host cell phenotypes (Leemans *et al.,* 2001) and are capable of infecting and transforming sheep peripheral blood mononuclear cells *in vitro* and *in vivo* (Brown *et al.,* 1998; Leemands *et al.,* 1999 a,b). Phylogentically, *T. lestoquardi* is more closely related to *T. annulata* than any other sheep or cattle *Theileria* and *Babesia* spp. (Schnittger *et al.,* 2000, 2003; Sparagano *et al*., 2006). In this concept, we amplified the V4 hyper variable region of *Theileria* 18S rRNA gene using the PCR protocol (DNA extracted from sheep blood, UDG-mix, RLB F2 and biotin labelled RLB R2). Amplification was performed according to the *Babesia/Theileria* touchdown PCR programme (Oosthuizen *et al.,* 2009). An in-house membrane was prepared containing the relevant *Theileria* and *Babesia* genus- and species-specific probes. The PCR products were then analysed using the RLB hybridization technique (Nagore *et al.,* 2004). Our result indicate that all probes bound only to their respective target species, except probes positive to *T. lestoquardi* that 100% contemporaneously reacted with *T. annulata* and *T. lestoquardi* (Figure 2). Our findings, confirmed the existence of cross-reaction and closer antigenic relationship between *T. lestoquardi* and *T. annulata* (A.H. El Imam and K.M. Taha: unpublished data). Thus, we concluded that *T. annulata* relatively evolved a common ancestor with *T. lestoquardi*.

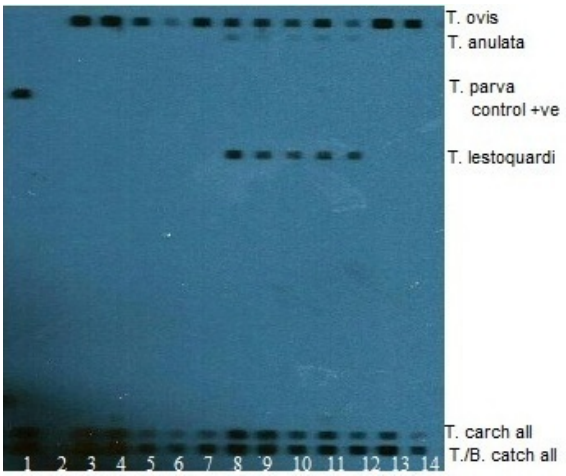

Figure 2. X-ray film of plotting RLB membrane, showing *T. lestoquardi* and *T. annulata* cross reaction.Captions: Lane $_1$, *T. parva* positive control, L_2 *Theileria/Babesia* negative control, L_{3-14} test samples.

11. Treatment

Little is known about the efficacy of the theilericidal drugs used for treatment of bovine theileriosis against *T. lestoquardi.* The chemotherapeutic efficacy of a number of compounds including parvaquone (Clexon) and buparvaquone (Butalex) have been tested for the treatment of the disease (Hooshmand-Rad, 1989). Although some of these drugs are likely to be effective (El Hussein *et al.,* 1993; Hashemi-Fesharki, 1997) they are not easily and quickly eliminated from the animal body and constitute a public health/hazard (McHardy, *et al.,* 1985). Recently, the therapeutic effect of the alkaloids extracted from a medical plant (*Peganum harmala*) has been investigated (Mirzaiedehaghi, 2006; Derakhshanfar and Mirzaei, 2008).

12. Control

The successful cultivation of *T. annulata* prompted the interest of researchers to attempt in vitro culture of *T. lestoquardi* schizont infected ovine cells and explore the possibility to generate an attenuated live vaccine. In addition, immunoprophylaxis trials of cell line vaccines have been successfully carried out in Iraq, Iran and in Sudan (Hooshmand-Rad, 1985; Hashemi-Fesharki, 1997; Ahmed *et al.,* 2013). In the last decade, the molecular characterization of sporozoite *T. lestoquardi* antigen-1 (SLAG-1) protein for inclusion in a sub-unit vaccine (Skilton *et al.,* 2000), parasite vacuolar H+ATpase as a potential molecular marker of attenuated *T. lestoquardi*-infected cell lines (Ali *et al.,* 2008) and 73-kDa protein (Namavari *et al.,* 2008) could be used in vaccine trials. It would be of interest to test whether the synergistic effect of combining recombinant SPAG1 and an attenuated cell line for vaccination against *T. annulata* (Darghouth *et al.*, 2006), also operates for *T. lestoquardi* immunization. The control of MOT has been achieved mainly by prevention of tick infestation using acaricides, although drug treatment of individual cases of valuable stock is now an important control method. In endemic areas, tick control is either not practiced, or used only occasionally to reduce excessive tick burdens as indigenous sheep rarely show disease.

In view of the relatively limited knowledge of sheep theileriosis and the importance of the disease it causes, an effective system of information exchange and some co-operation and co-ordination in research towards its control have been instituted. Globally, the collaborative effort among a number of international established research groups (Piro Vac, http://www.theileria.org/pirovac/ index. htm) to control and to combat MOT is promising.

13. Economic Impact

Due to the economic losses they cause, the most important representatives of the *Theileria* genus are the cattle-infecting species *T. parva* and *T. annulata*. In the case of *T. lestoquardi*, indigenous sheep are at risk in a situation where they are subjected to intensive tick control or when they are moved from disease free to endemic areas (Friedhoff, 1997; Tageldin *et al.,* 2005; El Imam *et al*., 2015). Globally, high morbidity and mortality rates in sheep and goats were reported (El Hussein *et al*., 1998; Taha *et al.,* 2011; Tageldin *et al.,* 2005; El Imam *et al*., 2015). The disease economic importance can therefore be predicted, especially in countries where export of sheep and sheep products are a major component of their foreign income. Animals that recover from *T. lestoquardi* may suffer from weight loss, reduced milk production and delayed maturity (Aisha *et al*., 2014). These animals also remain a carrier and may contribute to disseminating infection. Consequently, these losses have a major impact on animal welfare and stock-holder prosperity worldwide. A study performed in Tunisia indicated that the cost of the carrier state in cattle was greater than the losses caused by overt tropical theileriosis (Gharbi *et al.,* 2011). However, extensive studies on the economic impact of MOT and the impact of the carrier state over the clinical disease are needed.

14. Conclusion

In endemically unstable environments or when susceptible sheep are introduced to these infected environments, tick control or some other disease control measure is essential. Eradication is not a practical proposition due to environmental, managerial and resource constrains and to the lack of a strategy to generate infection-free animals, vector or environment (remove the ticks). Chemotherapeutic agents, such as parvaquone, buparvaquone and halofuginone, are available to treat *T. lestoquardi* infections but not curative and leading to the development of carrier states. In addition, the commercial production and dissemination of a live vaccine is not implemented, and there are difficulties in ensuring batch control. Perhaps delivery would not imperatively require a cold chain, *T. annulata* live vaccine can be also used at room temperature, and it needs to be investigated. It is envisioned that improved production and distribution of an effective live attenuated vaccine will contribute to controlling this important disease. The search for effective control measures towards an endemically stable situation with reduced reliance on chemotherapy and promotion of flock immunity or infected free ticks is difficult and long-term but a worthwhile goal. Precise information on the economic impact of MOT throughout the world is not available and is required.

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Inheritance Pattern of Metric Characters Affecting Grain Yield in Two Bread Wheat (*Triticum aestivum* L.) Crosses Under Rainfed Conditions

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Abstract

The present investigation was undertaken to study the inheritance pattern and genetic analysis of some indicators of grain yield in F_1 and F_2 populations of two bread wheat crosses. The results reflected a satisfactory range of variability within and among the studied generations. Significant positive selection differentials were observed for all characters. High heritability estimates, coupled with high to moderate expected response to selection, were observed in most traits. High predicted genetic gain was observed for grain yield and above ground biomass. The estimated number of genes, involved in the genetic control of these characteristics, varied between 2 to 5, showing partial to over-dominance, suggesting that the selection must therefore be delayed until the F_4 or F_5 generation. Significant heterotic values in positive direction were found for all characters except thousand-kernel weight in both crosses and number of grains per spike in $Acsad_{899}$ × Mahon-Demias cross. Inbreeding depression was observed in both crosses for the number of spikes, above-ground biomass and grain yield. The obtained results indicated that the studied crosses would be of interest in the breeding program. It was suggested that the selection for all characters, except for thousand-kernel weight, should be effective in early segregating generations due to additive gene effects.

Keywords: Heritability, Heterosis, Potence ratio, Semi-arid, Wheat, Yield.

1. Introduction

In Algeria, bread wheat (*Triticum aestivum* L.) is one of the most important food crops. It is mainly grown in the northern part of the country under rain fed conditions (Fellahi *et al*., 2013), where the production is subject to the fluctuation in the total yearly precipitation in addition to its distribution over the crop cycle (Chennafi *et al*., 2006). Drought is a major limiting factor of cereal crops production, posing a serious threat to food security worldwide (Cattivelli *et al.*, 2008). Water deficit is a multidimensional stress affecting plants at various levels of their organization (Blum, 1996). When subjected to water deficit, wheat demonstrates various morphological, physiological, biochemical, and molecular adaptive responses (Nezhadahmadi *et al*., 2013). During the post-anthesis stage, drought reduces plant size, leaf area, grain filling duration, grain number, thousand-kernel weight and grain yield (Kaur and Behl, 2010; Nouri *et al*., 2011). Physiological responses to water stress include stomata closure, development of oxidative stress, decrease in photosynthesis activity, alteration of the integrity of cell wall, production of metabolites to toxic levels, which cause plant death (Bray, 2002).

The genetic improvement of drought tolerance is an important objective in wheat breeding programs, in arid and semi-arid regions (Mullet and Whitsitt, 1996). Plant breeders need to develop varieties which are resilient to moisture stress conditions and able to express high grain

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yield potential (Noorka *et al.*, 2009). This requires a search for and a selection of traits such as biological yield, thousand-kernel weight, number of spikes, number of grains per spike and harvest index, which were found to be highly associated with grain yield (Richards *et al.*, 2002). Increasing the genetic potential of these traits is a continuous process. To accumulate a desirable gene pool in improved varieties, it is necessary to understand the mode of inheritance, the magnitude of gene effect and the mode of gene action in relation to the targeted environment for which varieties are to be developed (Farshadfar *et al.*, 2000). Previous genetic studies reported that both additive and non-additive gene actions were involved in the expression of most agronomic characters (Hannachi *et al.*, 2013). Nevertheless, the selection of promising parents to obtain superior hybrids depends, primarily, on the predominance of additive effect (Gowda *et al.*, 2010; Beche *et al.*, 2013). Keeping in view the importance of some metric characters as grain yield determinants under rain fed growth conditions, the goal of this research was to investigate the inheritance pattern of these metric traits in two bread wheat (*Triticum aestivum* L.) crosses utilizing P_1, P_2, F_1 and F_2 populations data sets.

2. Materials and Methods

2.1. Experimental Material and Design

The experience was carried out at the experimental field of the Algerian National Institute of Agronomic Research (INRAA), unit of Setif, during three successive crop seasons: 2010/11, 2011/12 and 2012/13. The experimental site coordinates are 36°15′N, 05°37′E and 981 m above sea level. In the 2010/11 cropping season, four genotypes $Acsad_{899}$, Mahon-Demias, $Acsad_{1069}$ and Rmada were crossed to obtain F_1 seeds of the following crosses $Acsad_{899}$ × Mahon-Demias and $Acsad_{1069}$ × Rmada. In the second season, the hybrid seeds of the two crosses were sown to grow the F_1 plants, which were selfed to produce F_2 seeds.

During the third season, seeds of four populations, P_1, P_2, F_1 and F_2, of each of the two crosses were sown in a randomized complete block design, with three replications. Plot had two rows of 10 m length, spaced 20 cm apart, with a plant to plant distance maintained at 10 cm by appropriate plant thinning. Ten competitive plants from parents, F_1 and thirty plants from F_2 generations were randomly harvested from each replication and measurements of above-ground biomass, thousand-kernel weight, spike number, number of grains per spike and grain yield were made.

2.2. Statistical Analysis and Estimation of Genetic Parameters

2.2.1. Variance Components, Heritability and Minimum Number of Genes

Estimates of phenotypic (σ^2_p), genotypic (σ^2_g) and environmental (σ^2_e) variances were obtained from the data of the parents and their offspring generations according to the method outlined by Cruz *et al.* (2012). Broad sense heritability (h^2_{bs}) was calculated using the components of variance derived from the parental and the offspring generations' data according to Acquaah (2007).

$$h_{bs}{}^2(\%) = \frac{\sigma^2_g}{\sigma^2_p} \times 100$$

where, σ^2_g = genotypic variance, σ^2_p = phenotypic variance.

The minimum number of genes involved in the control of each trait was estimated according to Burton (1951).

2.2.2. Selection Differential and Expected Response to Selection

Selection differential (*S*), the expected response to selection (*RS*), the expected response to selection expressed as % of the base population mean (*%RS*) and the expected genetic gain (*PGG*) were calculated using the formulas reported by Cruz *et al.* (2012).

$$S = (\bar{X}_s - \bar{X}_o)$$

$$RS = S \times h^2_{ns}$$

$$RS\ (\%) = 100 \times (\frac{RS}{\bar{X}_o})$$

$$PGG = (\bar{X}_o + RS)$$

2.2.3. Heterosis, potence ratio and inbreeding depression

The percent increase or decrease of F_1 hybrids over mid parent value was calculated to estimate heterosis, following Fonsecca and Patterson (1968). The dominance estimate (*P*) was determined using the potence ratio method (Peter and Frey, 1966). Inbreeding depression (*ID*) was estimated by calculating the percent decrease of F_2 population mean over F_1 hybrid mean:

$$Heterosis = 100 \times \left(\frac{\bar{X}F1 - \bar{X}MP}{\bar{X}MP}\right)$$

$$P = 2 \times \left(\frac{\bar{X}F1 - \bar{X}MP}{\bar{X}BP - \bar{X}MP}\right)$$

$$ID\ (\%) = 100 \times \left(\frac{\bar{X}F1 - \bar{X}F2}{\bar{X}F1}\right)$$

where $\bar{X}_{F1}$ = the first generation mean, $\bar{X}_{F2}$ = the second generation mean, $\bar{X}_{MP}$ = mid parent mean, $\bar{X}_{BP}$ = best parent mean. All statistical analyses were carried out using Genes software (Cruz, 2013) and Microsoft Excel spreadsheets.

3. Results

3.1. Mean Performances of the Evaluated Populations

Means and variances for the analyzed traits of the two crosses are presented in Table 1. Parents showed difference for all traits, in both crosses, except for thousand-kernel weight in $Acsad_{1069}$ × Rmada cross. Mean values of the first filial generation of the $Acsad_{899}$ × Mahon-Demias cross were between parental values for the number of grains per spike. In both crosses, the performances of F_1's were better than those of the best parents for the number of spikes per plant, above-ground biomass and grain yield. However, the F_1 mean performance of the number of grains per spike was higher than that of the best parent in $Acsad_{1069}$ × Rmada cross only. Furthermore, F_1 generation showed significant

decline over respective parent for thousand-kernel weight in both crosses. These results indicated the presence of heterotic effects for these characters. Means of the second filial generations were between parental values for thousand-kernel weight, number of grains per spike, biomass per plant and grain yield in $Acsad_{899}$ × Mahon cross. F_2 population mean exceeded the mean of the best parent for number of spikes per plant in both crosses and for thousand-kernel weight, above-ground biomass per plant and grain yield in $Acsad_{1069}$ × Rmada cross. These results indicated the presence of transgressive segregants in the F_2 generation for number of spikes per plant and thousand-kernel weight. Both traits are important determinants of grain yield, under semi-arid conditions. In addition, F_2 generation, of $Acsad_{1069}$ × Rmada cross exhibited lower number of grains per spike than the lowest parent. The largest variances for all evaluated traits were found in the F_2 generation. These results were somewhat expected, since the observed variability of the F_2 generation is due to environmental and genetic sources (Falconer and MacKay, 1996).

Table 1. Means ($\bar{X}$) and variances (σ^2) for the studied characters of four wheat populations (P_1, P_2, F_1 and F_2) derived from two bread wheat crosses.

Generation	Parameter	Characters				
		TKW	NS	NGS	BIO	GY
$Acsad_{899}$ × Mahon-Demias						
P_1	$\bar{X}$	39.47	5.87	48.82	24.00	11.41
	σ^2	17.78	4.88	150.48	105.24	33.32
P_2	$\bar{X}$	40.79	9.23	33.77	33.40	12.77
	σ^2	2.06	9.21	60.48	173.21	26.51
F_1	$\bar{X}$	33.11	17.67	35.07	56.93	20.40
	σ^2	1.14	10.33	3.70	82.97	9.12
F_2	$\bar{X}$	36.27	9.80	35.01	31.97	12.72
	σ^2	22.02	22.71	108.90	279.82	64.49
$Acsad_{1069}$ × Rmada						
P_1	$\bar{X}$	34.58	5.50	49.34	22.29	9.58
	σ^2	4.81	3.43	214.82	126.99	25.70
P_2	$\bar{X}$	34.69	7.40	48.97	24.17	12.40
	σ^2	5.25	10.52	146.67	107.87	35.90
F_1	$\bar{X}$	29.87	14.00	53.04	54.73	22.18
	σ^2	4.55	3.00	5.97	46.97	12.20
F_2	$\bar{X}$	37.20	8.00	44.60	30.37	13.81
	σ^2	18.66	11.66	199.02	274.79	59.80

TKW: Thousand-kernel weight (g), NS: Number of spikes per plant, NGS: Number of grains per spike, BIO: Biomass per plant (g), GY: Grain yield per plant (g).

3.2. Variance Components, Heritability and Minimum Number of Genes

Estimates of the components of variance, heritability, potence ratio and the minimum number of genes are presented in Table 2. In the present study, the values of the components of genetic variance were generally greater than the environmental component of variance for all traits except the number of grains per spike in $Acsad_{899}$ × Mahon-Demias, where the two components are of similar magnitude (Table 2). Therefore, the observed phenotypic variance can be associated, in most part, to the effect of their genetic differences, with negligible environmental effects. Contributions of genotypic variance to the total variance, in $Acsad_{899}$ × Mahon-Demias cross, were 74.9, 61.7, 49.9, 60.3 and 69.7 % for thousand-kernel weight, number of spikes per plant, number of grains per spike, above ground biomass and grain yield, respectively (Figure 1). These figures were 74.3, 57.2, 53.1, 70.1 and 64.0 % in $Acsad_{1069}$ × Rmada cross for the above mentioned traits (Figure 1). In the present study, estimates of broad sense heritability ranged between 49.87 and 74.88 % (Table 2). Both low and high h^2_{bs} values were observed in $Acsad_{899}$ × Mahon-Demias cross for the number of grains per spike and thousand-kernel weight, respectively. Generally, values of broad sense heritability were either equal to or greater than 50% for all characters understudy, indicating possibilities for improvement through selection in the genetic material produced.

The minimum number of genes is a useful indicator of the polygenic nature of the character studied. The minimum number of genes controlling thousand-kernel weight, spikes number, number of grains per spike, above ground biomass and grain yield in $Acsad_{899}$ × Mahon-Demias cross were 3.48, 2.90, 2.75, 4.86 and 4.85, respectively (Table 2). In the $Acsad_{1069}$ × Rmada cross, these figures were 2.24, 4.21, 5.39, 2.82 and 3.47, respectively. These results indicated that the characters under study are controlled by a relatively small number of genes and a few selection cycles would be necessary to obtain the required accumulation of the favorable alleles controlling them.

Table 2. Estimates of phenotypic (σ_p^2), genotypic (σ_g^2) and environmental (σ_e^2) variance components, broad sense heritability (h^2_{bs}), and minimum number of genes (mng) for the studied characters of four populations (P_1, P_2, F_1 and F_2) derived from two bread wheat crosses.

Genetic component	Characters				
	TKW	NS	NGS	BIO	GY
$Acsad_{899}$ × Mahon-Demias					
σ_p^2	22.02	22.71	108.90	279.82	64.49
σ_g^2	16.49	14.02	54.31	168.72	44.97
σ_e^2	5.53	8.69	54.59	111.10	19.52
h^2_{bs} (%)	74.88	61.74	49.87	60.30	69.72
F_2 range	23.81/ 45.26	4.00/ 22.00	20.91/ 55.49	11.00/ 92.00	2.08/ 43.85
mng	3.48	2.90	2.75	4.86	4.85
$Acsad_{1069}$ × Rmada					
σ_p^2	18.66	11.66	199.02	274.79	59.80
σ_g^2	13.87	6.67	105.67	192.59	38.30
σ_e^2	4.79	4.99	93.36	82.20	21.50
h^2_{bs} (%)	74.30	57.20	53.09	70.09	64.05
F_2 range	29.67/ 45.44	4.00/ 19.00	13.34/ 80.87	9.00/ 75.00	2.37/ 34.99
mng	2.24	4.21	5.39	2.82	3.47

TKW: Thousand-kernel weight (g), NS: Number of spikes per plant, NGS: Number of grains per spike, BIO: Biomass per plant (g), GY: Grain yield per plant (g).

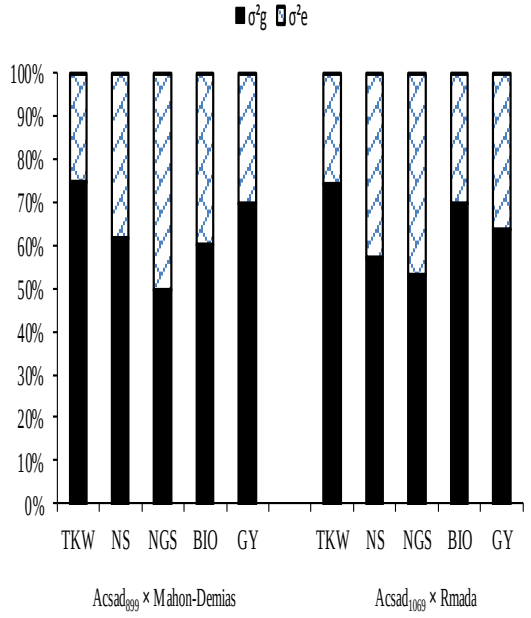

Figure 1. Relative contribution of the genetic and environmental variance components to the phenotypic variance for the measured traits in two bread wheat crosses.

3.3. Selection Differential, Expected Response to Selection and Expected Genetic Gain

The estimates of the selection differential for the studied characters ranged from 5.12 g for thousand-kernel weight in Acsad$_{899}$ × Mahon-Demias cross to 25.47 g for biomass per plant in Acsad$_{1069}$ × Rmada cross (Table 3). Positive selection differentials indicate the possibility of selecting genotypes with good performances. Knowledge of the expected response to selection and the consequent expected genetic gain are essential to identify the appropriate selection criteria (Acquaah, 2007). In Acsad$_{899}$ × Mahon-Demias cross, values of the expected response to selection for thousand-kernel weight, number of spikes per plant, number of grains per spike, above-ground biomass and grain yield were 3.83 g, 4.96 spikes, 8.50 grains per spike, 14.99 g and 8.04 g, respectively. Expressed as a percentage, the expected response to selection for grain yield took the highest value of 63.25%, suggesting an expected genetic gain, after the first cycle of selection, of 20.77 g. The lowest value, 10.58%, of the expected response to selection, expressed as percentage of the mean of base population, was recorded for thousand-kernel weight, leading to an expected genetic gain of 40.09 g.

In Acsad$_{1069}$ × Rmada cross, values of the expected response to selection for thousand-kernel weight, number of spikes per plant, number of grains per spike, above-ground biomass and grain yield were 4.51 g, 2.96 spikes, 9.08 grains per spike, 17.84 g and 7.21 g, respectively. The expected genetic gain values were 41.71, 10.96, 53.68, 48.21 and 21.02 for thousand-kernel weight, number of spikes per plant, number of grains per spike, above-ground biomass and grain yield, respectively (Table 3).

Table 3. Base population mean ($\bar{X}_o$), mean of the selected plants ($\bar{X}_s$), selection differential (S), expected response to selection (RS), expected response to selection expressed as percentage of the base population mean (%RS), and predicted gain genetic (PGG) for the studied characters of four populations (P_1, P_2, F_1 and F_2) derived from two bread wheat crosses.

Genetic component	Characters				
	TKW	NS	NGS	BIO	GY
Acsad$_{899}$ × Mahon-Demias					
$\bar{X}_o$	36.27	9.80	35.01	31.97	12.72
$\bar{X}_s$	41.38	17.83	52.06	56.83	24.26
S	5.12	8.03	17.04	24.87	11.54
RS	3.83	4.96	8.50	14.99	8.04
RS (%)	10.58	50.61	24.27	46.90	63.25
PGG	40.09	14.76	43.51	46.96	20.77
Acsad$_{1069}$ × Rmada					
$\bar{X}_o$	37.21	8.00	44.60	30.37	13.81
$\bar{X}_s$	43.28	13.17	61.71	55.83	25.07
S	6.07	5.17	17.11	25.47	11.26
RS	4.51	2.96	9.08	17.84	7.21
RS (%)	12.11	36.93	20.36	58.78	52.20
PGG	41.71	10.96	53.68	48.21	21.02

TKW: Thousand-kernel weight (g), NS: Number of spikes per plant, NGS: Number of grains per spike, BIO: Biomass per plant (g), GY: Grain yield per plant (g).

3.4. Heterosis, Potence Ratio and Inbreeding Depression

Heterosis over mid-parent and inbreeding depression percentage values in both crosses are presented in Figure 2 for the studied characters. Positive heterosis values for the number of spikes per plant, above-ground biomass and grain yield were observed in both crosses. A positive heterosis estimate was also recorded for the number of grains per spike in Acsad$_{1069}$ × Rmada cross. The heterosis values were low for grain yield and number of spikes but high for above ground biomass, in both crosses (Figure 2). Moreover, low and negative heterosis values were recorded for thousand-kernel weight in both crosses and for number of grains per spike in Acsad$_{899}$ × Mahon-Demias cross. Inbreeding depression was positive for number of spikes per plant, above-ground biomass and grain yield, and negative for thousand-kernel weight in both crosses (Figure 2). The maximum inbreeding effect was recorded for the number of spikes per plant in Acsad$_{899}$ × Mahon-Demias cross, while the minimum was exhibited by thousand-kernel weight in Acsad$_{1069}$ × Rmada cross. The positive estimates indicated that mean values of the F_2 generation were reduced compared to F_1 means. The degree of dominance of the various traits, presented as potence ratio, is reported in Table 4. Thousand-kernel weight potence ratio absolute value was

greater than unity, in both crosses, suggesting over dominance. Over dominance acted in opposite directions, reducing this trait in $Acsad_{899}$ × Mahon-Demias and increasing it in $Acsad_{1069}$ × Rmada (Table 4). A partial dominance towards the higher parent was observed for the number of spikes per plant, biomass per plant and grain yield; and toward the lowest parent for number of grains per spike, in both crosses. Potence ratio absolute values of these traits were smaller than unity.

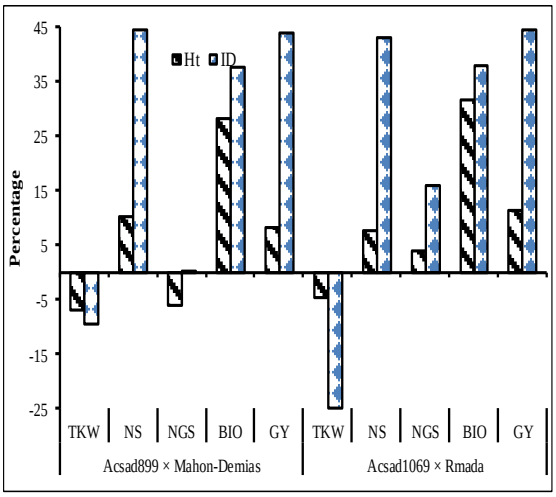

Figure 2. Percentage of heterosis (Ht) and inbreeding depression (ID) for the studied characters obtained in two bread wheat crosses.

Table 4. Potence ratio for the studied characters in two bread wheat crosses.

Characters	Potence ratio	
	$Acsad_{899}$ × Mahon-Demias	$Acsad_{1069}$ × Rmada
Thousand-kernel weight (g)	-2.75	2.24
Number of spikes per plant	0.60	0.36
Number of grains per spike	-0.33	-0.22
Above-ground biomass (g)	0.08	0.35
Grain yield per plant (g)	0.22	0.44

4. Discussion

Developing varieties resilient to moisture stress conditions and expressing high yield potential are an important objective in wheat breeding programs targeting arid and semi-arid regions. Various morphological, yield-related and physiological traits have been suggested as indicators for identifying drought tolerant genotypes (Richards *et al.,* 2002; Garcia del Moral *et al.,* 2003; Nouri *et al.,* 2011). The success of any plant breeding program depends on several factors including the amount of genetic variability, heritability and expected genetic gain of the trait under improvement. A higher amount of genetic variability accompanied by greater heritability and expected genetic gain will result in a significant progress through selection (Nouri *et al.,* 2011). To accumulate a desirable gene pool in improved germplasm, it is essential to understand the extent of genetic variation and the inheritance pattern of the metric traits in relation to the targeted environment for which varieties are to be developed (Farshadfar *et al.,* 2000). The results of the present study indicated a large phenotypic variation between parents and filial generations for the analyzed traits. Genetic components were greater than the environmental component of variance, suggesting negligible environmental effects. Several researchers, reviewed by Mohamed (2014), reported a sizeable variability in wheat cross populations for agronomic traits.

The results indicated that F_1's were better than the best parents for spike number, above-ground biomass and grain yield, suggesting the presence of heterotic effects. Transgressive segregants for thousand-kernel weight and inbreeding depression for biomass, grain yield, grains per spike and spike number were observed in the F_2 generation. Koumber and El-Gammaal (2012) reported similar results. Broad sense heritability values were equal to or greater than 50% for the traits under study, suggesting possibilities for improvement through selection. Heritability values, observed in this study, are of the same magnitude as those reported by Khan *et al.* (2007) and Khaled (2013). Knowledge of the heritability is important to breeders, because it measures the degree to which a character may be transmitted from parents to offspring and it indicates the extent to which the improvement is possible through selection (Acquaah, 2007). The studied traits are controlled by a relatively small number of genes, suggesting that few selection cycles would be necessary to obtain the required accumulation of the favorable alleles controlling them. The expected response to selection, expressed as a percentage of the base population mean, varied from 10.58% for thousand-kernel weight to 63.25% for grain yield.

Farshadfar *et al.* (2001) mentioned that moderate to high estimates of the expected response to selection suggest that the early selection could be effective as a strategy to improve the traits under study, whereas the delayed selection would be more effective to improve characters which exhibited a low expected response to selection values. Results indicated that over dominant inheritance was involved in thousand-kernel weight control, while partial dominance was involved in the genetic control of the other traits. Both additive and non-additive gene actions were involved in the expression of most agronomic characters (Hannachi *et al.,* 2013). Mohamed (2014) reported over dominance in the inheritance of these traits in two wheat crosses. Rashid *et al.* (2007) indicated that the grain yield was controlled by additive gene effects. Hassan *et al.* (2007) and Akhtar and Chowdhry (2006) reported that a partial dominance was involved in biomass, spike number and thousand-kernel weight.

The results suggested, based on h^2_{bs}, expected response to selection which expressed the significance of additive gene effects, that the early selection could be effective to improve the number of spikes, above ground

biomass, grain yield, and the number of grains per spike while delayed selection could be applied to improve thousand-kernel weight. In fact, a sizeable contribution of the dominance effect suggests postponing the selection of the character under improvement to advanced generations when a sufficient reduction of the dominance variance component is reached. However, in the presence of high dominance effect it is still possible to select superior plants in the progenies of a cross for a trait of interest.

5. Conclusion

In the present study, different characters were evaluated by estimating various genetic parameters in segregating and non-segregating generations in two bread wheat crosses under semi-arid environment. The results indicated the presence of desirable transgressors, in both crosses, for a number of spikes per plant; desirable transgressors for thousand-kernel weight, above-ground biomass and grain yield, and undesirable transgressors for number of grains per spike in $Acsad_{1069}$ × Rmada cross. Estimates of broad sense heritability took intermediate to high values for all characters under study, expressing the significance of additive gene effects and indicating possibilities for improvement through selection. The expected response to selection varied among traits and was of a similar magnitude in both crosses. Over dominance was observed in the expression of thousand-kernel weight, and partial dominance was observed for a number of spikes, above-ground biomass, grain yield, and number of grains per spike, in both crosses. The results suggested that the early selection could be effective to improve the number of spikes, above-ground biomass, grain yield, and number of grains per spike while delayed selection could be applied to improve thousand-kernel weight.

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Susceptibility Tests on Insecticides Used to Control Mosquitoes in Jordan

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Abstract

Insecticide susceptibility tests were conducted on five mosquito species collected from two localities in the Jordan Valley (Deir Alla and Ghor as Safi) in 2014. Diagnostic concentration of Temephos (0.25mg/L) was used against the larvae, while diagnostic concentrations of Deltamethrin (0.025%) and Lambdacyhalothrin (0.03g/m2) were used against the adults. The procedures of WHO were followed. A total of 25 larvae were used for adult or larval test for each replicate. All experiments were conducted at 27±2°C. Results showed that all larvae of the tested species in both locations were susceptible to Temephos. The Deir Alla population of *An. superpictus* adults showed a transit susceptibility (96% pooled mortality) to Deltamethrin. The Ghor as Safi populations of *Cx. theirelli* and *Cx. laticinctus* showed resistance to Deltamethrin with 87 and 86% pooled mortality, respectively. *Cx. pipiens* was resistant to Lambdacyhalothrin at the diagnostic doses (78% pooled corrected mortality). The least pooled corrected mortality (76%), among all populations and species, was found in Deir Alla population of *Cx. theirelli*, when treated with Lambdacyhalothrin. Further tests are needed to study the response of a larger number of populations covering different locations in Jordan. A control program should be prepared taking in consideration the resistance to insecticide by alternation with different groups including Bti or juvenile hormones.

Keywords: Mosquitoes, Insecticides, Susceptibility, Resistance, Jordan.

1. Introduction

Mosquitoes are common annoyance insects and are known to be vectors of some important diseases like malaria. Control programs implemented by the personnel of Malaria Control Program (MCP) at the Ministry of Health (MOH) to control mosquito larvae from 1953 to 1966, included the use of Solar Oil and DDT (dichlorodiphenyltrichloroethane) on a weekly basis. From 1967 to 1968, only the Solar Oil was used. From 1969 up to date, Temephos at the concentration (0.5 ppm) was applied weekly with an interruption of spraying for 3 to 4 months a year during the cold months. For controlling adult mosquitoes, Residual House Spraying (RHS), including Indoor Residual Spraying (IRS) with DDT, using a dosage of 2g/m2, was applied since 1959, with two rounds a year until 1983, and then one round a year until 1993. Deltamethrin 2.5% EC was used for wall and space spraying from 1994 up to date by MCP very strictly and in limited areas around diagnosed malaria cases when epidemiologic investigation showed a risk of local transmission.

Research on Jordanian mosquitoes included their ecology, systematics, biology and control. Al-Khalili (1997) surveyed the mosquitos' species in Jordan and provided significant data about their distribution. In addition, he constructed identification keys with original drawings for species in Jordan and surrounding areas. A list of 54 mosquito species occurring in Jordan and the surrounding countries was given. In 1996 and 1997, Al-Khalili *et al.* (2000) conducted a countrywide survey of mosquitos' larvae and raised the number of species known in Jordan from 23 to 28. Their distribution, habitats, collecting dates, species associations and number of larvae examined were provided. Al-Jaran and Katbeh-Bader (2001) studied the biology of the Jordanian population of *Culiseta longiareolata* (Macquart) which is not known to feed on human blood. Khyami-Horani *et al.* (1995) studied the susceptibility of *Culex pipiens molestus* to standard strains of *Bacillus thuringiensis* (Bti -IPS 82, 15000 I.T.U. against *Aedes aegypti* L.) and

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Bacillus sphaericus (Bs-SPH 88, 1700 I.T.U. against *A. aegypti*) in Jordan. Khyami-Horani *et al.* (1996) evaluated the mosquito larvicidal toxicity of endospore-forming bacilli isolated from Jordan. Katbeh-Bader *et al.* (1999) studied the effect of temperature on the susceptibility of *Culiseta longiareolata* (Macquart) to two standard strains of biocontrol bacteria. Khiyami *et al.* (1999) isolated endospore-forming bacilli toxic to *C. longiareolata* in Jordan. They found 10 out of 80 endospore-forming bacilli, isolated from various habitats in Jordan, to be highly toxic to the 4th instar larvae of *C. longiareolata*.

The available literature shows that no data have been published on the susceptibility of the Jordanian mosquitoes to insecticides. Therefore, the aim of this study is to evaluate the susceptibility of five mosquito species to insecticides that have been used to control them for a long time in Jordan.

2. Materials and Methods

Mosquito larvae were collected from two localities in the Jordan Valley: Deir Alla (Abatah site: 31° 03' 53"N, 35°29' 50") and Ghor as Safi (Assal site: 31° 11' 36"N, 31°38"E). Both locations are below the sea level. Susceptibility tests were conducted on a group of the collected larvae soon after the collection, while the other group was reared to adult stage to be used in the susceptibility tests. Mosquito species, insecticides used, their concentrations and localities are provided in Table 1.

The procedures recommended by World Health Organization (WHO) were followed for adult mosquito tests (WHO, 2013), using the diagnostic doses and concentrations. In this procedure, six sheets of clean white paper (12 x 15 cm), rolled into a cylinder shape, were inserted into six holding tubes (one per tube) and fastened into position with a steel spring-wire clip. The tubes were attached to slides. A total of 150 active female mosquitoes were aspirated (in batches) from a mosquito cage into the six holding tubes through the filling hole in the slide to give six replicate samples of 25 mosquitoes per tube (4 treated and 2 control). Once the mosquitoes were transferred, the slide unit was closed and the holding tubes were set in an upright position for one hour. Each of the 4 exposure tubes was lined with a sheet of insecticide-impregnated paper, while the 2 control exposure tubes were lined with oil-impregnated papers; each was fastened into position with a copper spring-wire clip. The empty exposure tubes were attached to the vacant position on the slides and with the slide unit open, the mosquitoes were blown gently into the exposure tubes. Once all the mosquitoes were in the exposure tubes, the slide unit was closed and the holding tubes were detached and set to one side. Mosquitoes were kept in the exposure tubes, which were set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour. Then the mosquitoes were transferred back to the holding tubes. The exposure tubes were detached from the slide units. A pad of a cotton-wool soaked in sugar water was placed on the mesh-screen end of the holding tubes. Mosquitoes were maintained in the holding tubes for 24 hours (the recovery period). At the end of recovery period, the number of dead mosquitoes was counted and recorded. An adult mosquito was considered to be alive if it was able to fly, regardless to the number of legs remaining. Any knocked down mosquitoes, whether or not with lost legs or wings, were considered moribund and were counted as dead. All tests were conducted at 27±2°C.

The procedures recommended by WHO (2005) were followed for larval mosquitoes tests. Batches of 25 third or fourth instar larvae were transferred by means of screen loops small disposable test cups, each containing 249 ml of water and 1 ml of the insecticide solution to give the required concentration. The test containers were held at 27±2°C and, after 24 h of exposure, larval mortality rate was recorded. Dead larvae were those that did not move when probed with a needle. Moribund larvae were those incapable of rising to the surface or not showing the characteristic diving reaction when the water was disturbed. If the control mortality was between 5 and 20%, the mortalities of the treated groups were corrected according to Abbott's formula:

$$\text{Mortality (\%)} = \frac{X - Y}{X} 100$$

where X = percentage survival in the untreated control and Y = percentage survival in the treated sample.

Table 1. List of mosquito species, stages, insecticides concentrations and localities in Jordan.

Mosquito species	Stage	Insecticides concentration	Locality
An. sergenti	Larvae	Temephos 0.25mg/L	Ghor as Safi
An. superpictus	Larvae	Temephos 0.25mg/L	Deir-Alla
Cx. laticinctus	Larvae	Temephos 0.25mg/L	Ghor as Safi
Cx. laticinctus	Larvae	Temephos 0.25mg/L	Deir-Alla
An. sergenti	Adult	Deltamethrin 0.025%	Ghor as Safi
An. superpictus	Adult	Deltamethrin 0.025%	Deir-Alla
Cx. laticinctus	Adult	Deltamethrin 0.025%	Ghor as Safi
Cx. theirelli	Adult	Deltamethrin 0.025%	Ghor as Safi
Cx. laticinctus	Adult	Deltamethrin 0.025%	Deir-Alla
Cx. pipiens	Adult	Lambdacyhalothrin 0.03g/m^2	Ghor as Safi
An. superpictus	Adult	Lambdacyhalothrin 0.03g/m^2	Deir-Alla
Cx. theirelli	Adult	Lambdacyhalothrin 0.03g/m^2	Deir-Alla

3. Results and Discussion

Results of susceptibility tests are presented in Table 2 which shows that all larvae of the tested species, in the two locations, were susceptible to Temephos. Their pooled corrected mortality rates ranged between 99-100%. However, the Deir Alla population of *An. superpictus* adults showed a transit susceptibility to

Deltamethrin (96% mortality). The Ghor as Safi populations of *Cx. theirelli* and *Cx. laticinctus* showed a relative resistance to Deltamethrin (87 and 86% mortality, respectively). *Cx. pipiens* was found to be resistant to Lambdacyhalothrin (78% mortality) at the diagnostic doses. The least pooled corrected mortality (76%), among all species, was found in Deir Alla population of *Cx. theirelli* when treated with Lambdacyhalothrin.

The high susceptibility of the larvae to Temephos, despite the fact that it was applied since 1969 till now on a weekly basis, may be due to the interruption of spraying for 3 months a year during the cold months and/or to the migration of the susceptible mosquitoes from the untreated populations in Jordan or in Palestine and mixing with the Jordanian populations. However, this needs further investigation taking into consideration the control programs implemented in Palestine. The status of pesticide resistance in arthropod pests in Palestine was reviewed, including houseflies and mosquitoes. *Cx. pipiens* was found to be resistant to Chlorpyrifos, Cypermethrin and Permethrin between the years 1994 and 1996 (Horowitz et al., 1998).

Mosquitos' resistance to insecticides depends on the type and frequency of the insecticides used. According to WHO (2013), if the observed mortality rate is between 90 and 97%, the presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests using the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions) and/ or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently showed a mortality rate below 98%, then the resistance is confirmed. Therefore, additional tests are needed on the Dayr Alla population of *An. superpictus* because the corrected mortality rate was 96%.

If the mortality rate is less than 90%, confirmation of the existence of resistant genes in the tested population with additional bioassays may not be necessary, as long as a minimum of 100 mosquitoes of each species was tested (WHO, 2013). In the present tests, *Cx. theirelli* and *Cx. laticinctus* showed resistance to Deltamethrin, as the mortality rates were 87 and 86%, respectively. *Cx. pipiens* and *Cx. theirelli* also showed resistance to Lambdacyhalothrin with 78 and 76% mortalities, respectively. Therefore, further investigation of the mechanisms and distribution of resistance should be undertaken according to (WHO, 2013).

Resistance of mosquitoes to insecticides varies according to the environmental conditions, the species studied and the control programs implemented, which may vary in type and frequency of insecticide use. Therefore, comparing obtained results to test results of mosquitoes that were exposed to different combinations and/ or frequencies of insecticidal applications is irrelevant.

However, when a mosquito species becomes resistant to a certain insecticide in any country, it may become resistant in another country, if exposed to the same insecticide under similar conditions. Results of susceptibility tests can be found in many countries around the world. For example, *C. pipiens pipiens* populations on Cyprus were sampled between 2002 and 2008 to evaluate the insecticidal resistance to Temephos, Chlorpyrifos, and Permethrin and to study susceptibility levels to the *Bacillus thuringiensis* subsp. *israelensis* De Barjac and the juvenile hormone analog, Methoprene. Susceptibility to the three conventional chemical insecticides varied among different collections. Most collections showed moderate or low resistance. The 2004 Akrotiri collection had the highest Temephos resistance ratio, 167-fold at the LC_{95}, although later sampling showed that the population returned to susceptibility after the treatments were stopped (Vasquez *et al*., 2009). Resistance to the organophosphates Temephos and Chlorpyrifos, the carbamate Propoxur, the pyrethroid Permethrin, and the organochloride DDT was investigated in Tunisian populations of *C. pipiens pipiens* collected between 1990 and 1996 (Cheikh *et al*., 1998). Resistance to Temephos was uniformly low and reached 10-folds in the most resistant population. In contrast, resistance to Chlorpyrifos was highly variable and reached the highest level (>10,000-folds) recorded worldwide. The Chlorpyrifos-resistant populations were also highly resistant to Propoxur. Some populations showed high resistance to Permethrin (up to 5,000-folds) and moderate resistance to DDT (≃20- folds). Araújo *et al.* (2013) studied the susceptibility of Brazilian *Aedes aegypti* populations displaying Temephos resistance to *B. t. israelensis.* They found that their data showed a lack of cross-resistance between Temephos and *Bti*, suggesting that *Bti* can be used in an integrated control program to fight *Ae. aegypti* and counteract the Temephos resistance that was found among all populations analyzed.

Table 2. Susceptibility of larvae and adult mosquitoes to various recommended concentrations of insecticides in Jordan in 2014.

Mosquito species	Stage	Insecticides concentration	Locality	Pooled corrected mortality %	Status
An. sergenti	Larvae	Temephos 0.25mg/L	Ghor as Safi	100	Susceptible
An. superpictus	Larvae	Temephos 0.25mg/L	Deir-Alla	100	Susceptible
Cx. laticinctus	Larvae	Temephos 0.25mg/L	Ghor as Safi	99	Susceptible
Cx. laticinctus	Larvae	Temephos 0.25mg/L	Deir-Alla	100	Susceptible
An. sergenti	Adult	Deltamethrin 0.025%	Ghor as Safi	98	Susceptible
An. superpictus	Adult	Deltamethrin 0.025%	Deir-Alla	96	Transit
Cx. laticinctus	Adult	Deltamethrin 0.025%	Ghor as Safi	100	Susceptible
Cx. theirelli	Adult	Deltamethrin 0.025%	Ghor as Safi	87	Resistant
Cx. laticinctus	Adult	Deltamethrin 0.025%	Deir-Alla	86	Resistant
Cx. pipiens	Adult	Lambdacyhalothrin 0.03g/m^2	Ghor as Safi	78	Resistant
An. superpictus	Adult	Lambdacyhalothrin 0.03g/m^2	Deir-Alla	100	Susceptible
Cx. theirelli	Adult	Lambdacyhalothrin 0.03g/m^2	Deir-Alla	76	Resistant

4. Conclusion

All larvae of the tested species were susceptible to Temephos. The Deir Alla population of *An. superpictus* adults showed a transit susceptibility to Deltamethrin. The Ghor as Safi populations of *Cx. theirelli* and *Cx. laticinctus* showed resistance to Deltamethrin. *Cx. pipiens* was found to be resistant to Lambdacyhalothrin at the diagnostic doses. Conducting susceptibility tests on a larger number of Jordanian populations covering diverse habitats is recommended. Alternative control methods to chemicals to which mosquitoes were found resistant should be considered. Insecticides used to control agricultural pests may reach mosquitoes, as non-target pest, when exposed to such insecticides, which accelerates their resistance. Therefore, changing insecticides is recommended when resistance is detected. Establishing susceptible mosquito colonies to be used as reference strains is needed for future tests.

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Ameliorative Effect of Different Concentrations of Mushroom (*Pleurotus tuberregium)* on Lipid Profile of Wistar Albino Rats Induced by Lead Nitrate

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Abstract

The cardiovascular effects of lead (Pb) are not only limited to increased blood pressure and hypertension, but they are also associated with an increased incidence of coronary heart disease, stroke, and peripheral arterial disease. The present study evaluates the effects of Pb poisoning in 90 Albino rats and its amelioration using different concentrations of mushroom/growers mash feed mixture. The results of the present work showed that mushrooms (*Pleurotus tuberregium*) were able to ameliorate the effect of lead toxicity by reducing total cholesterol, Low Density Lipoprotein/High Density Lipoprotein-cholesterol (LDL/HDL-ch) and Coronary Risk Index (CRI) (except in the group of rats fed with 90% feed+ 10% *P. tuberregium*) and to increase HDL-ch in the experimental rats (except in the group of rats fed with 90% feed+ 10% *P. tuberregium*) when compared to the control.

Keywords: Albino Rat, Lead Toxicity, *Pleurotus tuberregium*, Lipid Profile, Total Cholesterol.

1. Introduction

Heavy metals are chemical elements with a specific gravity that is at least five times the specific gravity of water (Babalola *et al.,* 2010). Examples of heavy metals commonly found in the environment include lead, cadmium, mercury, zinc, arsenic, bismuth, etc. These metals are particularly dangerous because they tend to bio-accumulate in the body tissues and organs (Babalola *et al.,* 2010). Lead is found in our food, water, air and soil. Lead emitted by power plants, smelters and boilers is frequently deposited in the soil, where it is taken up by crops (Lynda *et al.,* 2011). Lead exposure mainly occurs through the respiratory and gastrointestinal systems. Absorbed lead (whether inhaled or ingested) is stored in soft tissues (Anuradha, 2007). The toxicity of lead may largely be explained by its interference with different enzymes by binding to their protein or by displacing other essential metal ions. A wide range of biological effects of lead can cause a disruption of the biosynthesis of haemoglobin, a rise in blood pressure, a kidney damage, a brain damage, a miscarriage and subtle abortions, a disruption of the nervous system, declined fertility of men through a sperm damage, a behavioural disruption, a carcinogenic effect; it also causes an oxidative stress in the body, impairs learning, memory and audio-visual functions in children (ATSDR, 2007). Industrialized countries have made progress by phasing lead out of gasoline, banning lead in many consumer goods and replacing lead pipes with copper pipes.

Lead poisoning is presently becoming the most common disease of environmental origin and is increasing very rapidly in developing countries (Ademuyiwa *et al.,* 2002). Environmental toxicants, including lead and other metals, are potentially preventable exposures that may explain population variation in cardiovascular disease rates (Bhatnagar, 2006). The cardiovascular effects of lead, however, are not limited to increased blood pressure and hypertension, but it is also associated with an increased incidence of coronary heart disease, stroke, and peripheral arterial disease (Mark and Ellen, 2002). For a deeper insight into the lead exposure and its effects on lipid profiles, we investigated the ameliorative effect of

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different concentration of mushroom (*Pleurotus tuberregium*) on lipid profile of Wistar albino rats induced by lead nitrate.

2. Materials and Methods

2.1. Experimental Design

The 90 albino rats were subjected to acclimatization for 21 days pre-experimental stage. Thereafter, all the experimental groups were exposed to 0.1 g/l of lead daily for 21 days. At the end of the exposure period, lead salt was discontinued for 21 days and 42 days post-experimental stage. Samples were then collected and subsequently analyzed at every 21 day interval. Ninety (90) albino rats were divided into six groups with three replicates (with 5 rats per group) in this group order:

Group:

1. 90% of feed + 10% of *Pleurotus tuberregium*
2. 70% of feed + 30% *Pleurotus tuberregium*
3. 50% of feed + 50% *Pleurotus tuberregium*
4. 30% of feed + 70% *Pleurotus tuberregium*
5. Feed + 0.1 g/L lead
6. Control

2.1.1. Collection of Samples for Lipid Profile Analysis

Blood samples were collected from the heart of dissected albino rats using 5 ml disposable sterile syringes and transferred into sterile bottles containing Potassium Ethylene diamine tetra acetic acid (EDTA) anticoagulant. The sample bottles were then briefly kept in ice and transferred to the Haematological laboratory unit at University of Benin Teaching Hospital (UBTH), where they were analyzed for total cholesterol, total triglyceride, High Density lipoprotein-cholesterol (HDL-ch), Low Density lipoprotein-cholesterol (LDL-ch),LDL/HDL, Coronary Risk Index (CRI) which were determined by Sysmex Cell counter (Sysmex, Japan) using the methods of (Dacie and Lewis, 1991).

2.1.2. Statistical Analysis

Data collected for the study were analyzed using general descriptive statistics, one Way Analysis of Variance (ANOVA) at 95% probability level of significant. Duncan's multiple range tests were used to compare the different experimental groups. Computer software Statistical Package for Social Scientists (SPSS) and Microsoft Excel were used for the statistical analyses.

3. Results and Discussion

3.1. Total Cholesterol

The highest mean value recorded for total cholesterol was 101±0.51 mol/l. This value was recorded in the control group while the minimum mean value (75±0.3 mol/l) of total cholesterol was recorded in the group of rats fed with 90% feed+ 10% *P. tuberregium*. However, all other values were lower than the control mean values in the study (Figure 1). The results of plasma lipid distribution of rats fed with the experimental diets and lead showed that there were no significant differences between the mean value of plasma total cholesterol for treated groups and the control groups (p>0.05). However, all other mean values of plasma total cholesterol were lower than the control mean values in the present study. This could be because it was only a marsh diet, unlike the other diets which were supplemented with mushroom. Several studies reported the ability of edible mushrooms in reducing plasma total cholesterol level. A study on rats suggested that mushroom β-glucans were effective cholesterol lowering polysaccharides (Cheung, 1996). Ogundana and Fagadel (1982) reported 7.9% as crude fibre concentration of *P. tuberregium*. Hence, the crude fiber content might have important implications in lowering plasma cholesterol levels. From the results of the present work, it was observed that as the quantity of mushroom increases the total cholesterol were reduced. This agrees with the findings of Cheung (1996) and Oyetayo (2006). A Review of the physiological, biochemical and biotechnological applications of mushroom done by Sanjay and Singh (2013) showed that mushroom decreases concentration of total cholesterol, in the Swiss albino rats' blood sera.

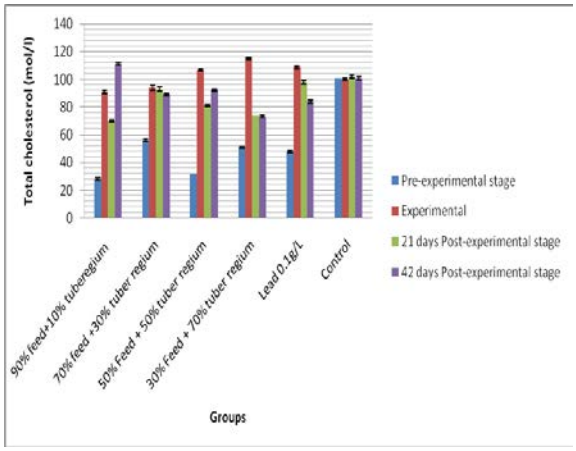


Figure 1. Total Cholesterol of Albino rats feed with various mixture of Pb and *P. tuberrigium*

3.1.1. Total Triglyceride

The highest mean value recorded for total triglyceride was 61±0.19 mol/l. This value was recorded in the group of rats fed with 70% feed+ 30% *P. tuberregium* while the minimum mean value (31 ± 0.42 mol/l) of total triglyceride was recorded in the control group of rats. However, all other values were higher than the control mean values in this study (Figure 2). The mean values of plasma triglyceride distribution was such that treated group showed no significant difference (p>0.05) from the control. Earlier reports by Chorvathova *et al*. (1993) where 4% *Pleurotus ostreatus* diet was fed in hyperlipo and protienemia rats; the results showed no significant effect on the plasma triglyceride levels but Oyetayo (2006) and Sanjay and Singh (2013) reported that plasma triglyceride distribution was such that rats fed mushroom diets had significantly lower plasma triglyceride than the control. However, this was not the same in the present study, as there was high plasma triglyceride. The higher triglyceride concentrations obtained in the present report were associated with low HDL-ch levels observed in the mushroom diets fed rats. High HDL-ch increased the rate of triglyceride catabolism (Oyetayo, 2006) and elevated triglyceride levels, which are frequently associated with

low HDL-ch levels (Oyetayo, 2006). High plasma triglyceride in the blood can increase the risk of heart diseases.

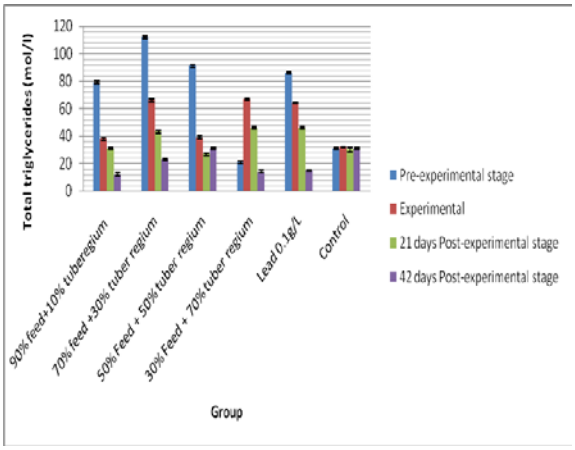


Figure 2. Total Triglycerides of Albino rats feed with various mixture of Pb and *P. tuberrigium*

3.1.2. High Density Lipoprotein-Cholesterol (HDL-ch)

The knowledge of the cholesterol sub-fractions is more meaningful than simple plasma total cholesterol level. The higher the Low Density Lipoprotien-cholesterol (LDL-ch), the greater the athreosclerosis risk and conversely the higher the HDL-ch, the lower the risk. This is true for humans from different racial and ethnic backgrounds and is observed at all adult ages (Baron, 2000).

The highest mean value recorded for HDL-ch was 43.75 ± 4.04 mol/l. This value was recorded in the groups of rats fed with 70% feed+ 30% *P. tuberregium* and in the group where 0.10 g/L of lead was administered while the minimum mean value (25.75 ± 0.00) of HDL-ch was recorded in the group of rats fed with 90% feed+ 10% *P. tuberregium*. However, all other values were higher than the control mean values in the present study except for the group of rats fed with 90% feed+ 10% *P. tuberregium* (Figure 3).

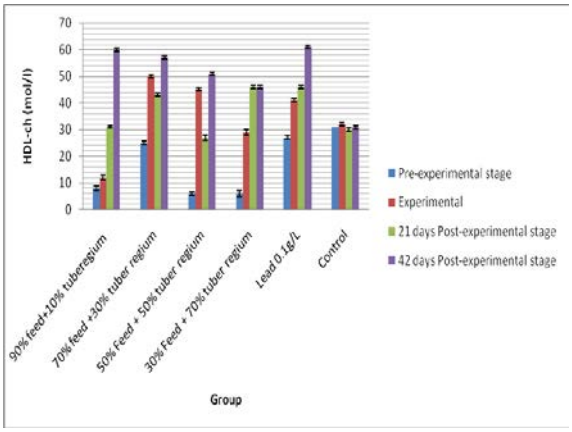
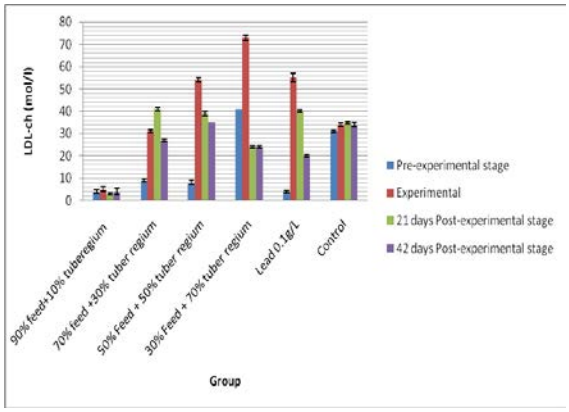


Figure 3. HDL-ch of Albino rats feed with various mixture of Pb and *P. tuberregium*

The mean values of HDL-ch for the treated groups were not significantly different (p>0.05) from the control group. The higher values of HDL-ch were in line with the work of Sanjay and Singh (2010), who reported that when rats feed were supplemented with 450 mg mushroom extract and 800 mg L-carnitine, high density lipoprotein had a higher values (p > 0.05) when compared to those of the control sets. HDL-ch concentrations vary inversely with plasma triglyceride concentration and directly with the activities of lipoprotein lipase (Murray *et al.*, 2003).

3.1.3. Low Density lipoprotein-cholesterol (LDL-ch)

The highest mean value recorded for LDL-ch was 40.5±0.0 mol/l. This value was recorded in the group of rats fed with 30% feed+ 70% *P. tuberregium* while the minimum mean value (4.0 ± 0.82) of LDL-ch was recorded in the group of rats fed with 90% feed+ 10% *P. tuberregium.* However, the control mean values were only lower than the group of rats fed with 50% feed+ 50% *P. tuberregium* and the group of rats fed with 30% feed+ 70% *P. tuberregium* in the present study (Figure 4). Sanjay and Singh (2010) reported that there was a



reduction in the low density lipoprotein when rats feed was supplemented with 450 mg mushroom extract and 800 mg L-carnitine when compared to those of the control sets.

Figure 4. LDL-ch of Albino rats feed with various mixture of Pb and *P. tuberrigium.*

3.1.4. LDL/HDL

The highest mean value recorded for LDL/HDL-ch was 2.0 ± 0.01. This value was recorded in the group of rats fed with 90% feed+ 10% *P. tuberregium* while the minimum mean value (0.626±0.04) of LDL/HDL was recorded in the group of rats fed with 70% feed+ 30% *P. tuberregium.* However, all other values were lower than the control mean values except for the group of rats fed with 90% feed+ 10% *P. tuberregium* (Figure 5). The LDL/HDL cholesterol ratio which is thought to be the athereogenic index of lipoproteins (El-Gengaili *et al.*, 2004) were lower in rats fed with mushroom diets (except for the group of rats fed with 90% feed+ 10% *P. tuberregium* and the group of rats fed with 30% feed+ 70% *P. tuberregium*) fed rats than the control group. This could be due to the high concentration of polyunsaturated fatty acid linoleic acid present in mushrooms. Cheung (1996) reported that about 72% linoleic acids constitute mushroom fat. A study on women by Muller (2003) showed that serum LDL/HDL cholesterol ratio was

influenced more favourably by exchanging saturated fat for unsaturated than reducing saturated fat composition of diets. The ratio is the predictive relation of coronary heart disease; the lower the ratio, the less athreogenic the lipoprotein profile is thought to be (Murray *et al*., 2003).

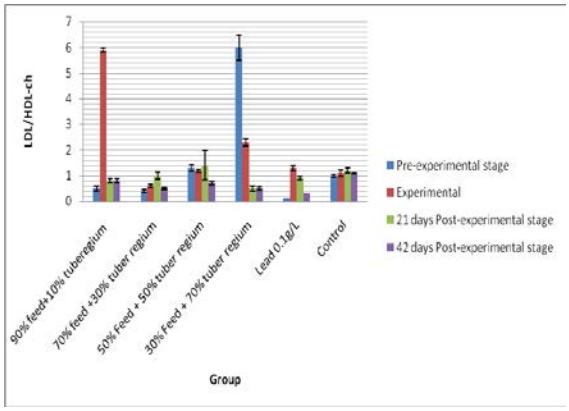


Figure 5. LDL/HDL of Albino rats fed with various mixture of Pb and *P. tuberrigium*

3.1.5. Coronary Risk Index (CRI)

The Coronary Risk Index (CRI) is a ratio of total cholesterol to HDL cholesterol (Oyetayo, 2006). Cholesterol research scientists and doctors are using this cholesterol ratio for predicting the chances of developing heart disease. This is the ratio between total cholesterol and HDL. The lower the ratio (that is less than 4), the better the outcome is. The highest mean value recorded for CRI was 3.825 ± 0.15. This value was recorded in the group of rats fed with 90% feed+ 10% *P. tuberregium* while the minimum mean value (2.0±0.02) of CRI was recorded in the group of rats fed with 0.1 g/l. However, all other values were lower than the control mean values except for the group of rats feed with 90% feed+ 10% *P. tuberregium* and the group of rats fed with 30% feed+ 70% *P. tuberregium* (Figure 6). This may be because of the little amount of mushroom present in the diets compare to the control group. Ogundana and Fageda (1982) reported that *P. tuberregium* is about 14.6% crude protein which may be the reason for the low coronary risk index in this study.

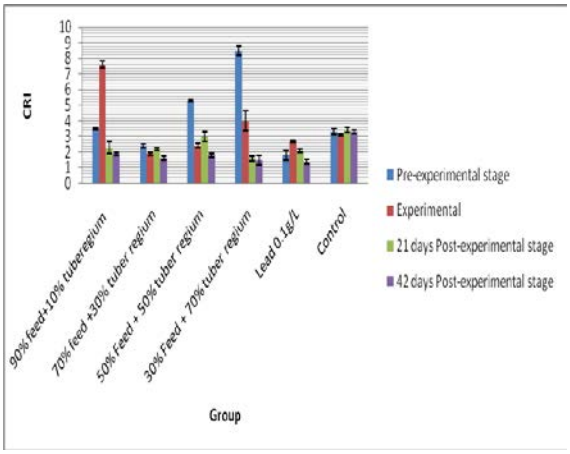


Figure 6. CRI of Albino rats fed with various mixture of Pb and *P. tuberrigium*

4. Conclusion

Even though previous studies suggest that lead exposed persons have altered lipid profile, increased total cholesterol and decreased HDL cholesterol, which can cause a high risk of cardiovascular diseases (Shyam *et al.*, 2012), the results of the present work showed that mushroom *(P. tuberregium*) was able to ameliorate the effect of lead toxicity by reducing total cholesterol, Low Density Lipoprotein/ High Density Lipoprotein-cholesterol (LDL/ HDL- ch) and Coronary Risk Index (CRI) (except for the group of rats fed with 90% feed+ 10% *P. tuberregium*) and to increase HDL-ch in the experimental rats (except for the group of rats fed with 90% feed+ 10% *P. tuberregium*) when compared to the control.

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Recent Collection of Sandflies of the Genus *Phlebotomus* (Diptera: Psychodidae) from Jordan, with a Checklist of Previous Records

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Abstract

Five species of sand flies *(Phlebotomus alexandri, Phlebotomus major syriacus, Phlebotomus mascitti canaaniticus, Phlebotomus papatasi* and *Phlebotomus sergenti*) were collected from several localities from Jordan. *Phlebotomus papatasi* was the most common species. A checklist of previous records for sand flies of the genus *Phlebotomus* is given.

Keywords: Jordan, Sandflies, *Phlebotomus*,Distribution.

1. Introduction

Sand flies (*Phlebotominae*) are hematophagous insects of the subfamily Psychodidae within the order of Diptera. Many species are involved in the transmission of viral and protozoan diseases that can affect human health. In Jordan, Leishmaniasis is the major disease that is transmitted by sand flies of the genus *Phlebotomus* (Oumish *et al.*, 1982; Saliba *et al.*, 1985; Kamhawi *et al.*, 1993; Janini *et al.*, 1995; Khoury *et al.*, 1996; Mosleh *et al.*, 2009).

Janini *et al.* (1995a) investigated the status of sand flies as vectors of cutaneous leishmaniasis in the southern Jordan Valley during 1992. Of 686 *Phlebotomus papatasi* females collected from burrows of the Fat Sand Jird, 14 harboured promastigotes in their guts. Their findings present the first direct evidence of the role of *P. papatasi* as a vector of *Leishmania major* in Jordan.

Since then, no studies have been carried out on the distribution of sand flies in Jordan. The present study investigates the current spatial distribution of sand flies in Jordan, with a list of these flies belonging to the genus *Phlebotomus.*

2. Materials and Methods

Twenty five localities in Jordan were examined for the presence of sand flies (Table 1, Figure 1).

Sand flies were collected either by sticky traps that consist of 21x30 cm white paper sheets coated by castor oil and held with a metal stand above ground surface, CDC light traps were set 1 h before sunset and collected after sunrise next morning or by aspirator tube from inside houses. Insects were then washed from the sticky traps, sorted, and examined for species determination. Identification of collected material was based on Lewis (1982) and Lane *et al.* (1988).

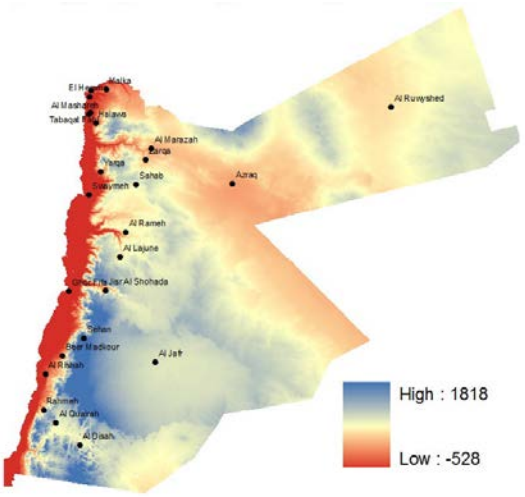

Figure 1. Map of Jordan showing localities from which sand flies were collected

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Table 1. Localities and their coordinates from which sand flies were collected.

Locality	N	E
Al Adasyeha	32° 40' 08''	35° 37' 30''
Al Disah	29° 41' 50''	35° 23' 40''
Al Jafr	30° 18' 52''	36° 10' 00''
Al Lajune	31° 14' 20''	35° 52, 49''
Al Marazah	32° 24' 47''	35° 35' 19''
Al Mashareh	32° 25' 49''	35° 35' 30''
Al Quairah	29° 46' 53''	35° 18' 10''
Al Rameh	31°49' 00''	35° 43' 13''
Al Rishah	30° 13' 29''	35° 13' 07''
Al Ruwyshed	32° 30' 38''	38° 12' 09''
Azraq	31° 51' 26''	36° 49' 40''
Beer Madkour	30° 26' 27''	35° 17' 08''
El Hemma	32° 26' 34''	35° 35' 47''
Ghor Fifa	30° 55' 38''	35° 27' 36''
Halawa	32° 22' 47''	35° 41' 32''
Jisr Al Shohada	30° 57' 42''	35° 44' 29''
Malka	32° 39' 40''	35° 43' 09''
Osarah	32° 23' 10''	35° 41' 15''
Rahmeh	29° 54' 46''	35° 08' 34''
Sahab	31° 54' 04''	36° 01' 47''
Sehan	32° 11' 28''	35°43' 40''
Swaymeh	31° 48' 15''	35° 35' 44''
Tabaqat Fahl	32° 26' 46''	35° 36' 10''
Yarqa	31° 58' 48''	35° 42' 00''
Zarqa	32° 02' 54''	36° 04' 05''

3. Results

A total of five species of sand flies belonging to the genus *Phlebotomus* was identified.

Phlebotomus alexandri (Sinton, 1928)

Material examined (23): El Hemma (1), 19.7.2009. Halawa (1), 11.8.2009. Jisr Al Shohada (3), 17.8.2009. El Hemma (3), 1.10.2009. Tabaqat Fahl (1), 1.10.2009. Al Marazah (1), 1.10.2009. Al Mashareh (5), 1.10.2009. Al Rameh (1), 26.11.2013. Rahmeh (2), 14.5.2014. Al Adasyeha (4), 20.5.2014. Ghor Fifa (1), 10.9.2014.

Remarks: Figure 2 shows the distribution of *Ph. alexandri.* This species was collected previously from Tabaqat Fahl (Lane *et al.*, 1988), and from Jarash, Amman. Petra, El Hemma, Tabaqat Fahl, Wadi Dhulail, Ras en Naqb, Swaima, Aqaba, South Shounah, and Tafileh mountains (Kamhawi *et al.*, 1995). It was found to be associated with different types of biogeographical regions including the Meditternian, Irano-Turanian and the Saharo-Arabian.

Phlebotomus major syriacus (Adler & Theodor, 1958)

Material examined (68): El Hemma (1), 19.7.2009. Halawa (5), 11.8.2009. Al Mashareh (13), 1.9.2010. Halawa (1). 15.9.2009. Azraq (1), 29.10.2009. Malka (39), 8.10.2009. Sehan (1), 21.11.2013. Malka (5), 20.5.2014. Osarah (1), 26.5.2014. Sahab (1), 21.8.2014.

Remarks: *Phlebotomus major syriacus* was found in the northern Ghor area to Azraq. It was found along with *Phlebotomus alexandri* in El Hemma in the Northern Ghor. However, it is mostly associated with the Ajlun and Irbid highlands (Figure 3). Lane *et al*. (1988) listed a number of localities for this species, including Barha, Hawarrah, Umm Qais, Ras el Naqb and South Shounah. Other reported localities include Ajlun, Salt, Irbid, Jarash, Petra, El Hemma, Umm Qais, Ras en Naqb and Swaima (Kamhawi *et al*. 1995).

Phlebotomus mascitti canaaniticus (Adler and Theodor, 1931)

Material examined (3): Halawa (2), 11.8.2009. Al Adasyeha (1), 20.5.2014.

Remarks: *Phlebotomus mascitti canaaniticus* was collected during this study in low numbers from the upper northern Jordan Valley and Ajlun area. It was reported from Ajlun, Petra, El Hemma and Swaima (Kamhawi *et al.*, 1995). Figure 4 shows the distribution of *Ph. m. canaaniticus.*

Phlebotomus papatasi (Scopoli, 1786)

Material examined (1085): El Hemma (2), 29.5.2009. El Hemma (15), 19.7.2009. El Hemma (7), 17.8.2009. El Hemma (9), 30.8.2009. El Hemma (17), 1.10.2009. Tabaqat Fahl (1), 29.5.2009. Tabaqat Fahl (12), 19.7.2009. Tabaqat Fahl (21), 17.8.2009. Tabaqat Fahl (3), 30.8.2009. Tabaqat Fahl (9), 1.10.2009. Al Marazah (2), 29.5.2009. Al Marazah (5), 19.7.2009. Al Marazah (6), 17.8.2009. Al Marazah (6), 30.8.2009. Al Marazah (11), 1.10.2009. Al Mashareh (12), 29.6.2009. Al Mashareh (18), 31.7.2009. Al Mashareh (37), 17.8.2009. Al Mashareh (18), 30.8.2009. Al Mashareh (37), 1.10.2009. Al Mashareh (3), 27.10.2009. Al Mashareh (1), 31.12.2009. Al Mashareh (13), 1.9.2010. Swaymeh (18), 27.7.2009. Swaymeh (30), 3.8.2009. Azraq (7) , 29.10.2009. Jisr Al Shohada (61), 17.8.2009. Yarqa (56), 7.9.2009. Al Quairah (17), 29.9.2009. Malka (50), 8.10.2009. Halawa (22), 11.8.2009. Halawa (29), 24.8.2009. Halawa (104), 15.9.2009. El Hemma (8), 18.11.2009. Sehan (5), 21.11.2013. Al Rameh (4), 26.11.2013. Beer Madkour (7), 15.4.2014. Rahmeh (15), 14.5.2014. Malka (38), 20.5.2014. Al Adasyeha (26), 20.5.2014. Osarah (3), 26.5.2014. Halawa (3), 27.5.2014. Azraq (21), 28.5.2014. Al Rishah (5), 3.6.2014. Al Disah (17), 4.6.2014. Al Quairah (13), 4.6.2014. Al Lajune (7), 9.6.2014. Jisr Al Shohada (19), 9.6.2014. Zarqa (15), 22.6.2014. Al Jafr (3), 24.6.2014. Al Damkhi (2), 30.6.2014. Al Mowaqar (3), 6.7.2014. Al Ruwyshed (4), 14.8.2014. Aqraba (2), 18.8.2014. Sahab (3), 21.8.2014. Ghor Fifa (2), 10.9.2014.

Remarks: *Phlebotomus papatasi* was the most common species with a distribution that covers most of the study sites. This species was found in almost all types of habitats in the Jordan Valley, southern Jordan as well as in Azraq area to the East. It was collected from Barha, Bushra, Hawarrah, Umm Qais, Ras el Naqb, Swaima, Azraq and South Shounah (Lane *et al.*, 1988). Kamhawi *et al.* (1995) included localities from several localities extending from Irbid to Aqaba and the Jordan Valley (Figure 5).

Phlebotomus sergenti (Parrot, 1917)

Material examined (49): El Hemma (1), 1.10.2009. Tabaqat Fahl (2), 1.10.2009. Al Marazah (1), 1.10.2009. Al Mashareh (4), 1.10.2009. Yarqa (8), 7.9.2009. Malka (13), 8.10.2009. Halawa (5), 11.8.2009. Halawa (3), 24.8.2009. Sehan (1), 21.11.2013. Malka (2), 20.5.2014. Osarah (1), 26.5.2014. Halawa (2), 27.5.2014. Al Disah (1), 4.6.2014. Al Quairah (4), 4.6.2014. Jisr Al Shohada (1), 9.6.2014.

Remarks: *Phlebotomus sergenti* was found in the Jordan Valley, Balqa highlands and Wadi Rum area (Figure 6). *Ph. sergenti* is known to be highly anthropophilic, but can also be found in rural habitats. Collected from Barha, Bushra and Azraq (Lane *et al*., 1988).

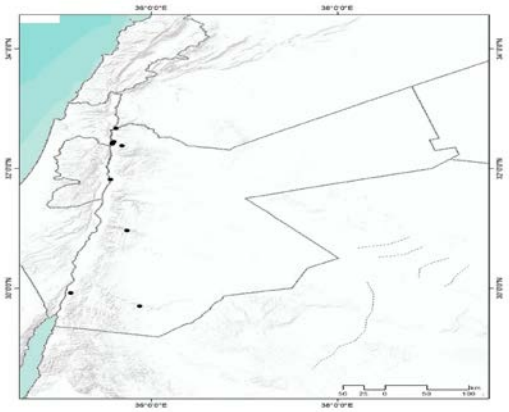

Figure 2. Distribution of *Phlebotomus alexandri* in Jordan

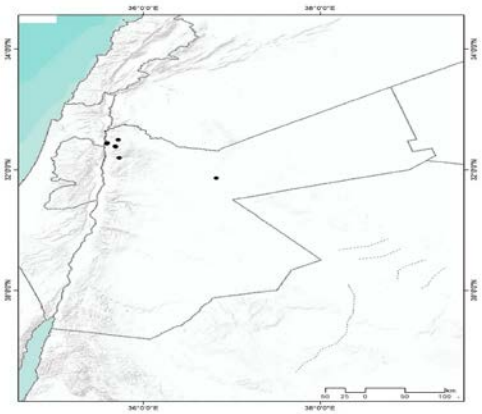

Figure 3. Distribution of *Phlebotomus major syriacus* in Jordan

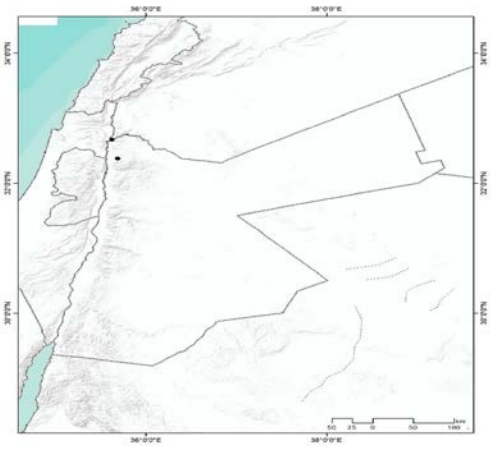

Figure 4. Distribution of *Phlebotomus mascitti canaaniticus* in Jordan

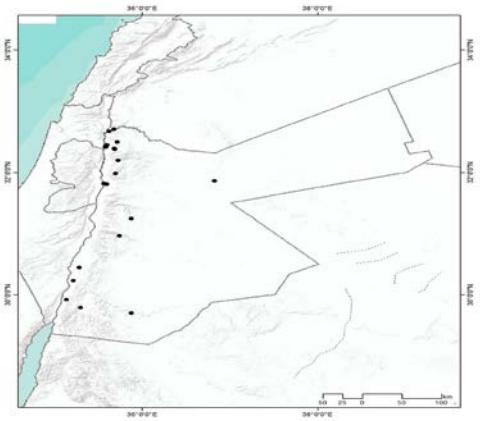

Figure 5. Distribution of *Phlebotomus papatasi* in Jordan

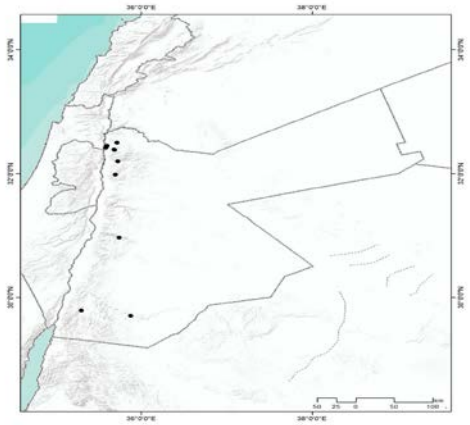

Figure 6. Distribution of *Phlebotomus sergenti* in Jordan

4. Review of sand flies of Jordan of the genus Phlebotomus

In Jordan, the genus *Phlebotomus* includes 11 species (Table 2). The first record of sand flies from this country was indicated in Adler and Theodor (1929), where they reported *Ph. sergenti* and *Ph. papatasi*. The first study by locals on the sand flies was published by Oumeish *et al*. (1982) who reported the presence of one species, *Ph. papatasi*, from several locations. Later, Lane *et al*. (1988) conducted a comprehensive study that covered several bioclimatological regions, and reported 13 species of sand flies (six *Phlebotomus* and 7 *Sergentomyia*). Kamhawi *et al*. (1988) reported on the sand flies of Aqaba area, and recorded eight species, three of which belong to the genus *Phlebotomus*. In 1991, Kamhawi *et al*. studied the sand flies of Ras el Naqab, and reported a total of nine species (5 *Phlebotomus* and 4 *Sergentomyia*). In a large-scale study, Kamhawi *et al*. (1995) studied the sand flies fauna of the country and reported a total of 21 sand fly species including additional records (*Phlebotomus jacusieli, Ph. tobbi, Ph. perfiliewi galilaeus, Ph. mascittii, Ph. arabicus, Ph. halepensis, Sergentomyia palestiniensis* and *S. toizi*).

Eleven species of sand flies were recorded in the southern Jordan Valley, including *P. kazeruni*, *P. tohbi* and *Sergentomyia squamipleuris*. *Phlebotomus papatasi* was the most abundant species collected from domestic habitats (Janini *et al*., 1995). Table 2 lists the known species of *Phlebotomus* sand flies in Jordan to which we added the records noted above.

Table 2. Records of species of the genus *Phlebotomus* in Jordan

Species	Localities	References
Ph. alexandri (Sinton, 1928)	Barha, Tabaqat Fahl, Hawarrah, Ras el Naqb, South Shounah, Umm Qais	Lane *et al.* (1988)
	Aqaba	Kamhawi *et al.*
	Ras el Naqb	(1988)
	Amman, Aqaba, Jarash, El Hemma, Petra, Ras el Naqb, South Shounah, Swaima, Tabaqat	Kamhawi *et al.*
	Fahl, Wadi Dhulail	(1991)
		Kamhawi *et al.*
		(1995)
Ph. arabicus Theodor, 1953	Ajlun, El Hemma, Petra, Ras en Naqb.	Kamhawi *et al.*
		(1995)
Ph. halepensis Theodor, 1958	Ajlun, El Hemma, Petra, Ras en Naqb.	Kamhawi *et al.*
		(1995)
Ph. jacusieli Theodor, 1947	Ajlun, Amman, El Hemma, Irbid, Jarash, Petra, Ras en Naqb, Shoubak.	Kamhawi *et al.*
		(1995)
Ph. kazeruni Theodor & Mesghali,	Azraq, Ras el Naqb, Aqaba	Lane *et al.* (1988)
1964	Aqaba	Kamhawi *et al.*
	Ras el Naqb.	(1988)
	Irbid, Jarash, Shoubak	Kamhawi *et al.*
		(1991)
		Kamhawi *et al.*
		(1995)
Ph. major syriacus Adler & Theodor,	Jordan	Perfil'ev (1968)
1958	Barha, Hawarrah, Ras el Naqb, South Shounah, Umm Qais	Lane *et al.* (1988)
	Ajlun, El Hemma, Irbid, Jarash, Petra, Ras en Naqb, Salt, Swaima, Umm Qais	Kamhawi *et al.*
		(1995)
Ph. mascittii Grassi, 1908	Ajlun, El Hemma, Petra, Swaima	Kamhawi *et al.*
		(1995)
Ph. papatasi (Scopoli, 1786)	Awajan, Sweilah	Lewis (1982)
	Swaima	Oumeish *et al.*
	Mowoqqar	(1982)
	Ajlun, Amman, Azraq, Barha, Bushra, Hawarrah, Irbid. Jarash, Ras el Naqb, South Shounah,	Saliba *et al.* (1985)
	Swaima, Umm Qais	Lane *et al.* (1988)
	Aqaba	Kamhawi *et al.*
	Ras el Naqb.	(1988)
	Aqaba, Azraq, El Hemma, Ghor el Safi, Ras en Naqb, South Shounah, Swaima, Tabaqat	Kamhawi *et al.*
	Fahl, Wadi Dhulail	(1991)
		Kamhawi *et al.*
		(1995)
Ph. perfiliewi galilaeus Theodor, 1958	Irbid, El Hemma.	Kamhawi *et al.*
		(1995)
Ph. sergenti Parrot, 1917	Awajan.	Lewis (1982)
	Mowoqqar	Saliba *et al.* (1985)
	Barha, Bushra and Azraq	Lane et al. (1988)
	Ras el Naqb	Kamhawi *et al.*
		(1991)
Phlebotomus tobbi Adler and Theodor,	El Hemma, Irbid, Ras en Naqb, Umm Qais	Kamhawi *et al.*
1934		(1995)

5. Discussion

Five species of sand flies were identified during the present study. Our results are consistent with those reported by Lane et al. (1988). For example, *Ph. major syriacus* was the dominant *Phlebotomus* species in Ajlun Mountains and Balqa, where it was collected from domestic as well as rural habitats. Also, *Ph. sergenti* was the least common species, while *Ph. papatasi* was the dominant species.

The distribution of sand flies may vary according to season. Lane *et al.* (1988) recorded *Ph. alexandri, Ph. sergenti* and *Ph. major syriacus* in Tabaqt Fahel in the Jordan Valley, while we reported *Ph. alexandri, Ph. papatasi* and *Ph. sergenti* from the same locality. Similarly, we recorded *Ph. papatasi* and *Ph. major syriacus*, while Lane *et al.* (1988) found *Ph. papatasi* and *Ph. sergenti* from Al Hemma.

Phlebotomus papatasi is considered the most important vector for *L. major* in Jordan (Saliba *et al.*, 1985; Janini *et al.*, 1995). Its distribution overlaps with the distribution of human cases. On the other hand, *Ph. sergenti* is the main vector for *L. tropica* in Bani Kananah area. This is in accordance with the present distribution of *Ph. sergenti*. Elsewhere, Orshan *et al.* (2010) stated that *Ph. segenti* is the most common outdoor species in the Judean Desert, while Sawalha *et al.* (2003) found that *Ph. perfiliewis* was the most common species in the areas of the West Bank of Jordan.

Further studies should address the distribution and bionomics of the sand flies of Jordan for several seasons. Identification manual for the sand flies of Jordan is urgently needed in addition to training entomologists for the use of identification keys.

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Partial Purification and Characterization of 2,4-Dichlorophenoxyacetic Acid Degrading Bacteria Harboring Alpha Ketoglutarate Dioxgenase Enzyme

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Abstract

The catabolic adaptability demonstrated by microorganisms is important in the bioremediation of polluted environments. 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase is the first enzyme in the catabolic pathway for the 2,4-dichlorophenoxyacetic acid (2,4-D). In the present study, dioxygenase enzymes were inducible and the serial adaptation of four studied bacterial isolates steadily evolved strains exhibited higher and significantly different rates of 2,4-dichlorophenoxyacetic acid metabolism. For biomass production relative to the original starting strain, 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase was partially purified to apparent homogeneity from four bacterial isolates that were studied, using ammonium sulphate precipitation and gel filtration on sephadex G-100. 2,4-dichlorophenoxyacetic acid concentration resulted in a lower yield of the cell biomass of the *in vitro* culture of each of the bacterial isolates and consequently a lower enzyme yield. There was a gradual increase in the enzyme activity from the different bacterial isolates at pH 5.5, maximum enzymes activity at pH 7.5 and no activity at pH 10.0. Optimum temperature for enzyme activity was at 35^{o}C but activity was reduced to zero at 60^{o}C. In conclusion the four bacterial isolates and their cell-free extracts have the potential to bio-mineralized 2,4-dichlorophenoxyacetic acid..

Keywords: 2,4-Dichlorophenoxyacetic Acid; Dioxygenase; α-Ketoglutarate; Catalytic Properties; Enzyme Activity.

1. Introduction

Chlorinated aromatic compounds are environmental pollutants due to their widespread use in the ecosystem. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a chlorinated phenoxy compound which functions as a systemic herbicide and is used to control many types of broadleaf weeds. The wide use of this compound has prompted interest in its biodegradation (Igbinosa, 2006; Igbinosa *et al.*, 2007a,b). *Alcaligenes eutrophus* and other bacteria can degrade 2,4-D through 2,4-dichlorophenol, 3,5-dichlorocatechol, and *ortho* cleavage of this catechol (Fukumori and Hausinger, 1993). Alternatively, *Axotobacter chroococcum* first removes the chloride in the 2 position to produce 4-chlorophenoxyacetate, 4-chlorophenol, and 4-chlorocatechol, again with *ortho* cleavage of this catechol (Balajee and Mahadevan, 1990). 2,4-D biodegradation, by this pathway branch, may produce a byproduct antibiotic protoanemonin, which can be degraded to cis-acetylacrylate by a dienelactone hydrolase of *Pseudomonas* sp. strain B13 (Brückmann *et al.*, 1998). *Comamonas testosteroni* JH5 can cleave 4-chlorocatechol by a *meta* pathway, forming 5-chloro-2-hydroxymuonate semi aldehyde which can be further transformed by a 2-hydroxymuconic semi-aldehyde dehydrogenase or a 2-hydroxymuconic semi-aldehyde hydrolase. Dehydrogenation is the major route, however; the hydrolase step is also of physiological significance. Both branches lead to 5-chloro-2-oxopent-4-eneoate (Selifonov, 1992). *Pseudomonas cepacia* P166 can move the chloride in 5-chloro-2-oxopent-4-eneoate through 5-chloro-4-hydroxy-2-oxopentanate to chloroacetate. Chloroacetate accumulates transiently, and stoichiometric dehalogenation is observed (Arensdorf and Focht, 1995).

Alcaligenes eutrophus JMP 134 is the most extensively studied 2,4-D degrader (Don and Pemberton, 1981). *A. eutrophus* JMP 134 catalyzes the conversion of 2,4-D to 2,4-dichlorophenol (2,4-DCP) by the action of the product of 2,4-dichlorophenoxyacetic acid/α-ketoglutarate dioxygenase (*tfdA*). TfdA catalyzes a hydroxylation reaction and it has been repeatedly reported to be 2,4-D monoxygenase, which in fact is not the case (Hausinger and Fukumori, 1995). Rather, *tfdA* is a Fe(II) and α-ketoglutarate-dependent dioxygenase. 2,4-D/α-ketoglutarate dioxygenase is an Fe(II) and α-ketoglutarate-dependent enzyme that catalyzes the first step in degradation of the herbicide 2,4-D. This enzyme

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couples the oxidative decarboxylation of α-ketoglutarate to the hydroxylation of a side chain carbon atom. The resultant hemiacetal spontaneously decomposes to form 2,4-dichlorophenol (2,4-DCP), succinate, glyoxalate and carbon dioxide (Hogan *et al.,* 2000). The enzyme *tfdA* possesses multiple essential histidine residues, whereas catalytically essential cysteine and lysine groups do not appear to be present (Hogan *et al.,* 2000). *TfdA* resembles numerous other *a*-ketoglutarate-dependent dioxygenases from plants, animals, fungi, and bacteria that catalyze similar hydroxylation reactions at inactivated carbon center. Petroleum degrading bacteria were isolated and biochemical characterizes by Sanni and Ajisebutu (2003a, b). Igbinosa *et al.* (2007a, b) assessed their potentials in the mineralization of 2,4-dichlorophenoxyacetic acid in an attempt to develop active bacterial strains for bioremediation of 2,4-D contaminated system. The present paper describes the partial purification and biochemical characterization of 2,4-D / α-ketoglutarate dioxgenase from four petroleum degrading bacteria.

2. Materials and Methods

2.1. Bacteria Strains and Culture Condition

Characterization of the bacterial strains (SOGU 11, SOGU 16, SERU 2 and SERU 11), identified as *Achromobacter* sp, *Corynebacterium* sp, *Pseudomonas* sp and *Arthobacter* sp, respectively, using a standard culture-based and biochemical reaction, was previously described by Sanni and Ajisebutu (2003a, b). All bacterial isolates were grown in Luria-Bertani (LB)-medium, which contained, per liter: 5g yeast extract, 10g casein peptone, 10g NaCl, pH was adjusted to 7.0 before autoclaving. Subsequently, 10% inocula from LB cultures were transferred to 50 mL freshly prepared Minimal Salts Medium (MSM) containing, per liter, 2.75g K_2HPO_4; 2.25g KH_2PO_4; 1.0g $(NH_4)_2SO_4$; 0.2g $MgCl_2.6H_2O$; 0.1g NaCl; 0.02g $FeCl_3.6H_2O$ and 0.01 $CaCl_2$ (pH 6.8 to7.0) with 5 mM 2,4-D as sole carbon and energy sources. Erlenmeyer flask containing 500 mL of the medium was incubated at 30°C on a rotary shaker at 200 rpm.

2.2. Serial Adaptation of Isolates Between Glucose and 2,4-Dichlorophenoxyacetic Acid (2,4-D)

The standardized bacterial isolates were inoculated into minimal salt medium enriched with 5 mM 2,4-D. The flasks containing the medium were incubated on a horizontal shaker at 200 rpm for 24 h at room temperature (28 ± 2°C). The cultures were later harvested as previously described by Igbinosa (2006) and the bacterial isolates were designated as A_o generation. These A_o generation bacteria were re-cultured in 5mM glucose-enriched medium, harvested and then used to inoculate another fresh medium enriched with 2,4-D. The cultures were then incubated and harvested to generate the A_1 generation. This procedure was repeated to produce the A_2, A_3, and A_4 generations and, in each case, the cultures were harvested by centrifuging at 3,500 × *g* for 15 min, washed in sterile normal saline solution (0.85% w/v NaCl) and finally stored in sterile normal saline solution. The different generations were standardized to an optical density of 0.1 values using a spectrophotometer at 540 nm wavelength. The standardized A_o, A_1, A_2, A_3 and A_4 generations were then cultivated in another batch of minimal salts medium enriched with 5 mM 2,4-D. The flasks were incubated on horizontal shaker (200 rpm) at room temperature and sampled for growth of the bacterial isolates at different time intervals by taking the optical density reading. This was done using a spectrophotometer at 540 nm wavelength and the growth pattern monitored.

2.3. Assay of 2,4-D/α–ketoglutarate Dioxygenase Enzyme

The cells of each bacterial isolate were grown at 30°C in Peptone Tryptone Yeast extract Glucose Medium (PTYG Medium) This medium consists of 0.25g peptone, 0.25g tryptone, 0.5g yeast extract, 0.5g glucose, 0.03g $MgSO_4$, 0.0035g $CaCl_2$ per liter (pH 7.2) with 2.5 mM 2,4-D. Each of the bacterial cells was harvested by centrifugation at 6,000 × *g* for 20 min and suspended in a solution containing 25 ml of 20 mM Tris, 1 mM EDTA, and 0.4 mg/l leupeptin (pH 7.2). The assay was carried out according to Fukumori and Hausinger (1993). A 0.2 mL of each of the suspended bacterial isolate solution above was added to 1.0 mM α–ketoglutarate, 50.0 µm ascorbate, 50.0 µm $(NH_4)_2$ Fe $(SO_4)_2$, and 1.0 mM 2,4-D in 10.0 mM imidazole buffer (pH 6.75), incubation was at 30°C for 1 min. The reaction was terminated with the addition of Ethylenediamine tetra acetic acid (EDTA). The absorbance was measured at 510 nm after the addition of 10.0 µL of 8% potassium ferricyanide. One unit of enzyme activity was defined as the amount of enzyme that formed 1.0 µmol of phenol derivative per minute at 30°C.

Enzyme activity unit [µmol phenol min^{-1} ml^{-1}]

$$= \frac{OD_{510} \times Df}{10\ \mu mol \times V \times t}$$

where OD_{510} = absorbance reading

V = Volume of enzyme used

t = time of incubation = 1 min

Df = Dilution factor of the enzyme solution

2.4. Protein Determination

Protein concentrations of the crude extract were determined by the method of Lowry *et al*. (1951). Protein concentration was extrapolated from the standard curve using Bovine Serum Albumin (BSA) as standard.

2.5. Extraction of the Crude Enzyme

The clear crude extract was brought to 15% ammonium sulphate saturation by the addition of solid ammonium sulphate (84 g/L). The precipitate formed was collected by centrifugation at 12,000 × *g* for 20 min, and was discarded. The supernatant was taken up to 65% saturation by adding ammonium sulphate (350 g/L) over a period of 1 h with continuous stirring and left overnight. The resulting precipitate was collected by centrifugation at 6,000 × *g* for 30 min and immediately dialyzed against several changes of 50 mM citrate buffer (pH 5.0) containing 10mM $Na_2S_2O_3$. The dialysate was centrifuged at 6,000 × *g* for 30 min to remove insoluble material (Igbinosa, 2006).

2.6. Determination of Molecular Weight of the Crude Enzyme

The molecular weight of the native enzyme was estimated by gel filtration on a sephadex G-100 column (40 × 2.5cm) in 20 mM Tris, 1.0 mM EDTA, 100 mM NaCl (pH 7.2). The standard proteins used in calibrating the column were bovine serum albumin Mr, 68,000; 7.0 mg/mL, egg albumin Mr, 45,000; 10.0 mg/mL, trypsinogen Mr, 24,000; 10.0 mg/mL, catalase Mr, 58,000; 10.0 mg/mL. A volume of 2.0 mL of each of these protein standards was passed through the column in successions, at a flow rate of 40.0 mL/h. Fractions of 3.0 mL were collected and elution was monitored by measuring absorbance at 280 nm for each protein standard. The void volume (V_o) of the column was determined with Blue dextran 2000 (2.0 mg/mL) and its elution was monitored by measuring absorbance 600 nm. According to Andrew (1964), the K_{av} value of each eluted protein was calculated from the equation.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume of protein, which is measured from the start of the sample application to the inflection point (or the half height) of the rising part of the elution peak.

V_o = void volume, which is the elution volume of blue dextran.

V_t = total gel bed volume, calculated as the product of the cross sectional area and the bed height.

The K_{av} values of the protein standard were plotted against their corresponding molecular weight on a semi log graph sheet. The molecular weight of the enzyme was extrapolated from its K_{av} value.

2.7. Determination of Kinetic Parameters

The kinetic parameters (V_{max} and K_m) were determined for the crude enzyme 2,4-D/α-ketogluarate dioxygenase activity at various concentrations of 2,4-D from 0.01 mM - 2.0 mM. The apparent K_m and V_{max} of the enzyme for 2,4-D were determined by plotting the reciprocals of the initial velocities against the reciprocal of 2,4-D concentration (Lineweaver-Burk 1934).

2.8. Effect of pH and Temperature on Enzyme Activity

The effect of pH on the enzyme was determined by carrying out the assay of the enzyme using the following 3 buffers at different pH values, 0.2 M citrate buffer (pH 4.0 - 6.0), 0.2 M phosphate buffer (pH 6.5 - 8.5), and 0.2 M Borate buffer (pH 8.0 - 10.0) but at constant ion strength of 0.05 M, in assay mixture.

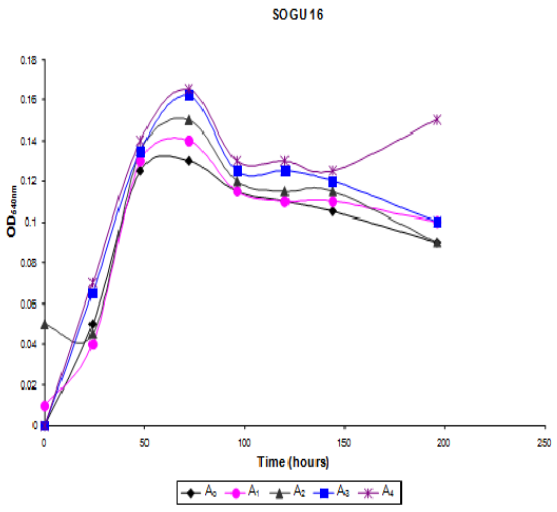
2.9. Stability of Enzyme Activity

For the pH stability test, the enzyme was incubated at different pH at 30°C for up to 40 h and the residual activity was measured after readjustment of the pH to 7.0. To determine the effect of temperature on the stability of the enzyme, the enzyme solution was incubated in 100mM (pH 7.0) buffer for up to 40 h at various temperatures between (4, 20, 40 and 60°C), then the residual enzyme activity was assayed. To examine the optimum temperature for enzyme activity, this was measured at various temperatures (20, 30, 35, 40, 45, 50 and 60°C).

3. Results and Discussion

3.1. Serial Adaptation of the Isolates to 2,4-D

The ability of each bacterial isolate to grow in the 2,4-D enriched media may be due to a selection process leading to the emergence of strain, which is subsequently able to resist the toxic effect of the compound. The successive growth yield in SOGU16 (*Corynebacterium* sp) showed highest yield of 0.165 cells/h that was observed in 5th generation at 72 h with the least yield being 0.00 cells/h that was observed in the 1st generation at zero hour (Fig. 1). In SOGU11 (*Achromobacter* sp), maximum yield of 0.165 cells/h was observed in the 5th generation at 72 h and a minimum yield of 0.01 cells/h in the 1st generation at zero hour (Figure 1). In SERU11 (*Arthrobacter* sp), isolate maximum growth yield of 0.150 cells/h was observed in the 5th generation at 120 h and a minimum yield of 0.05 cells/h in the 1st generation at zero hour (Figure 2). In SERU2 (*Pseudomonas* sp), isolate maximum growth yield of 0.185 cells/h was observed in the 3rd and 4th generation at 48 h while a minimum growth yield of 0.00 cells/h was observed in the 1st generation at zero hour (Figure 2). In all the isolates, there was no significant variation between the different generation times ($p < 0.05$). The ability of bacterial isolate to grow in the 2,4-dichlorophenoxyacetic acid enriched media may be due to a selection process leading to the emergence of strain (mutants) which are subsequently able to resist the toxic effect of the compound. It was reported that Dichloroacetic acid (DCA) inhibit *Pseudomonas putida* pp3 which are sensitive (DCA^S), but DCA resistant mutants (DCA^R) arise readily if the growth environments contains between 5 and 100 mM DCA. The results of the growth yield of different generations of the bacterial isolates obtained by serial adaptation to the 2,4-dichlorophenoxyacetic acid revealed that the subsequent generations obtained showed steady increase in rate of metabolism for biomass production through the series of generations. In general, the more distant the generation was from the original stains, the higher the yield became, which were significantly different ($p < 0.05$) from each other.



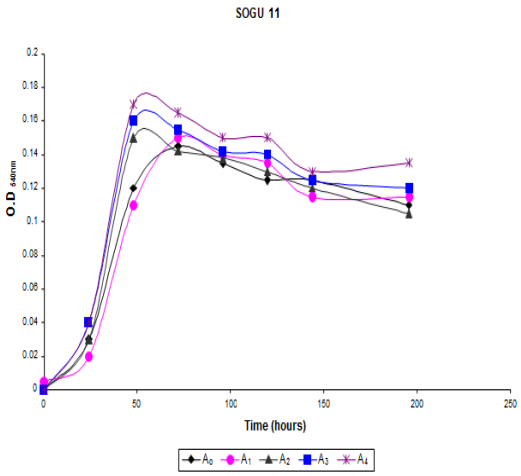


Figure 1. Trends in the growth of the S OGU16 and isolates at different generations (Ao A4, shows 1st to 5th generation, respectively)

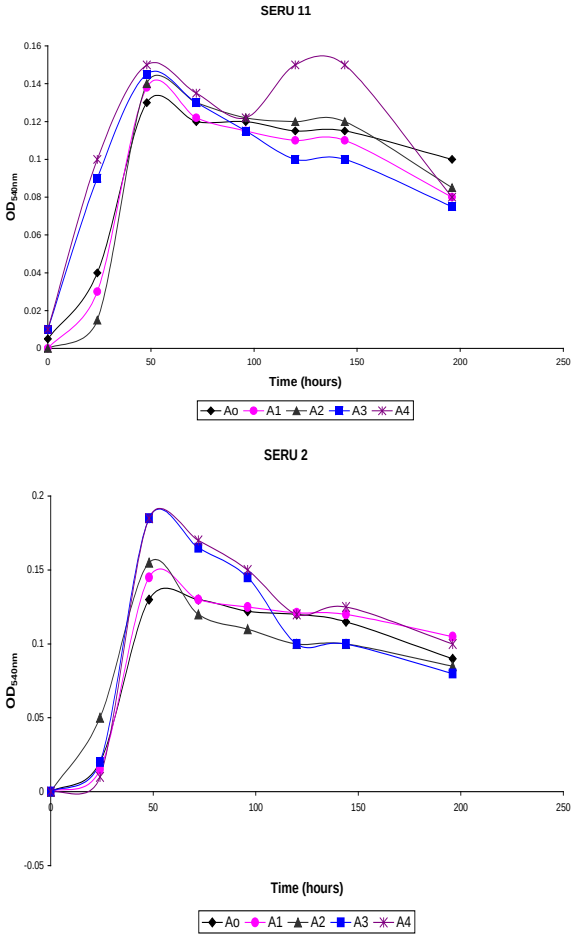


Figure 2. Trends in the growth of the SERU 11 and SERU 2 isolates at different generations (Ao – A4, shows 1st to 5th generation, respectively).

3.2. Partial Purification of the Crude Enzyme

A summary of the result of the partial purification of the 2,4-D\α-ketoglutarate dioxygenase from the four different bacteria isolates (SOGU 16, SOGU 11, SERU 11 and SERU 2 *Corynebacterium* sp, *Achromobacter* sp, and *Arthrobacter* sp, *Pseudomonas* sp respectively) are presented in Table 1. The presence of protease inhibitor during the early stages of 2,4-D/α–ketogulatarate dioxygenease partial purification enhanced the stability of the enzyme. The partial purification gave a low specific activity of the enzyme. The loss of activity during the affinity chromatography step seemed to differ from those of previous reports for *Alcaligenes eutrophus* JMP134 (Fukumori and Hausinger, 1993) and *Psendomonas cepacia* CSV90 (Bhat *et al.*, 1993). This might be attributed to the fact that some of the proteins removed might be involved in the stability of the enzyme as reported by Fersht (1989), although the step gave a good increase in the partial purification fold. The specific activity of the partial purified bacterial isolate, as shown on (Table 1), was lower than that obtained for *A. eutophics* JMP134 (16.9 units/mg protein) (Fukumori and Hausinger 1993). However, the partial purified 2,4-D/α-ketoglutarate dioxygenase was stable at 4°C. This was similar to *A. eutrophus* JMP134 and *P cepacia* CSV90 as reported by Fukumori and Hausinger (1993) and Bhat *et al.* (1989), respectively.

Table 1. Partial purification of 2,4-D/α-ketoglutarate dioxygenase

Procedure	Total volume (ml)	Total protein (mg)	Total activity (µmol/min)	Specific activity µmol/ min/mg rotein)	Purification fold	% yield
Crude Extract						
SOGU 16	600	950.0	1750	1.84	1	100
SOGU 11	600	878.1	1725	1.96	1	100
SERU 11	600	980.2	1758.0	1.79	1	100
SERU 2	600	967.4	1980.1	2.05	1	100
Ammonium Sulphate precipitation (70 %)						
SOGU 16	100	318.4	1210	3.80	2.07	69.0
SOGU 11	110	305.2	1208.6	3.96	2.02	70.0
SERU 11	110	310.0	1150.1	3.71	2.07	65.0
SERU 2	110	295.0	1301.0	4.41	2.15	66.0
Sephadex G-100						
SOGU 16	75	245.1	1011.5	4.132	2.24	58.0
SOGU 11	75	110.1	1094.6	5.21	2.83	63.0
SERU 11	80	195.6	980.0	5.01	2.80	56.0
SERU 2	65	189.2	966.8	5.11	2.50	49.0

3.3. Effect of pH on Enzyme Activity

The residual enzymatic activity of the different bacterial isolates after incubation at various pH values was determined by using standard assay condition. The enzymes from the different bacterial isolates showed a gradual increase from pH 5.5 to a maximum at 7.5; there was no activity at pH 10.0. Hausinger and Fukumori (1995) reported that optimum pH for 2,4-D is 6.5 to 7.5. Also, an optimum pH of 8.0 was reported for 2-monochloropropionic acid and 2,2-dichloropropionic acid (Slater, 1992). It would appear that chlorination process

is favored in an alkaline medium. It is suggestive that a change in pH alters the state of ionization of charged amino acids that may play crucial roles in substrate binding or the catalytic event itself, as well as decreasing the saturation of the enzyme with substrate as a result of decrease in affinity.

3.4. Effect of Temperature on Enzyme Activity

The optimum temperature for the enzyme activity was 35°C. The activity reduced to zero at 60°C. The optimum temperature of 2,4-D dioxygenase activity was between 30°C and 35°C, which fell within the range reported by Hausinger and Fukumori (1995). Microorganisms, found effective in bioremediation, have been shown to perform well in the temperature range of 10°C- 40°C (Cookson, 1995). The effect of temperature on the enzyme catalyzed reaction revealed that the enzyme has its optimum catalysis at temperature of 35°C. Temperatures of 30°C and 35°C have been reported for an optimum activity of dioxygenase enzyme (Bhat, 1993).

3.5. Stability of Enzyme Activity

Maintaining low temperatures during bacterial growth and enzyme isolation is important for obtaining high levels of activity. It was observed that the colonies grown at higher temperatures were turbid, whereas those grown at a moderate temperature remained clear. The partially purified 2,4-D/α-ketoglutarate dioxygenase was found to be very sensitive to thermal inactivation. At a protein concentration of 0.5 µm, 2,4-D/α-KG dioxygenase enzyme remained stable only up to 30°C and is completely inactivated within 10 min at 40°C. Loss of the enzyme activity, at relatively high temperatures, may also be related to the denaturation of the enzyme at such temperatures since enzymes are proteins and may be denatured at certain temperatures (Martin *et al.*, 1983; Hausinger and Fukumori, 1995).

3.6. Catalytic Properties of 2,4-D/α-Ketoglutarate Dioxygenase

Activity of 2,4-D/α-KG dioxygenase requires ferrous ion and is enhanced by ascorbic acid. The ferrous ion requirement could not be met by other divalent metal ion including Co(II), Cu(II), Li(II), Mg(II), Mn(II), Ni(II), and Zn(II) when added as sulfate salt at a concentration of 100 µm. There was a time-dependent decrease in the enzyme activity and ferrous ion alone was unable to sustain enzyme catalysis over long-time periods. The rate of activity loss was greatly reduced by the inclusion of ascorbic acid in the assay. The specific requirement for α-KG and Fe(II), and the stimulation of activity by ascorbate, are typical characteristics of α-KG-dependent-dioxygenases (Abbott and Udenfriend, 1974).

3.7. Molecular weight Estimation by Sephadex G–100 Chromatography

The molecular weight of 2,4-D/α-ketoglularate dioxygenase in the different bacterial isolates was estimated by running the enzyme through a standardized sephadex G–100 column and estimated to be $M_{r,}$ 36,000 ± 1,400; $M_{r,}$ 35,000 ± 765; $M_{r,}$ 40,000 ± 1,850 and $M_{r,}$ 36,000 ± 950 for SOGU 16, SOGU 11, SERU 11 and SERU 2, respectively, for the native enzyme. These findings differ from the findings in the work of Fukumori and Hausinger (1993) who reported the native enzyme to be M_r, 50,000 ± 2,500 and from those in the work of Bhat *et al.* (1993) where the molecular weight of the native enzyme was given as $M_{r,}$ 56,000 by light scattering method. It was suggested that the conversion was found to occur after cell disruption rather than during the cell cultivation. This same procedure was used here to demonstrate that the molecular weight of the protein present in *A. eutrophus* JMP134 cell extract was $M_{r,}$ 32,000.

3.8. Kinetic Parameters

The kinetic parameters of each of the bacterial isolates were estimated. The double-reciprocal plots were linear and gave patterns, which intersected at various points to the left of the ordinate above the abscissa. The apparent K_m and V_{max} values for 2,4-D and α-ketoglutarate as substrate and co-substrate, respectively, are summarized in Table 2. In addition to 2,4-D, the enzyme catalyzes the α-ketoglutarate dependent release of the expected bacterial enzyme (Table 2). The enzyme exhibits a great affinity for 2,4-D. This was similar to *A. eutrophus* JMP 134, as reported by Fukumori and Hausinger (1993). Similarly, α-ketoglutarate is the preferred co-substrate because the enzyme exhibits a high affinity for it, as observed in the present study.

Table 2. K_m and V_{max} values of 2,4-D and α-ketoglutarate as substrate of 2,4-D/ α-ketoglutarate dioxgenase for the different bacteria isolates

Isolates	2,4-D		A-KG	
	K_m (µm)	V_{max}	K_m (µm)	V_{max}
SOGU 16	18.0 ± 1.5	5.01 ± 0.45	3.50 ± 0.64	1.51 ± 0.05
SOGU 11	16.8 ± 1.0	4.5 ± 0.35	3.20 ± 0.55	1.0 ± 0.03
SERU 11	17.5 ± 1.3	4.0 ± 0.32	4.00 ± 0.80	1.45 ± 0.04
SERU 2	19.8 ± 1.8	5.50 ± 0.65	3.80 ± 0.75	1.50 ± 0.06

Results were the average of five determination ± the standard error of mean (SEM)

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Choking Among Infants and Young Children

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Abstract

This retrospective study aims to determine the epidemiological features of deaths caused by chocking among infants in one of the general teaching hospitals in Jordan with a focus on weaning practices and its relation to sucking as major factors underlying the mechanism of choking in infants and young children. The study utilized a retrospective design to review the records of forensic cases due to a foreign body aspiration examined at the forensic department at the Jordan University Hospital. A total of 27 cases of choking in the pediatric age group were retrieved from the reports of the autopsy cases dissected. All cases of children who died due to chocking by foreign bodies were under 11 years old. Choking by food materials constituted (44.4%) of cases under 3 years of age while choking by non-food material was less prevalent under 3 years of age, comprising 18.5% of the cases. Health care personnel and parents need to be aware that the introduction of solid food, unlike exclusive breast or formula-milk feeding, can have serious consequences if occurring in inappropriate timing or consistency during early childhood physical and functional development. Parents need to be educated regarding the appropriate timing and process of weaning.

Keywords: Chocking, Weaning Practices, Infants , Young Children.

1. Introduction

Adequate respiration and nutrition are essential throughout a lifetime. Breathing occurs spontaneously without requiring an active effort by infants. Eating, on the other hand, requires that the infant coordinate sucking, swallowing, and breathing at the breast or bottle (Kidsafe, 2006). The urge to suck and mouth by young children is a natural developmental phase (Norris and Smith, 2002). Yet, chewing and swallowing food do not come naturally to infants. They are complex behaviors, having both reflex and learned components. Infants must learn to coordinate these actions and breathe, too (Stafford, 2006; Canadian Pediatric Society (CPS), 2004).

At the age of six months, the infant is developmentally ready to accept solid foods and the weaning process can be initiated (CPS, 2004; Arvedson, 2006). As weaning refers to the addition of new foods not the mere cessation of breastfeeding, it requires that the baby develop the chew-swallow reflex that accompanies a certain degree of neurological development (Stafford, 2006; Highton, 2001). Eating table food is a new behavior for infants and toddlers (Bobbie, 2005). According to the National Resource Center for Health and Safety in Child Care (2002), learning to chew and swallow takes time. This process of learning accompanies physical growth that is concurrent with social, cultural, sociological, and physiological development. This indicates the presence of critical and sensitive periods in the development of normal feeding behavior. Introducing complementary food to infants without considering these periods increases their vulnerability to choking hazards (Arvedson, 2006).

Choking is the interruption of respiration by internal obstruction of the airway, usually by food or small toys in young children (The European Child Safety Alliance, 2006; Kidsafe, 2006; Tarrago, 2000). This prevents oxygen from getting to the lungs and the brain leading to a brain damage or even death within four minutes (Norris and Smith, 2002). Children less than three years old are especially vulnerable to choking because they have small airways. They do not have a full set of teeth and cannot chew well as the older children, so large chunks of foods may lodge in the throat and cause choking. Furthermore, in infancy, the larynx is not only the narrowest part of the upper airway, but it is also relatively smaller than that in older children and adults, and this increases the risk of occlusion by a foreign body. Moreover, as part of the infant's development, the sucking pads in an infant's cheeks begin to disappear at the end of the first year (Highton, 2001). However, it may last for several years as

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witnessed by the use of bottles, dummies, and thumbs by many toddlers and preschoolers and it is shown to be the most common mouthing behavior at all ages (Norris and Smith, 2002). Children, especially younger ones, are at risk for choking as they have a tendency to place objects in their mouths and when they are learning to chew, they may attempt to suck or swallow foods whole (Bobbie 2005; Bren 2005). According to the 1997 U.S. Consumer Product Safety Commission annual study of children under age 9, one child chokes on food and dies in the United States every five days. The majority of victims are under age 5 (Stafford, 2006). Choking is a risk whenever food is consumed. A US study reports an incidence of death due to foreign body airway obstruction of 0.66 per 100,000 population (Mittleman and Wetli, 1982). Prevention of death from chocking has long been a concern of health care providers, whose role may involve education of parents, other caregivers and manufacturers about the risks and the prevention of choking.

Infants' weaning and feeding processes and practices have been emphasized by several authors as a risk factor for chocking (American Academy of pediatrics, 2001; Food Insight, 2002; Norris and Smith, 2002; Jafari *et al.*, 2003; Qureshi and Mink, 2003; Mikita and Callahan, 2006).

In Jordan, deaths from aspiration of foods and foreign material are not listed as a separate category. Furthermore, the relation between weaning and chocking has not been highlighted on either a national level or worldwide. Therefore, this study aims to determine the epidemiological features of such deaths in one of the general teaching hospitals in Jordan with a focus on weaning practices and its relation to sucking as major factors underlying the mechanism of choking in infants and young children. The results can be used by health care managers in developing and implementing training programs for health care providers (pediatricians and nurses), emphasizing their role in providing health education to parents and other care givers on strategies directed at preventing choking and associated deaths. .

2. Methodology

A total of 27 cases of choking in the pediatric age group were retrieved from the reports of the autopsy cases dissected at the forensic department at a general teaching hospital in Amman/ The Jordan University Hospital. The cases were analyzed and categorized according to their age, sex, nationality, history of exposure to foreign bodies, characteristics of obstructing bodies and the autopsy findings, especially the site of impaction and the stomach content. Confidentiality was maintained throughout the study.

3. Results

Table 1 shows that 16 (59.26%) of the diseased were males and 11 (40.74%) were females and the majority of the children (62.9%) were between 1 and 3 years of age. The second demographical factor is age. The age ranged between 7 months and 132 months with an average of 33.44 months age.

Table 1. Age group and male to female ratio

Age group (year)	N (%)	Male	female
< 1	6 (22.2)	4	2
1 - < 3	11 (40.7)	6	5
3 - < 5	4 (14.8)	2	2
5 - < 7	4 (14.8)	2	2
7 - < 9	1 (3.7	1	0
11	1 (3.7	1	0
Total	27 (100)	16	11

The witnessed cases were equal to the non-witnessed cases and three cases were not reported in table 2.

Table 2. The witnessed cases versus non-witnessed cases

History of exposure	Number of cases
Known, starts in the morning	2
Unknown, starts in the afternoon	7
Known, starts in the afternoon	6
Unknown, starts in the evening	5
Known, starts in the evening	4
Not reported	3

According to the time of death, 7 (25.9%) of the victims were dead on arrival, 10 (37.0%) sudden death, 7 (25.9%) within the way and 3 (11.1%) more than one day, as shown in table 3.

Table 3. Postmortem interval

Time of death	Number of cases (%)
Arrived dead	7 (25.9)
Immediate death	10 (37.0)
Within a day	7 (25.9)
More than one day	3 (11.1)

Food materials were the aspirated objects in 15 (55.6%) of the cases; non-food materials were in 12 (44.4%) cases. The obstructing food material in general is characterized by being solid, rounded or oval in shape, of moderate size and with smooth skinned surface. The chocking non-food agents are mostly of rubber or plastic materials and with smooth surfaces as shown in table 4.

Table 4. Food material versus non-food material

Foreign body type	Number of cases (%)
Food materials	15 (55.6)
Non-food materials	12 (44.4)

Larynx 8 (37.0%) was the most common site of the foreign body. The foreign bodies were seen in the respiratory tract at trachea in 5 (25.9%), larynx and trachea in 5 (25.9%), bilateral main bronchi in 2 (7.4%), right main bronchi in 2 (7.4%), trachea at the level of vocal cords in 2 (7.4%), trachea and bronchi in 1 (11.1%), larynx and oropharynx in 1 (3.7%) and not reported case 1 (3.7%) in table 5.

Table 5. Number of cases according to obstructing site

Obstruction site	Number of cases (%)
Trachea	5 (25.9)
Larynx	8 (37.0)
Larynx & trachea	5 (25.9)
Trachea and bronchi	1 (11.1)
Right and left main bronchi	2 (7.4)
Right main bronchi	2 (7.4)
Trachea at the vocal cords	2 (2.7)
Larynx and oropharynx	1 (3.7)
Not reported	1 (3.7)

The stomach contained the same obstructing body in eight cases choked by food materials, as shown in table 6.

Table 6. Number of cases according to the stomach content

Stomach content	Number of cases
Not reported	8
irrelevant	2
Fluid contents	4
Semisolid contents	4
Food particles	6
Citrus fruit seed, milk	1
Some curdled milk	1
Pumpkin and water melon seeds	1

There was one death attributable to medical conditions, tracheoesophygeal fistula which might have impaired swallowing and predisposed to aspiration of a foreign body. Another child was intubated and his death was due to brain hypoxia. In addition, 92.6% of these deaths occurred without a known medical history when they came to forensic department, see table 7.

Table 7. Number of cases according previous history of medical history

History medical illness	Number of cases
No Medical History	25
History of tracheoesophygeal fistula	1
History of death due to brain hypoxia	1

4. Discussion

In the present study, a slight male pre-dominance have been reported (59.26%). The age of cases investigated by the present study ranged between 7 months and 11years and the majority of the children were between 1 and 3 years of age. This age distribution goes in accord with that indicated by the American Academy of Pediatrics (Bruns and Thompson, 2010).

The academy reported that the children under three years of age are at a greater risk of choking and advised that once young children begin to eat table food educators and caregivers need to be aware of the dangers and risks of choking (Rimsza *et al*., 2002). Fortunately, no case was reported to be less than 7 months which indicates that care givers do not start to introduce food to their infants at this age.

According to the American Academy of Pediatric Dentistry (AAPD), early introduction (prior to 6 months of age) of solid food interferes with the intake of human milk or iron-fortified formula that the infant needs for growth. Weaning a child to drink from a cup is an individual process, which occurs over a wide range of time. However, the Academy recommends weaning by the child's first birthday (Bruns and Thompson, 2010; Bamber *et al*., 2014).

Choking by food materials constituted the majority of the cases under 3 years of age while choking by non-food material were more prevalent above 3 years of age. Studies reported that children less than three years of age are especially vulnerable to choking because they have small airways. They do not have a full set of teeth and cannot chew well as the older children, so large chunks of foods may lodge in the throat and cause choking (Sidell *et al*., 2013). Furthermore, in infancy, the larynx is not only the narrowest part of the upper airway, but it is also relatively smaller than that in older children and adults, and this increases the risk of occlusion by a foreign body (Collins, 1985). On the other hand, all of the non-food cases may be attributed to the lack of supervision and forceful suction in infants and young children, which becomes dangerous and even fatal whenever dangerous chocking foods and non-food materials are present inside the mouth such as seeds, objects, latex balloons etc. (Rimell, 1995; Mallick, 2014). This also may be related to that choked infants and children were living in low socioeconomic and heavily populated areas in the capital city of Amman and outside Amman.

The time choking occurred was at midday or 13:00 in the afternoon in almost half of the cases (48.1%). During this period, the parents are usually busy and may leave the infant/child unattended with some food material in front of him/her or, in older children who find something to play with, such as balloons, away from supervision. Tarrago (2000) indicated that a poor parental supervision may be a contributing factor. Children need to be reminded to take small bites and chew thoroughly. Children are more likely to choke when fed by a sibling, as food may not be properly cut or inappropriate foods may be given to the younger child.

There is a limited number of studies reporting on the location of foreign body in fatal cases. In the present study, obstructions were in the larynx and larynx & trachea in sudden death cases, in the larynx in cases of rapid death. Similar findings were reported by Tomaskea *et al*. (2006); the problem is the forceful suction (suction can be considered as a form of an excessive sucking). This is supported by the cases of choking by non-food materials especially balloons; the mouth of the balloons were found to be at an upward direction (Abdel-Rahman, 2000). This indicates that balloons were sucked forcefully to be inverted in such a manner. The orientation of the whistle inside the larynx can be explained in the same way. Norris and Smith (2002) indicated that sucking was the most commonly observed mouthing behavior, accounting for approximately two thirds of all observed

mouthing behaviors of children between 1 month and 5 years.

The obstructing food material in general is characterized by being solid, rounded or oval in shape, of moderate size and with smooth skinned surface. The stomach contained the same obstructing body in the most of cases choked by food materials. Similar descriptions of the obstructing objects were given by Tarrago (2000). According to Bobbie (2005), children under 5 years often have a trouble in managing unfamiliar, irregular and hard foods. When infants and young children are learning to chew, they may attempt to swallow the whole food.

In the present study, one child had an underlying medical condition, tracheoesophygeal fistula which might have impaired swallowing and predisposed to aspiration of a foreign body. Another child had a history of Cardiopulmonary resuscitation was done & patient intubated and his death was due to brain hypoxia.

The post-mortem intervals are summarized in Table 3, a prolonged survival interval was recorded in three cases; victims died within more than one day, seven cases were dead within a day and in the remaining cases death was reported to occur immediately after the choking incident.

In previous study, ten cases were identified out of a total autopsy population of 2165. Only one individual had an underlying diagnosis potentially contributing to aspiration. All but one case involved aspiration of food, with grapes being a feature of four cases. In cases with a prolonged survival interval, autopsy demonstrated bronchopneumonia and hypoxic-ischemic encephalopathy. In the remaining cases autopsy findings were non-specific (Bamber *et al.*, 2014).

In Turkey another study aimed to investigate the frequency and epidemiological features of deaths due to foreign body asphyxiation (FBA) in childhood, over 1990-2003. Of the victims, 14 (63.6%) were male and 8 (36.4%) females. There were 20 (90.9%) children between 1 and 3 years, and two other cases at 2/12 and 5 years of ages. All aspirations had occurred at home. Eight (36.4%) of the victims were dead on arrival, 11 (50%) on intervention, and 3 (13.6%) after complications. Food material was the most commonly aspirated foreign body in 81.8% of the cases, nuts being the most common (50%). Food asphyxiation remains a common problem particularly in children between 1 and 3 years of age in our region. (Goren *et al*; 2005).

In conclusion, the study finding of age distribution goes in accord with that indicated by the American Academy of Pediatrics Health. Care personnel and parents need to be aware that introduction of solid food, unlike exclusive breast or formula -milk feeding, can have serious consequences if occurring in inappropriate timing or consistency during early childhood physical and functional development. In order to reduce the incidence of morbidity and mortality from choking, suffocation and strangulation, health care providers should provide anticipatory guidance to parents based on the developmental stage of the child. Health care managers need to ensure that health care providers are well equipped with the skills and knowledge necessary to carry out this role.

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Production of Citric Acid by *Aspergillus niger* Using Sugarcane Molasses as Substrate

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Abstract

Citric acid ($CH_2COOH.COH.COOH.CH_2COOH$) is a tricarboxylic acid, soluble in water with a pleasant taste; it is an important acid used in food Industries. It exists in nature when carbohydrates are oxidized to carbon dioxide. Because of its high solubility, palatability and low toxicity it can be used in food, biochemical, and pharmaceutical industries. The aims of this study are citric acid production from fungi (*Aspergillus niger*) using by-product of sugar (sugarcane molasses) and to evaluate its concentration. Indigenous strains of *A. niger* were isolated from soil (depth 15cm), air and bread and identified using ordinary medium Sabouraud's dextrose agar medium supplemented with Rose Bengal. A pure culture of tested microorganisms were inoculated into different flasks containing different concentrations of molasses and incubated for 144 hrs at 28°C. The production of citric acid determined by the appearance of air bubble and colour's change; the mixtures were distilled at 175ºC for one and half hr. After the distillation process; the citric acid was detected and titrated to determine its percentage by adding bromocryesol green and NaOH (N 0.1), respectively. Citric acid production from the soil sample was of high amount, when compared with air, and bread. The soil sample produced 9.6 % of citric acid compared with air 6.7% and bread 7.7 %. The maximum citric acid production was produced on the 6th day of fermentation in all samples. By recycling and reusing waste material from cane molasses citric acid production can be easily achieved by using microorganisms that have the ability to produce citric acid efficiency such as *Aspergillus niger*.

Keywords: *Aspergillus niger*, Sugarcane molasses, Citric Acid production, Sucrose, Distillation, Fermentation.

1. Introduction

Citric acid is a weak organic acid with the formula $C_6H_8O_7$. It is a natural preservative conservative and is also used to add an acidic or sour taste to foods and drinks. In biochemistry, the conjugate base of citric acid, citrate, is important as an intermediate in the citric acid cycle, which occurs in the metabolism of all aerobic organisms. It consists of 3 carboxyl (R-COOH) groups (Berovic *et al*., 2007). The basic substrates for citric acid fermentation using submerged technique of fermentation are beet or cane molasses (Pazouki *et al*., 2000). Other different methods are being used for citric acid production, extraction of citric acid from fruits and its chemical synthesis, citric acid from whey and other dairy product wastes, citric acid from beet molasses as substrates. But the most commercially used method for the production of citric acid is by *Aspergillus niger* using cane molasses as an example of fungal over flow metabolism (Kabera *et al*., 2010). Many microorganisms, such as fungi and bacteria, can produce citric acid but *A. niger* remained the organism of choice for the production of citric acid due to its genetic stability, high yields, capacity of using cheaper raw material (like cane molasses) and absence of undesirable reactions (Murad *et al*., 2003). Many useful enzymes are produced using the industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. Alpha-galactosidase, an enzyme that breaks down certain complex sugars, is a component of beano and other products that decrease flatulence. Another use for *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for nuclear magnetic resonance (NMR) analysis (Hess *et al*., 2000).

The objectives of this study are: to produce citric acid from sugarcane molasses as a substrate using *A.niger* with charcterize and to determine citric acid yield and concentration.

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2. Material and Method

2.1. Area of study

This study was conducted at the Department of Microbiology and Molecular Biology, Faculty of Science and Technology, Al-Neelain University, Khartoum – Sudan. All experiments were accomplished aseptically in the laboratory of microbiology.

2.2. Collection of sample

Three samples were collected from air, bread, and soil in depth of 15 cm from Tuti islands' farms (Erika et al., 2013). Forty litres of sugarcane molasses sample were obtained from the Distillery Unit of Kennan Sugars (D.U.K.S) Company – White Province – Sudan. The sugarcane molasses were collected in clean, durable plastic container and stored at room temperature for further uses.

2.3. Isolation of test microorganism (Aspergillus niger)

One gram of the soil sample was placed in the test tube containing 10 ml of sterile distilled water to make a soil suspension and tenfold serial dilution was made by transferring one ml of the soil suspension to another test tube containing 9 ml of sterile distilled water. This step was repeated ten times to obtain a dilution of 10-10. An amount of 0.1 ml from each of the first three test tubes (10-1, 10-2, and 10-3) was taken and placed on the plate containing Sabouraud dextrose agar medium supplemented with rose bengal to inhibit the growth of saprophytes fungi other than *A. niger*. Another plate was opened inside the laboratory of microbiology to isolate *A. niger* from the air; the third plate was inoculated with the *A. niger* from infested bread; all plates were incubated aerobically at 25°C for 72 hrs. After the incubation period, the culture characteristics were observed and the growth was examined microscopically to confirm its purity using lactophenol cotton blue stain technique (Cheesbrough, 2008).

2.4. Physical characteristics of the sugarcane molasses sample

The physical characteristics of sugar cane molasses, such as moisture content, ash measurement and pH, were analyzed following standard methods (APHA, 2000).

2.4.1. Moisture content and ash measurement

The moisture content and ash measurement of molasses was performed by taken 10 grams of molasses sample and oven dried in a crucible at 104°C for 30 minutes (Hubert, 2006). Then the results were calculated using the following equations:

$$\text{Moisture content}(\%)=(A - X \div A) \times 100 \quad (1)$$

Ash (unit) = Weight of molasses before burning (A) - Weight of molasses after burning (X) (2)

where A is the weight of molasses before burning. While X is the weight of molasses after.

2.4.2. The pH value

The pH value was measured before and after inoculation of molasses samples using pH meter device (pH 213 Microprocessor-based Bench pH/mV/C Meters. Hanna Instruments).

2.5. Production of citric acid from raw sugarcane molasses

Isolates of *A. niger* were transferred to the 15 flasks containing raw sugarcane molasses media with different concentrations, i.e., each three flasks have the equal amount of molasses (20%, 30%, 40%, 50%, and 60%) by taking 100 ml, 150 ml, 200 ml, 250 ml, and 300 ml of sugarcane molasses and the volume was completed to 500 ml using sterile distilled water. The flasks were autoclaved at 115°C for 10 minutes. An amount of 50 ml of distilled water was added to the fungal pure culture to make a fungal suspension and then 10 ml from this suspension was transferred to the sugarcane molasses media. All flasks were incubated at 28°C for 144 hrs till 10 days. After incubation, the suspension was distilled to monitor the growth and observe the results (Elholi and Al-Delaimy, 2003).

2.6. Production of citric acid from sugarcane molasses with determined concentration of sucrose

In another experiment, different concentrations of sucrose (10%, 25%, 35%, and 50%) were measured using a hand refractometer device. After sterilization, an amount of 0.5 grams of urea powder was added to each flask containing sugarcane molasses with known concentration. Then 3 ml from pure *A. niger* were added to the media and the culture was incubated at 28°C for144 hrs till 10 days. After incubation, the suspension was distilled to monitor the growth and observe the results (Dubey, 2003).

2.7. Detection of citric acid

The detection of citric acid was done chemically by the addition of three drops of bromocrysol green indicator to the 10 ml of distillation yield (Soccol1 *et al*., 2006).

2.8. 2.8. Determination of citric acid concentration

Citric acid was determined by titration using 0.1N NaOH and Phenonphthalein as indicator and calculated as percentage according to the following formula (Soccoll *et al.*, 2006):

- Normality of Citric acid = normality of NaOH× NaOH volume ÷ volume of Citric acid
- Concentration of Citric acid = Citric acid normality ×equivalent ×100 ÷ volume of distillation
- (Equivalent = 96, volume of distillation = 10)

3. Results and Discussion

3.1. Isolation of Aspergillus niger

Three isolates were isolated from three different sources air, bread, and soil using sterile culture media, the isolates were purified, examined microscopically to show its purity and characterized by its culture characteristics.

3.2. Physical characteristics of the sugarcane molasses sample

The physical characteristics of sugarcane molasses were determined and calculated. The present study shows that the percentage moisture content was 65%. The ash was calculated as 6.50%.While the pH shown 6.0±0.2.

These findings were in disagreement with the findings of Gasmalla *et al.* (2012) who reported that the pH value of obtained molasses was 5.8±0.35. The ash was 12.69% on wet weight basis. Also these findings were in disagreement with the findings of Osunkoya and Okwudinka (2011) who reported that the pH value of obtained molasses was 5.1. The ash was 8.24%.

3.3. Production of citric acid from raw sugarcane molasses

As can be seen in Table 1, the best yield by all strains was 37.5 ml, 37.0 ml, 35.0 ml which were obtained at concentration of 20%. At concentration 30% they were 37.0 ml, 37.5 ml, and 33.5 ml, while at concentration 40% the two strains that were isolated from soil and bread gave a similar yield 35.0 ml and the air isolate strains gave 20.0 ml citric acid. The present study was almost in agreement with Sikander *et al*. (2002) who reported four *A. niger* isolates produced citric acid with the concentration of 18.86±1.8 – 42.56±2.0 g/l on 150g/l molasses sugar.

The lowest yields were obtained at the concentration of 50% as 10.0 ml, 5.5 ml, and 8.0 ml citric acid. At 60% sugarcane molasses concentration the microorganism did not exhibited any growth due to the effect of hypertonic solution. These findings were in disagreement with Sikander *et al*. (2002) who stated that three *A. niger* cultures gave concentrations of citric acid ranged between 58.14±2.7 – 78.18±18 g/l on 150g/l molasses sugar. Also, the present study was in agreement with Peksel and Kubicek (2003) who reported that the concentration and type of sugar influence the yield of citric acid production by *A. niger*. The present findings also were in agreement with Laboni *et al*. (2010) who reported that the citric acid production increased with the increase of the fermentation period and the maximum citric acid production was found on day 13. Also, the present results were in disagreement with Helen *et al.* (2014) who stated that the production of citric acid by *A.niger*, cultured on *Parkia biglobosa* fruit pulp, showed that the highest yield (1.15 g/L) of citric acid was obtained at pH 2 and it declined as the pH increased from being acidic to alkaline (pH8) with the yield of (0.86 g/L).

During the fermentation process there was a gradual reduction (Figure 1) of pH noticed in all the experiments and it indicated the production of citric acid. These findings were in agreement with Thangavelu and Murugaiyan (2011) who stated that, in control production medium, the initial pH 6.5 is gradually reduced to 1.5 during fermentation.

Table 1. Production of citric acid from raw molasses

Sugarcane Molasses concentration%	Solids/ g	Soil yield (ml)	Bread yield (ml)	Air yield (ml)
20	28.50	37.5	37.0	35.0
30	42.75	37.0	37.5	33.5
40	57.00	35.0	35.0	20.0
50	71.25	10.0	5.5	8.0
60	85.50	No production	No production	No production

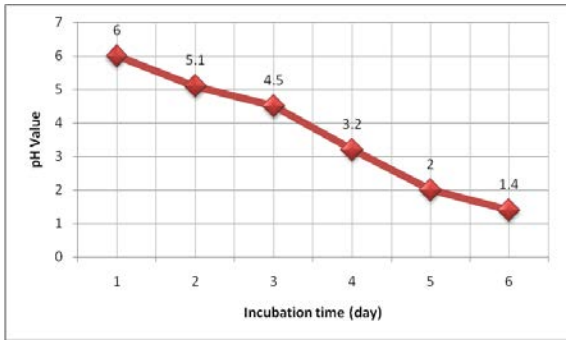

Figure 1. Citric acid indicated by pH reduction

3.4. Production of citric acid from molasses with determined concentration of sucrose

As can be seen in Table 2, the yield of citric acid was high when using the *A. niger* which was isolated from soil at all concentrations compared with other isolates (air, bread), followed by bread isolates, then air isolates which was the lowest yield. These findings were in disagreement with Kareem *et al.* (2010) who stated that the inoculation of *A. niger* on medium supplemented with sucrose (15% w/v) gave the highest citric acid value (36.6 g/kg). Also these findings were in agreement with the same author who stated that *A. niger*, when inoculated on medium containing pineapple peels, gave 17.23 g/kg at 5 days fermentation period. The increase in citric acid production and biomass values was accompanied with a steady decrease in sugar along the incubation time.

The addition of urea as a nitrogen source did not affect the production of citric acid; this is in agreement with Sadia *et al.* (2011) who reported that all concentrations (0.1 to 0.6%) of ammonium sulphate, peptone and yeast extract, used as a nitrogen source, were found to be inhibitory to fungal growth, sugar utilization and citric acid production. Also, the findings of this study were in agreement with Laboni *et al.* (2010) who stated that in the presence of prescott salt, citric acid production was found lower than it is with the absence of prescott salt and mixed substrate prepared with molasses and pumpkin media proved to be the best and potential for citric acid production. The present study was in disagreement with Nehad (2002) who reported that the natural oils with high unsaturated fatty acids content when added at concentrations of 2% and 4% (v/v) to Beet Molasses (BM) medium caused a considerable increase in citric acid yield from *A. niger*. The maximum citric acid yield was achieved in surface culture in the presence of 4% olive oil after 12 days incubation.

Table 2. Production of citric acid from molasses (sucrose + urea)

Sucrose%	Solids/g	Urea /g	Soil yield (ml)	Bread yield (ml)	Air yield(ml)
10	14.25		10.6	10.0	9.0
25	35.63	0.5	18.0	15.0	13.0
35	49.88		19.5	17.0	10.5
50	71.25		9.0	7.5	8.5

3.5. Determination of citric acid concentration

As can be seen in Table 3 and figure 3, the percentage of citric acid was determined. The concentration of citric acid varied due to the sucrose percentage; it showed high at the concentration of 35% (9.6%) for soil isolate, and (7.7%) for bread isolate, similar to the concentration 25% (7.7%) for soil isolate. At the concentration 25% of sucrose, bread and air isolates showed a similar concentration of citric acid (6.7%). At 10% of sucrose the soil and bread isolate exhibited a similar result as (4.8% citric acid percentage) followed by 3.8% of air isolate. The lowest concentrations of citric acid were shown at concentration 50% (2.9%) for both soil and bread isolates and 1.9% for air isolate.

Table 3. Titration of citric acid of three different isolates

Sucrose %	Citric acid %		
	Soil isolate	Bread isolate	Air isolate
10	4.8	4.8	3.8
25	7.7	6.7	6.7
35	9.6	7.7	4.8
50	2.9	2.9	1.9

Comparing the three isolates, the percentage of citric acid which was estimated as the lowest percentage was obtained by the *A. niger* isolated from air, shown in Figure 2.

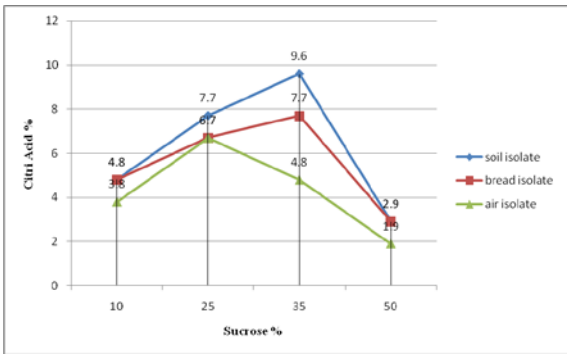

Figure 2. The citric Acid concentration by different *A. niger* isolates

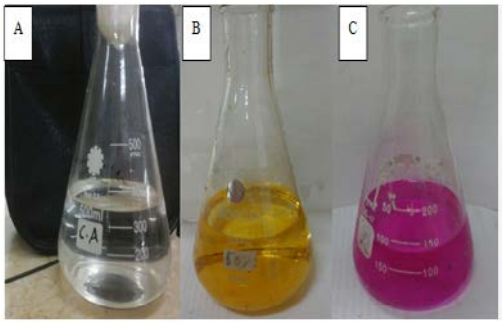

Figure 3. A. Citric acid yield, B. Citric acid after addition of three drops from bromogresol green, C. Citric acid after titrated by NaOH (0.1)

4. Conclusion

By recycling and reusing waste material from sugarcane molasses, citric acid production can be easily done by using microorganism that has the ability to produce citric acid efficiency such as *Aspergillus niger*. The result of this study indicates that the use of Sugarcane molasses for fungal production of citric acid might represent an efficient method of cost reduction in the production and concomitantly producing organic acid of valuable importance.

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Molds Associated with Olive Fruits Infested with Olive Fruit Fly (*Bactrocera oleae)* and their Effects on Oil Quality

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Abstract

Olive is the most widely grown fruit tree in Jordan; it is annually attacked by the olive fruit fly, *Bactrocera oleae* (Rossi), whose larvae usually cause great economic losses in fruit yield. *Alternaria solani, Aspergillus niger, Cladosporium herbarum, Fusarium solani, Penicillium digitatum, P. italicum* and *Rhizopus stolonifer* were found associated with the fly infestation with a sample frequency ranging from 6.7-33.3%. *Penicillium digitatum* was the most dominant species. All molds were vulnerable for a hot water treatment at 50 and 70ºC as indicated by their low spore germination and colony-forming unit except for *P. digitatum* and *P. italicum* which were the most heat-tolerant. When the olive oil was inoculated with pure cultures of these molds, the mold fungi were able to colonize olive oil. *Rhizopus stolonifer* was the greatest colonizer and besides *Alternaria solani*, both had obviously reduced the oil peroxide value over the control without greatly affecting the oil free fatty acid content.

Keywords: *Bactrocera oleae*, Jordan, *Olea europea* L., Olive fruits, Spore germination.

1. Introduction

Olive, *Olea europea* L., is the most widely grown fruit tree in Jordan occupying about 77% of the total area planted with fruit trees and 34% of the whole planting area covering now more than 130 thousand hectares (Anonymous, 2013). Olive plantation is a traditional part of the Jordanian agriculture (Al-Shdiefat *et al*., 2006; 2009) having a social, economic and environmental importance; it occupies the same position in the surrounding Mediterranean Basin countries (Loumou and Giourga, 2003).

Olive fruits are a valuable commodity worldwide; they are consumed as whole, as a table olive that is stuffed and sliced and as olive oil produced by milling fruits that must be prepared using safe conditions based on international olive oil standardization (Tokuşoğlu *et al.,* 2012).

Contamination of the fruits by hazardous microorganisms may occur through insect pest infestation, when they fall on the ground or by workers during handling (Asehraou *et al.,* 1992; Chliyeh *et al.,* 2014). One of the most important insect pests that usually attack olive in Jordan is olive fruit fly (*Bactrocera oleae* (Rossi) formerly *Dacus oleae* (Diptera: Tephritidae) whose larvae usually cause great annual economic losses in fruit yield through making fruit tunnels and enhancing the infection of fungal molds (Al-Raddad and Mustafa, 2008).

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease and death in humans and other animals (Bennett and Klich, 2003). Many types of mycotoxins are produced by mold fungi including aflatoxin, citrinin, alkaloids, fumonisins, ochratoxin A, patulin, trichothecenes, and zearalenone (Abarca *et al*., 2003; Abrunhosa *et al*., 2002; Bennett and Klich, 2003). Mycotoxins could act as nephrotoxic teratogenic, immunotoxic, genotoxic, mutagenic and carcinogenic agents and cause other human health hazards, all of which lead to life-threatening diseases (Creppy *et al.,* 1985; El Adlouni *et al.,* 2000; Bhat *et al.,* 1997).

Some olive mills usually use hot water to wash olive fruits before processing, which may drastically raise milling temperature. The milling temperature is kept under 27°C which is crucial to olive oil quality. Sometimes, hot water is added to olive paste during oil extraction for increasing the oil release from the tissues especially from olive fruits yielded under rainfed and non-supplementary irrigation conditions. If the temperature exceeds 27°C, the more volatile aromas are lost and oil oxidation rate is increased, thus reducing oil quality. The chemical content of polyphenols, antioxidants, and vitamins of the oil is also reduced by higher temperatures

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(Boskou, 2006) but these temperatures may have the advantage of controlling molds associated with olive fruit.

Therefore, the present study was conducted to isolate and identify the various mold fungi that are usually associated with olive fruit decay, to evaluate their ability of resisting hot temperature and colonizing olive oil and their influence on some oil quality parameters.

2. Material and Method

2.1. Mold Isolation, Culture and Identification

About 200 fully-mature and ripped olive fruits, previously infested with olive fruit fly, showing internally mold growth, were collected during Nov. 2014 from some trees belonging to some olive cultivars; Nabali, Nassohi, Rassei and Nabali Mohassan grown at the Agricultural Research Station (31° 16' N, 35° 45' E and ca 920 meters above sea level) of Faculty of Agriculture, Mutah University, Karak, Jordan. The part of olive fruit sample, showing the mold growth (0.5cm-in-diamter piece), was cut off and transferred into potato dextrose agar medium-containing Petri dish, 3 pieces of the same fruit/ plate (Asehraou *et al.*, 1992). All culture dishes were then incubated at 25ºC in darkness in a growth chamber for one week. A week later, all fungal colonies growing out from the pieces were identified under 100 and 400X slide stereoscopic microscope.

2.2. Mold Type Frequency

The percentage of each identified mold species was determined by counting the number of fruits showing positive isolation per fungal species divided by the total number of collected samples and multiplied by 100%.

2.3. Influence of Hot Water on Mold Spore Germination

To estimate the influence of hot water treatments at different temperatures on mold growth and development indicated by Colony Forming Unit (CFU) and spore germination %, a sterile tap water was heated to 30, 50 and 70±5ºC using a hot plate. A half ml-volume of hot water was transferred into one ml-in-volume test tubes (3 replicates per a treatment) and inoculated with a drop of fungal suspension made by washing the mold culture plate for each of the isolated mold fungi (Table 1) with a sterile tap water and set at a concentration of about 30000 spores per ml. Then, the test tubes were set at the same temperature for 2 minutes. One drop of water containing mold spores was taken from each test tube and added on a slide and spore germination % was then determined by counting the number of germinated spores. A 0.25 ml of each test tube was transferred into potato dextrose agar medium-containing Petri dish (3 plates/ a treatment). All culture dishes were then incubated at 25±2°C in darkness in a growth chamber for one week. After that, the number of emerging fungal colonies was counted per plate and the CFU/ml was determined per a plate and the average of CFU/ml for each treatment was calculated.

2.4. Mold-Olive Oil Interactions

A fresh virgin olive oil (10ml-volume per a 15ml glass test tube) was inoculated with a loopfull of each mold (Table 1) plate culture (3 replicates per treatment). All mold-inoculated oil test tubes were incubated at 25±2°C in darkness in a growth chamber for about three months. Percentage of mold colonization (in terms of mycelial growth and sporulation) of the oil was estimated; oil Peroxide Value (PV) and the percentages of Free Fatty Acids (FFA%) were measured using standard methods (IUPAC, 1979; Wong, 1989)

3. Results and Discussion

Table 1 shows that seven species of mold fungi were isolated from olive fruits infested with olive fruit fly; *Alternaria solani* (causes brown rot), *Aspergillus niger* (black mold), *Cladosporium herbarum* (sooty mold), *Fusarium solani* (fruit rot), *P. digitatum* (green mold), *P. italicum* (blue mold) and *Rhizopus stolonifer* (causes soft rot) with a sample frequency ranging between 6.7-33.3%. Among them, *Penicillium digitatum* was the most frequent mold species isolated from the fruit samples (Table 1).

Table 1. Fungal species associated with olive fruit rot that were isolated and identified *in vitro*:

Fungal species	Sample Frequency %
Alternaria solani Sor.	10.0*
Aspergillus niger v. Tieghem	13.3
Cladosporium herbarum Fr.	16.7
Fusarium solani (Mart.) App. Et Wr.	13.3
Penicillium digitatum Sacc.	33.3
Penicillium italicum Wehmer	06.7
Rhizopus stolonifer (Her.) Vuill	20.0

* Over 200 rotted fruits with apparent mycelial growths were collected during November 2014.

At 50ºC hot water treatment, there was a significant reduction in CFU/ml and in the spore germination % of the mold fungi except for *P. digitatum* and *P. italicum* where no significant reduction in the spore germination % at 50ºC was recorded with 30ºC (Table 2). At the highest hot temperature of water (70ºC), all fungi showed remarkably lower CFU/ml and spore germination % than those at 30ºC except *P.digitatum* and *P. italicum* that gave more than 50% spore germination at 70 ºC (Table 2).

All mold fungi were able to colonize olive oil (Table 3) and the range was from 5.7-36.2%. Mold colonization % of olive oil was significantly the highest for *Rhizopus stolonifer* while the lowest value was for *Aspergillus niger*.

Peroxide Value (PV) was significantly lower in the olive oil treated with *Alternaria solani* and *Rhizopus stolonifer* than that in the oil treated with the other fungi or in non-treated oil (Table 3).

The olive oil treated with *P. digitatum* and *Rhizopus stolonifer* had significantly higher FFA% than the oil treated with *P. italicum.* The increase or decrease caused by fungi in FFA% over the untreated control was not significant (Table 3).

Table 2. Influence of hot water treatments on colony forming unit (CFU) and spore germination % of some mold fungi associated with olive fruits:

Fungal species	CFU/ml			Spore germination %		
	30 ºC	50 ºC	70 ºC	30 ºC	50 ºC	70ºC
Alternaria solani	36[1] a[2]	0 b	0 b	96 A	33 B	0 C
Aspergillus niger	61 a	37 b	33 b	66 A	10 B	5 B
Cladosporium herbarum	26 a	10 b	0 c	78 A	28 B	0 C
Fusarium solani	14 a	0 b	0 b	84 A	34 B	5 C
Penicillium digitatum	71 a	50 b	32 c	97 A	70 A	51 B
Penicillium italicum	111 a	39 b	51 b	98 A	83 A	60 B
Rhizopus stolonifer	47 a	6 b	0 b	76 A	0 B	0 B

[1] Average of three replicates per a treatment.

[2] Means within rows per a parameter followed by the same letter are not significantly different at 0.05 probability level.

Table 3. Effects of mold fungi on peroxide value (PV) and free fatty acid percentage (FFA%) of olive oil:

Fungal species	Mold colonization%	PV	FFA %
Alternaria solani	18.6[1] bc[2]	22.0 bc	1.00 ab
Aspergillus niger	05.7 d	31.0 ab	0.86 ab
Cladosporium herbarum	10.0 cd	30.0 abc	1.03 ab
Fusarium solani	15.3 c	28.0 abc	1.03 ab
Penicillium digitatum	23.7 b	28.5 abc	1.13 a
Penicillium italicum	26.4 b	26.2 abc	0.63 b
Rhizopus stolonifer	36.2 a	18.8 c	1.10 a
Negative Control	00.0 d	36.2 a	0.88 ab

[1] Average of three replicates per a treatment.

[2] Means within columns per a parameter followed by the same letter are not significantly different at 0.05 probability level.

4. Discussion

Seven species of mold fungi were identified from olive fruits followed the infestation of olive fruit fly including *Alternaria solani, Aspergillus niger, Cladosporium herbarum, Fusarium solani, P. digitatum, P. italicum* and *Rhizopus stolonifer* with a sample frequency ranging between 6.7-33.3%. The low rate of colonization could be due to the occurrence of some natural inhibitors in the olive oil, such as some polyphenols (Ruiz-Barba *et al.*, 1990; Asehraou *et al.*, 1992).

Penicillium digitatum was the most dominant mold and was frequently isolated. *Penicillium crustosum, P. roqueforti* and *P. viridicatum* were the dominant flora of black table olives and the most frequently encountered species in Aegean and Marmara areas of Turkey (Tokuşoğlu *et al.*, 2012). Three of the mold genera identified in the present study, *Aspergillus, Penicillium* and *Fusarium*, are considered the most frequent toxigenic fungi in Europe (Creppy, 2002). *Penicillium* and *Aspergillus* represented the majority of mesophilic fungi isolated Moroccan olive and olive cake (Roussos *et al*., 2006).

Most of the isolated molds were vulnerable to hot water treatment at 50 and 70ºC as indicated by the great reduction in their spore germination and colony forming unit. This is a valuable result since some olive mills usually use hot water to wash olive fruits, which has an additional value through its detrimental effect on spore germination and fungal growth. Physical, chemical and microbiological characteristics of olive oil can be affected by pre-milling storage time and the storage manner of the oil (El Haouhay *et al.*, 2015). Generally, olives are very sensitive to physical damage and alterations caused by the presence and activity of microorganisms like molds, yeast and bacteria (Gutiérrez *et al.*, 2009; Asehraou *et al*., 1992; El Haouhay *et al.*, 2015). The seven isolated mold fungi were able to colonize olive oil through growth and spore production. Among them, *Rhizopus stolonifer* was the greatest colonizer and had obviously reduced PV of the oil and the same effect was also caused by *Alternaria solani.* When olive fruits were inoculated with some mold fungi, fungal infection had indirectly resulted in a significant increase in the extracted oil acidity and PV. However, there was no significant difference in the acidity and PV among different fungal isolates (Torbati *et al.*, 2014).

Acidic pH even slight is most probably caused by the double action of the lipolytic microorganisms and/or lipases, which may release FFA. This can be supported by the high PV, which may indicate the level of the lipolysis (Asehraou *et al*., 1992). There was no considerable change in FFA three months after inoculating olive oil with different molds. Perhaps, a longer period of incubation may lead to significant changes in the oil FFA. Free fatty acids are related to the lipolytic action of some microorganisms, which are generally active inhibitors against most microorganisms (Asehraou *et al*., 1992).

Storage of olive fruits and oil under uncontrolled environmental conditions had a drastic effect on the quality and shelf life of olive oil as indicated by the increase in its acidity and oxidation (Chliyeh *et al*., 2014; El Haouhay *et al*., 2015). Besides, a significant and important reduction of polyphenol contents and antioxidant capacity were detected in olive oil. The development of molds on olives is responsible for the poor nutritional quality of olives because molds can disturb the synthesis of fatty acids (Biasone *et al.*, 2012; El Adlouni *et al*., 2000).

Insect pest infestation and bad storage conditions of olive fruits may enhance the activity of other microorganisms besides molds, e.g. *Clostridium* spp., *Pseudomonas* spp. and *Enterobacter* spp. (Angerosa *et al.*, 1999). Olive oil produced under these adverse conditions not only leads to poor quality but could also put human health at risk. Mold growth on black table olives may be prevented by treating the fruits with sorbic acid, methyleugenol and spice essential oil where sorbic acid was the most efficient (Kivanç and Akgül, 1990).

Therefore, it is essential to have an effective management of the conservation of olives before and after

harvest to avoid fungal mold infection and its adverse effects. Effective management of olive fruit fly would reduce its fruit infestation and secondary mold infections, which would improve olive oil yield and quality.

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Comparative Genotoxicity of Herbicide Ingredients Glyphosate and Atrazine on Root Meristem of Buckwheat (*Fagopyrum esculentum* Moench)

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Abstract

Herbicides have been extensively used in agriculture but people have not concerned about its adverse effects on plants and animals. The aim of the present study is to analyze the detrimental effects of two herbicides in *Fagopyrum esculentum* Moench (Variety: VL-7). The root tips were treated with four concentrations viz. 50, 100, 150 and 200 ppm of glyphosate and atrazine at room temperature for 3 hrs. Mitotic indices and chromosomal anomalies were calculated. It was observed that both the herbicides induced different types of chromosomal abnormalities comprising of scattering, precocious movement, stickiness, bridges, laggard etc. along with the increasing doses of herbicides. Scattering and stickiness are most prevalent abnormalities among others. The effect of glyphosate was more toxic than atrazine in the root meristem of *Fagopyrum esculentum*.

Keywords: *Fagopyrum esculentum* Moench, Herbicides, Chromosomal anomalies, Mitodepressive, Mitotic indices.

1. Introduction

Herbicides have been extensively used for a better exploitation of different plants in modern agriculture and landscape turf management. They are effective but have a strong biological activity against plants. Across the world, they are manufactured to cultivators who suffered heavy losses due to weed. However, the advantages come at a significant cost. They are persistent to degradation, and get bio accumulated in the environment affecting higher organisms and producing undesirable secondary consequences in plants. They can also be classified by their "site of action" or the specific biochemical site that is affected by the herbicides. Their properties increase the likelihood of transport including resistance to degradation and high water solubility.

One of the most widely commercialized herbicides in the world is glyphosate (Vivancos 2011). It is categorized under the systemic EPSPS (Enzyme-5 enolpyruvyl-shikimate-3-phosphate synthase) inhibitor herbicides inactivated by soil content that can control most annual and perennial plants. EPSPS is an enzyme which is found only in plants and micro-organisms. It controls the weeds by inhibiting the synthesis of aromatic amino acids such as tryptophan and tyrosine which is necessary for protein formation in susceptible plants (Pipke *et al.*,1987). It also affects other biochemical processes and although these effects are considered, they may be important in the total lethal action of glyphosate.

Atrazine is categorized under triazine class used to prevent pre and post emergence broadleaf weeds in variety of crops such as maize, sorghum, sugarcane and some extent in landscape vegetation. In the United States as of 2014, atrazine was the second most widely used herbicides after glyphosate found in the rural environment. It does not occur naturally. It is prepared from cyanuric chloride when treated sequentially with ethylamine and isopropyl amine. Plants can absorb atrazine through roots or through the foliage. Once absorbed, it is accumulated in the growing tips and the new leaves of the plant, inhibiting photosynthesis in susceptible plant species.

Many studies have been done to study the effect of the herbicides including cyanazine, gespax, goltix, aventox and atrazine on the mitotic activity, chromosomes and nucleic acids content in root tip cells of different plants in a large scale. (Wuu and Grant, 1966; Liang *et al.*, 1967; Stroev, 1970; Hakeem and Shehab, 1972; Liang and Liang, 1972; Dryanovska and Petkov, 1980 Badr, 1983, 1986; Badr *et al.*, 1985; Mousa, 1982a, Tomaskova and Mydilova, 1986; Airapetyan *et al.*, 1984; Papes *et al.*, 1989; Haliem, 1990; Ashour and Abdou, 1990). The previous authors stated that all the herbicides inhibited the cell division and produced chromosomal abnormalities and in some cases the inhibition was integrated with the reduction of the nucleic acids.

Buckwheat is a pseudocereal belongs to Polygonaceae family and its diploid chromosome no. is 16. But the

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chromosome sizes are small, which makes the cytogenetical analyses complicated (Neethirajan *et al*., 2011). It is a very easily grown plant; it prefers dry sandy soils but succeeds in most conditions including poor, heavy or acid soils and even sub-soils. It is frequently cultivated for its edible seeds and leaves. It is very nutritious food. The primary production of pseudocereal is its seeds which have relatively higher contents of carbohydrate and calories as compared to other cereal and it is also most desirable crop because all parts of plants are valuable such as seeds and leaves used for medicinal purposes, mainly in diabetes, celiac disease and gluten allergies. It is a good honey plant producing a dark, strong monofloral honey. It is easily contaminated with herbicides from the environment because of its medicinal use; during growth and rejuvenation process when its readymade products are produced. Testing of plant roots are very useful because root meristem is the place for exposure of chemicals which spread in environment, soil and water.

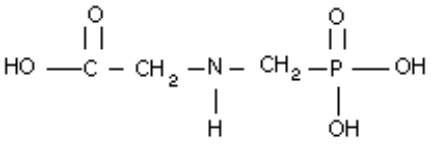
It is therefore of interest to conduct the effect of glyphosate and atrazine on cell division, chromosome behaviour and DNA and RNA contents in the root tip cells of *Fagopyrum esculentum* in turn responsible for mutagenecity and its outcome may help in understanding the possible constraints in the role of these two herbicides as toxic in plants.

2. Material and Method

For the cytological study, the seeds of buckwheat variety VL-7 were collected from National Bureau of Plant Genetics Resources, Shimla, Phagli, India. For the mitotic studies, seeds were germinated in petridish into an incubator for 1-2 days at 22-25^{0}C.

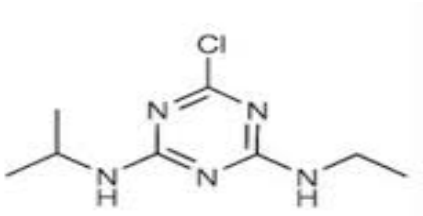
2.1. Herbicide compounds

1. Glyphosate- Molecular formula- $C_3H_8NO_5P$
(IUPAC Name: N- (phosphonomethyl) glycine**)**
Structural formula-



Molecular weight- 169.07 g·mol^{-1} (Health & Consumer Protection Directorate-General. 2002) Active purity percentage- > 80%

2. Atrazine- Molecular formula-$C_8H_{14}ClN_5$
(IUPAC Name: 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine**)** (World Health Organization 2011) Structural formula-



Molecular weight- 215.68 g·mol^{-1} (Plakas *et al*., 2006)

2.2. Herbicides treatment

After the emergence of root tips, different concentrations of glyphosate and atrazine solutions were prepared by using dilution method. Then, the seeds were treated by soaking method with different concentrations viz. 50,100,150 and 200 ppm of each herbicides for 3 hrs and one set of germinated seeds were also maintained as control with distilled water only. The root tips were then washed thoroughly in distilled water and fixed in carnoy's fixative (1 Glacial acetic acid: 3 alcohol) for 24 hours and were preserved in 90% ethanol.

2.3. Cytological analysis

The root tips were hydrolysed in 1N HCl for 2-3 minutes for softening and then stained with 2% acetocarmine for 30 minutes. Finally, slides were prepared by using squash technique for cytology and observation was done at 40X resolution in light microscope and photographs were taken by using PCTV Vision Photography Software.

Following the formulae used in the calculation of Active mitotic index and abnormality percentage –

$$\text{Active mitotic index (AMI) \%} = \frac{\text{Total no. of dividing cell}}{\text{Total no. of cell observed}} \times 100$$

$$\text{Total Abnormality percentage (TAP) \%} = \frac{\text{No. of Abnormal cell}}{\text{Total no. of cell observed}} \times 100$$

2.4. Statistical Analysis

Statistical analysis was performed using the SPSS 16.0 software. Three replicates for each treatment and one independent variable were used. A one way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT, $P < 0.05$) were performed for mean separation and the graph was plotted by using sigma plot 10.0 software. Actual mean and standard error were calculated. The data were subjected to analysis of variance.

3. Results

In buckwheat plant, the eight basic (Morris, 1951) chromosome number has been found (2n=2x=16). The cytological observation revealed that the mitotic index in control sets was found to be 12.62%, with normal chromosome behaviour, showing perfectly normal arrangement of 16 chromosome at metaphasic plate (Figure1A) and 16:16 separation during anaphase (Figure1B). However, after the treatment of the root tip cells with glyphosate and atrazine changes in normal behaviour of chromosome and rate of dividing cells were

observed. Mitotic indices and chromosomal abnormality percentages of both the herbicides treatment sets were inversely related to each other.

The ranges of Active Mitotic Index (AMI) and chromosomal aberrations (Total Abnormality Percentage-TAP) with respect to the doses of herbicides have been summarized in Table 1. Figure 2 shows a decline in the value of active mitotic indices along with the increasing concentration of herbicides, i.e., glyphosate and atrazine. In case of glyphosate treated set, the percentage of active mitotic index reduced from 10.98% to 4.64%, whereas in case of atrazine, it was reduced from 11.85% to 6.90% on increasing the concentration from 50 to 200 ppm. It has been investigated that a lower dose was not much effective for the plant tissues and abnormalities might be recovered but the highest dose, i.e., 200 ppm, severely damaged the plant cells and also destroyed the cells, causing a cell elongation and a cell distortion, So, the maximum abnormality percentage and the lowest active mitotic indices were observed at higher doses of treatment in both cases viz. 200 ppm glyphosate (TAB% 9.24, AMI% 4.64) and atrazine (TAB% 7.05, AMI% 6.90) shown in Table 1. Both the herbicides induced various types of chromosomal aberrations (Figure1) such as scattering, c-mitosis, precocious movement, unorientation, stickiness, laggard with forward movement, bridge, etc. But the glyphosate induced a higher percentage of chromosomal abnormalities as compared to atrazine (Figure 3).

Most of the aberrations were present predominantly at higher doses of treatment, eg., scattering, stickiness and unorientation were found at the highest doses (200ppm) treatment set of glyphosate and atrazine respectively (Table 1).

The total abnormalities increased from 3.52% to 9.24% and 2.73% to 7.05% as the doses increased from 50 ppm to 200 ppm in treated sets of glyphosate and atrazine, respectively.

Table 1. Abnormality induced by Glyphosate & Atrazine in root meristems of *Fagopyrum esculentum* Moench

Treatment	Doses (ppm)	AMI % (Mean ± SE*)	Metaphasic abnormalities (%) (Mean ± SE)						Anaphasic abnormalities (%) (Mean ± SE)				TAP (%) (Mean ± S.E)
			Cm	Sc	St	Pr	Un	Br	St	Un	Lg	Oth	
Glyphosate (*N*-(phosphonomethyl)glycine)	Control	12.62± 0.02^{a**}	--	--	--	-	--	--	--	--	--	--	--
	50	10.98± 0.04^{b}	0.36± 0.01^{a}	0.61± 0.12^{b}	0.36± 0.01^{b}	0.55± 0.17^{b}	0.37± 0.01^{a}	0.55± 0.19^{a}	0.55± 0.19^{a}	0.48± 0.11^{b}	0.37± 0.11^{b}	0.36± 0.01^{a}	3.52± 0.32d
	100	9.04± 0.27^{c}	0.52± 0.15^{a}	0.68± 0.01^{b}	0.46± 0.10^{b}	0.81± 0.23^{ab}	0.46± 0.11^{a}	0.46± 0.10^{a}	0.70± 0.22^{a}	0.69± 0.20^{b}	0.81± 0.11^{a}	0.35± 0.01^{a}	5.44± 0.14^{c}
	150	6.52± 0.25^{d}	0.59± 0.17^{a}	0.89± 0.12^{b}	0.69± 0.25^{ab}	0.69± 0.08^{ab}	0.79± 0.11^{a}	0.39± 0.09^{a}	1.09± 0.12^{a}	1.19± 0.16^{a}	0.79± 0.83^{a}	0.49± 0.09^{a}	7.63± 0.24^{b}
	200	4.64± 0.22^{e}	0.64± 0.07^{a}	1.45± 0.11^{a}	1.05± 0.07^{a}	1.13± 0.05^{a}	0.89± 0.22^{a}	0.49± 0.23^{a}	0.98± 0.25^{a}	1.38± 0.10^{a}	0.97± 0.03^{a}	0.38± 0.14^{a}	9.24± 0.15^{a}
Atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine)	Control	12.62± 0.02^{a}	--	--	--	--	--	--	--	--	--	--	--
	50	11.85± 0.14^{b}	0.35± 0.15^{b}	0.47± 0.01^{b}	0.35± 0.02^{a}	0.36± 0.10^{a}	0.37± 0.01^{a}	0.35± 0.15^{a}	0.35± 0.16^{c}	0.37± 0.01^{b}	0.35± 0.15^{a}	0.37± 0.01^{a}	2.73± 0.11^{d}
	100	10.97± 0.03^{c}	0.35± 0.06^{b}	0.47± 0.12^{b}	0.59± 0.12^{a}	0.47± 0.11^{a}	0.47± 0.12^{a}	0.35± 0.01^{a}	0.70± 0.07^{b}	0.58± 0.11^{b}	0.47± 0.12^{a}	0.58± 0.11^{a}	4.79± 0.14^{c}
	150	8.93± 0.09^{d}	0.52± 0.17^{ab}	0.57± 0.11^{b}	0.46± 0.12^{a}	0.87± 0.19^{a}	0.57± 0.11^{a}	0.45± 0.10^{a}	0.79± 0.09^{b}	1.13± 0.87^{a}	0.57± 0.11^{a}	0.46± 0.12^{a}	5.93± 0.19^{b}
	200	6.90± 0.15^{e}	0.72± 0.25^{a}	1.07± 0.19^{a}	0.71± 0.19^{a}	0.72± 0.21^{a}	0.71± 0.19^{a}	0.48± 0.13^{a}	1.32± 0.12^{a}	0.96± 0.13^{a}	0.37± 0.01^{a}	0.53± 0.16^{a}	7.05± 0.14^{a}

Where:Cm- C- Mitosis, Sc- Scattering, St- Stickiness, Pr- Precocious movement, Un- Unorientation, Br- Bridge, Lg- Laggard, Oth- Others *SE= Standard error **Means followed by lowercase letter are statistically significant at $p < 0.05$ in Duncan multiple range test.

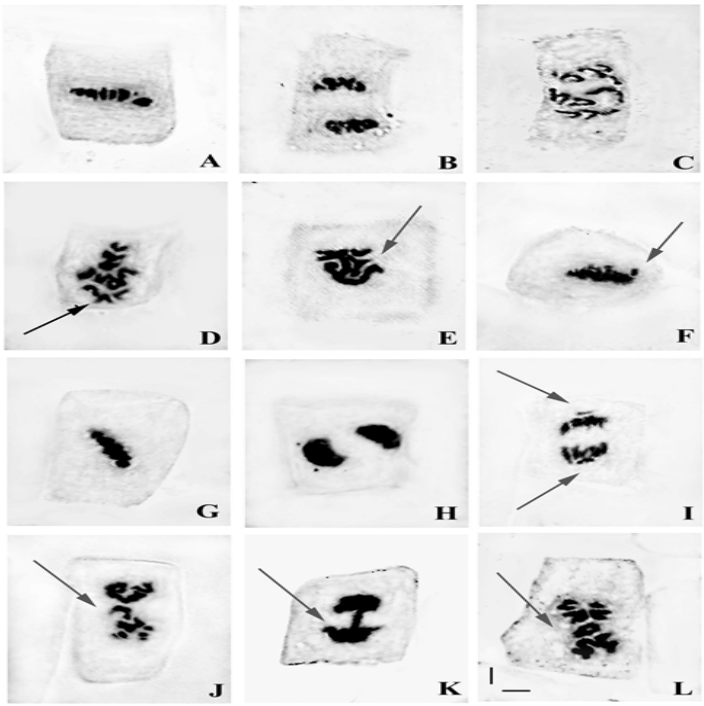


Figure 1. **A**.Normal Metaphase (2n =16);**B.**Normal Anaphase (16:16 separation);**C.** Scattering ;**D**.C- mitosis;E.Clumping at Metaphase;**F**.Precocious movement;**G**.Unorientation with stickiness at Metaphase;H**.**Unorientation with stickiness at Anaphase;I.Forward movement at Anaphase;**J**.Laggard formation;**K.**Single bridge at Anaphase;**L**.Disturbed polarity at Anaphase .
[Bar length = 6.42µm, width = 7.5 µm]

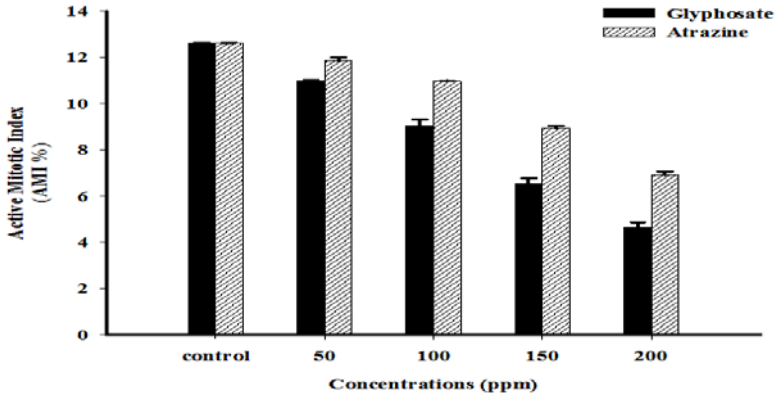


Figure 2. Comparative account of Active Mitotic Index (AMI) for Glyphosate and Atrazine in the root meristem of *Fagopyrum esculentum* Moench

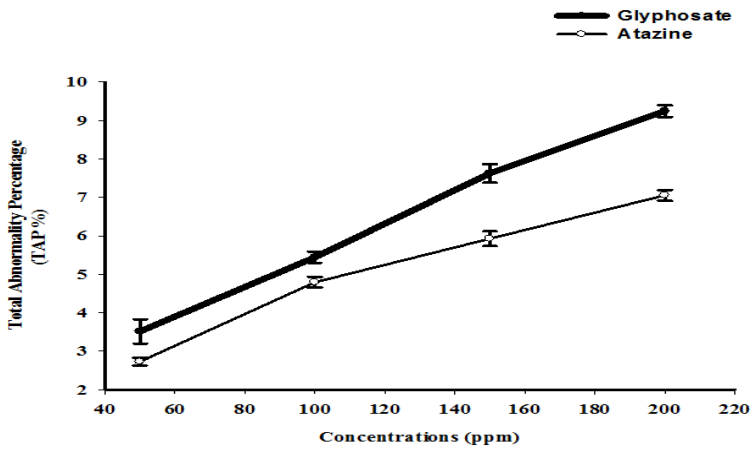


Figure 3. Comparative account of Total abnormality percentage for Glyphosate and Atrazine in the root meristem of *Fagopyrum esculentum* Moench

4. Discussion

The mitotic index refers to the frequency of cell division which is an important parameter for determining the rate of root growth (Liu *et al.*, 1992). The results of the present study show that there is a gradual reduction in mitotic index as a result of the increase in the herbicides concentration. This gradual reduction of mitotic index and higher rate of chromosomal abnormalities clearly show that herbicides are toxic for plant at chromosome level. Chemicals which affect the plants and induce chromosome damaging are called as clastogens and their action on chromosome is generally regarded to involve an action on DNA (Grant, 1978; Chauhan *et al.*,1990). In the current findings, scattering, stickiness and unorientation were most frequent abnormality after treating the root tip cells with glyphosate and atrazine, respectively. The higher concentration, i.e., 200 ppm, was effective in inducing a statistically significant increase in the frequency of abnormal mitosis. But the toxicity of glyphosate is more than atrazine which is clearly proved at cytological level.

The highest frequency of scattering and spindle dysfunctioning were recorded at 200 ppm of glyphosate and the reason was attributed to the loss of microtubules of the spindle fibres and also shows co-relation with the precocious c-mitosis types of chromosome anomalies (Figure 1 C). Another reason for the formation of precocious chromosome is due to early terminalisation of the chromosome or due to chemical breakage of the protein moiety of nucleoprotein backbone (Patnaik, 1984) (Figure 1 F).

Cytotoxic and mitodepressive effects of glyphosate and atrazine, used in the present case, were in conformity with the earlier findings. (Saxena *et al*., 2004; Gul *et al.*, 2006; Mustofa and Arikan, 2008). C-mitosis was the most frequent chromosomal abnormality observed during metaphase at 150 and 200ppm in both the treated sets (Table 1, Figure 1 D). Arrested metaphasic cells indicated that the herbicide in the present investigation cause disturbance or inhibition of spindle formation similar to the effect of colchicines and lagging chromosome (Figure 1 J) were the result of impairment of mitotic apparatus, treatment of 100 and 200 ppm in the treatment of glyphosate gave a higher frequency of laggards (Haliem, 1990; Sonia Sharma and Adarsh Pal Vig, 2012).

Chromosomal Stickiness was very frequent in both herbicides treatment at the 200 ppm (Figure 1G and H) and it is induced either by the effect of herbicides on the chromosomal protein which is attributed to the irregular folding of chromosome fibres or due to the action of herbicides on the polymerization process, resulting in the fragmentation of chromosome bridges forms sticky chromosomes (El- Gha-Mery *et al*., 2000). It might have occurred due to the sudden contraction of some of the spindle fibres using to toxic effect of glyphosate. (Mahakhode and Somkuwar, 2013).

Chromosome fragmentation is also observed in the root meristem of *Fagopyrum esculentum* due to these herbicides treatment, which results from the breaks of chromosome in which there is a loss of chromosome integrity. Amer and Ali (1969) also reported that Pentachlorophenol induced fragmentation of both mitotic and meiotic chromosome of *Vicia faba* (Grant, 1978).

Chromosome bridges were not observed in root tip cells of both the treatments with the lowest concentration where an abundance of breakage and clumping was recorded. The proportion of bridges generally increased on increasing concentration, i.e., at the highest dose viz. 200 ppm. It is formed due to the sticky behaviour of chromosomes which could not move towards pole regions at anaphase (Kumar and Rai, 2007) or due to the chromosomal stickiness and subsequent failure of free anaphasic separation or inversion of chromosome (Najjar and Soliman, 1980). The breakage of chromosomes at the same locus and their lateral fusion led to the formation of dicentric chromosome. It plays a main role in the bridge formation (Figure 1 K). It was pulled equally towards both the pole at anaphase and bridges were formed (Anis *et al.*, 1998).

Such chromosomal abnormalities may affect adversely the vigour, fertility and yield of exposed plant. Herbicides with such action can also alter the genetic constitution of crop, resulting in mutational change which could be very dangerous. Hence, the highest concentration of both herbicides may become genotoxic, chromotoxic and clastogenic for crop plants in the environment. Therefore, its higher concentration is not suggestive to all people especially the herbicide glyphosate because its toxicity is too high.

5. Conclusion

From the above foregoing discussion, the results obtained in the present study indicate cytotoxic activity of glyphosate was more as compared to atrazine on the basis of cytological study. So, if a higher concentration of herbicides is present in the environment and absorbed by the plants, it may adversely affect the genetic system causing damage to the chromosome in crop plants. Regular uses of herbicides in agricultural practices are a potential threat to the genetic constitution of crop plants and animals. Therefore, judicial uses of these herbicides are essential. An indiscriminate use of herbicide should be discouraged as far as practicable. Rather, it should be replaced with bio-herbicides and bio- control agents which do not pose adverse risks to crops as well as the ecosystem.

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Cigarette Smoking Risks on Blood Indices and Liver Enzymes of Male and Female Smokers in Kurdistan, Iraq

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Abstract

Smoking is the worst human behavior; it is practiced by people addicted to nicotine; smoking cigarettes causes many harmful diseases, such as anemia and liver sickness. The aim of the present study is to assess the relationship between gender and the effect of smoking cigarettes. For the purpose of this study, twenty-eight volunteers had participated in four different groups: Female smokers (n = 7), Female non-smokers (n = 7), Male smokers (n = 7), Male non-smokers (n = 7). The results showed that Red Blood Cells (RBCs) in Male smoker group significantly ($P < 0.05$) increased in comparison with the Female smoker group. Also, Aspartase amino transferase (AST) significantly rose in Male smoker group versus that in the Female smoker group. On the other hand, many blood indices and liver enzyme parameters in male smoker group were higher than those in the female smoker. In conclusion, cigarettes lead to a change in blood cellular and fluctuation in liver enzymatic activity in both male and female volunteers, but males were more sensitive to smoking consequences as compared with the females.

Keywords: Smoking; Blood cellular; Blood indices, Liver enzyme, Smoking Risks.

1. Introduction

Smoking is one of the basic causes of many diseases, and every 6 minutes one person dies in the world due to smoking risks (Mathers, 2006). Approximately, ten percent of the human mortality in 2012 resulted from smoking (Murray, 1997). To date, millions have died, and this rate will have reached 8 million by 2030 (Murray, 1997; Mathers, 2006). Tobacco can be used as burning as cigarettes, which affects hematological parameters and the liver enzyme activity (Khaled, 2014).

There are 4000 substances in a single cigarette, 200 of which are poisonous and 80 cause cancer; such poisonous substances include nitrogen oxide, nicotine, hydrogen cyanide, carbon monoxide and free radicals which result in disorders in the human body (Farhang, 2013). Also, smoking produces carbon monoxide that binds more firmly with hemoglobin compared to oxygen, leading to many diseases, such as blood pressure (Iqbal, 2003), anemia blood viscosity and hypoxia (Meberg, 1979; Bureau, 1983; Knottnerus, 1990; Bili, 1996) lung cancer, kidney cancer, pancreas cancer, colon cancer, liver cancer and oropharynx cancer (Fariborz, 2014), heart disease, stroke, and chronic obstacle pulmonary disease (Pankaj, 2014).

Furthermore, cigarette smoking alters the hematological system by rising in eosinophil, basophil, monocyte, lymphocyte, platelets and macrophage concentrations. It also increases haemoglobulin and RBCs in the blood (Besime, 2014). Nicotine, which is the most important among the smoking substances, stimulates hormone secretion, which leads to blood cells accumulation, an increase in blood vessel stickiness, and aggregation of platelets and blood cells (Pankaj, 2014). Additionally, it has been proven that the number of leucocytes increases according to the number of cigarettes smoked daily. The number of leukocytes decreases in the body after quitting smoking, and this is related to the period of smoking and the concentration of substances in cigarettes (Friedman, 1973; Yeung, 1984).

However, the most important diagnostic procedure to determine and evaluate liver diseases is the liver function test. Generally, it is composed of general proteins in the serum, including Albumin, Alkaline phosphate, and Bilirubin. One of these proteins is Albumin which is a negative protein that has an effect on inflammatory marker of anti-oxidants. The determination of the liver enzymes should be tested accurately, as these parameters can be affected by the environmental factors and they usually vary from one person to another (Khaled, 2014).

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Therefore, the present study focuses on the harmful consequences of tobacco on hematological parameters and serum live enzyme activity in male and female smokers.

2. Patients and Method

2.1. Case study

This investigation was conducted on twenty-eight volunteers, aged between 21 and 35 years old from Ashty hospital, Soran city. Also, their body mass index is approximately equal and all of them have similar healthy conditions.

2.2. Layout

The volunteers were divided into four groups: Group A, Female smoker (n = 7); Group B, Female non-smoker (n = 7); Group C, Male smoker (n = 7); Group D, Male non-smoker (n = 7). Data were acquired through a questionnaire that was developed for this purpose to measure many hematological parameters including the liver function test.

2.3. The Questionnaire

No.	Questions	Answer
1	Are your Smoking cigarettes currently?	Yes or no
2	Volunteer's Ages	21-35 years
3	Volunteer's Height	Cm
4	Volunteer's Weight	Kg
5	Your menstruation is normal (Females only)	

2.4. Measuring WBC, RBC HB, HCT, and MCV

On the left hand of each volunteer, a tight tourniquet was placed around his/her arm, then skin was cleaned by alcohol in median cubitalvein by venipuncture of forearm veins, 2 ml of blood were withdrawn into a syringe with all aseptic precautions in place and immediately transferred into an anticoagulant tube (K3 EDTA, Jordan), and was continuously shaken (shaker, Turkish) for a period, then WBC, RBC HB, HCT and MCV were measured by a full automated coulter count instrument (Medonic, Swedish).

2.5. Determination of Liver Function Test (LFT)

Under aseptic precautions, 3 mL of blood were taken and immediately transferred into gel tubes (Vacumed, Italy). After standing for 30 minutes, the blood was centrifuged at 4000 rpm for 10 minutes (Hettich, Germany), then the serum was stored in Eppendorf tube at -15^{o}C until assay of liver enzymes by auto human analyzer instrument (Bt35i, Italy)

2.6. Statistical analysis

Student T test was used to compare between the groups by using a computer based software, Statistical Package for Social Science (SPSS, version 16.0). Data were expressed as mean ± standard error of mean.

3. Results

3.1. WBC, RBC HB, HCT, and MCV of smokers and non-smokers

WBC, RBC HB, HCT and MCV were measured by full automated coulter count instrument for all volunteers smokers and non-smokers. Cigarette smoking caused a significant increase ($P < 0.05$) in RBC in males in comparison to that in females (Figure 1). Also, there was a statistical significant rise ($P = 0.000$) in HB (16.34 $\pm$ 0.394) and HCT (51.62 $\pm$ 1.503) of male smoker in concomitant with the female smokers (12.45 $\pm$ 0.548) and (38.81 +2.047), respectively. Additionally, MCV showed a statistically significant change ($P < 0.05$) between male smokers and male non-smokers (Table 1). The analysis also showed that white blood cell counts changed among all groups, but did not reach a statistical significance ($P > 0.05$) (Table 1)**.**

3.2. Liver function test of smokers and non-smokers

Liver function test measured liver enzymes by an auto human analyzer instrument for all smokers and non-smokers. The AST concentration significantly ($P < 0.05$) rose in male smokers in comparison with that in female smokers, whereas there were no significant differences among the other groups (Figure 2). In addition, serum ALT(29.78 $\pm$4.853), bilirubin (0.346 $\pm$0.027) and total bilirubin (1.192 $\pm$0.144) significantly ($P <0.05$) increased in male smoker group as compared to the female smoker group (14.92 $\pm$ 2.656), (0.22 $\pm$0.043), and (0.697 $\pm$0.067), respectively; but ALP level was not statistically significant ($P > 0.05$) between male and female groups or between pair groups (Table 2) .

Table 1. Blood indices in male and female smoker

Group	Female	Female non smoker	Male smoker	Male non smoker	Statistical evaluations T. test (*p*. value)			
parameter	Smoker (A)	(B)	(C)	(D)	A+B	A+C	C+D	B+D
WBC	7.285$\pm$ 0.631	7.257$\pm$ 0.630	9.000$\pm$ 1.263	8.157$\pm$ 0.751	0.975	0.248	0.577	0.377
HGB	12.45$\pm$ 0.548	13.44 $\pm$0.412	16.34$\pm$ 0.394	14.92$\pm$ 0.557	0.177	0.000	0.061	0.053
HCT	38.81$\pm$ 2.047	41.48$\pm$ 1.109	51.62$\pm$ 1.503	47.40$\pm$ 1.605	0.274	0.000	0.079	0.010
MCV	85.30$\pm$ 3.477	89.8857$\pm$3.7935	91.60$\pm$ 1.270	83.47$\pm$ 3.955	0.390	0.115	0.074	0.265

Table 2. Liver function test in male and female smoker

Group parameter	Female Smoker A	Female nonsmoker B	Male smoker C	Male non smoker D	Statistical evaluations T. test (*p*. value) A+B	A+C	C+D	B+D
ALT	14.92± 2.656	12.75± 1.652	29.78± 4.853	27.40± 10.46	0.513	0.049	0.831	0.260
ALP	153.0± 15.98	195.4± 21.66	166.0± 9.055	180.3± 25.42	0.142	0.478	0.583	0.670
Bilirubin	0.22± 0.043	0.260± 0.025	0.346± 0.027	0.262± 0.026	0.437	0.028	0.050	0.954
Total Bilirubin	0.697± 0.067	0.908± 0.118	1.192± 0.144	0.885± 0.151	0.135	0.009	0.170	0.911

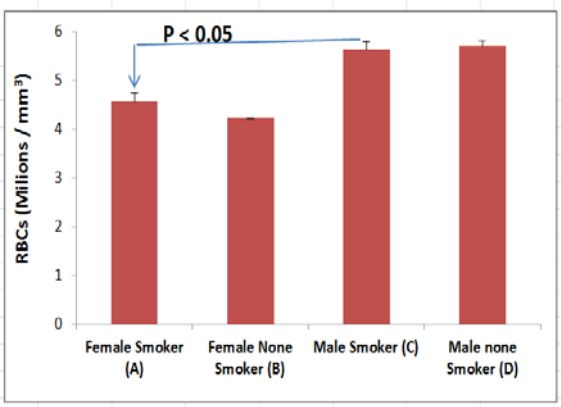

Figure 1. Red blood cell count (million / mm^3) in male and female smoker**.**

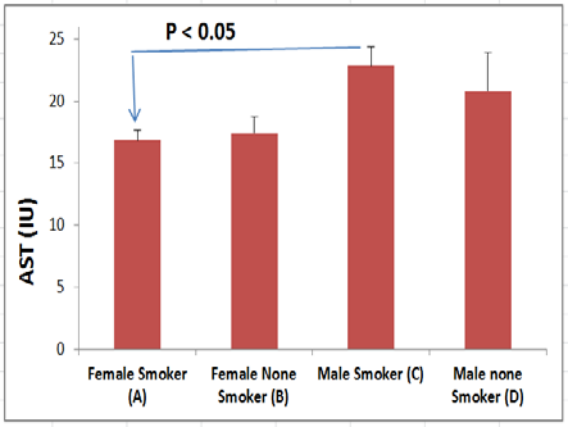

Figure 2. Serum AST concentration in male and female smoker

4. Discussion

The raising in RBC level, in the present study, was mainly due to the increase in the partial pressure of CO2 and CO gases in the blood and led to a decrease in binding O2 gas with the Hb, then the cells of juxtaglomerular of kidney sensed and resulted secretion erythropoietin hormone (Kaliev *et al.*, 2014), which directly contributes in RBCs production by stimulating the stem cell of the red bone marrow (Mader, 2004). But, the reason behind the high number of RBCs in male smoker group versus female smoker group (Figure 2), could be related to sex hormone differences.

Also, there was a significant (P < 0.05) rise in HB and Hct levels, especially in the male smoker group (Table 1). These two parameters are highly related to RBC counts, given that HB is the concentration of haemoglobin in grams, whereas HCT is the total volume of RBCs in percentage (Barret *et al.*, 2010). Thus, if the number of red cells is increased, the HB and HCT level would increase as they proportionally change with RBCs.

However, the size of red blood cells increased in the present study due to significant ($P < 0.05$) increases in MCV of the male smoker group (Table 2). We believe that cigarettes can affect blood cellular physiology in different mechanisms, including physical and chemical interaction mechanisms.

Furthermore, smoking cigarettes caused alteration in liver enzymes, but it was more obvious in the male smoker group (Figure 2, Table 2); many of those fluctuations are related to the rise in blood indices (Stocker, 1987; Micozzi,1989; Milman, 2001). Another possible mechanism is related to male sex hormone.

In conclusion, cigarette risks lead to a change in blood cellular and fluctuation in liver enzymatic activity regardless of gender; but males were more sensitive to smoking consequences, possibly due to hormonal changes based on gender.

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Decadal Variation of Nutrient Level in Two Major Estuaries in Indian Sundarbans

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Abstract

The impact of the nutrient level on the water quality in the Hooghly and Matla estuarine complex in Indian Sundarbans was assessed for three decades (1984-2014). Nitrate, phosphate and silicate were used as indicators of nutrient related water quality in the estuarine water. Our first order analysis reflects significant spatio-temporal variations of selected nutrients with relatively higher values in the Hooghly estuary (in the western Indian Sundarbans) compared to the Matla estuary (in the central Indian Sundarbans). Significant variations were observed in dissolved nitrate, phosphate and silicate concentrations between stations and years ($p < 0.01$). Such pronounced variations may be attributed to the location of highly industrialized and urbanized city of Kolkata, Howrah and Haldia port-cum-industrial complex adjacent to the Hooghly estuary. The sudden rise of selected nutrients during premonsoon, 2009 (irrespective of sampling stations) is directly related to AILA, a super-cyclone that occurred in the lower Gangetic delta during 22nd - 25th May, 2009.

Keywords: Indian Sundarbans, Nutrients, AILA, Spatio-temporal variation.

1. Introduction

Coastal waters and estuaries are facing a variety of adverse impacts affecting both the ecosystem and the human health through a sewage-wastewater discharge and the disposal practices that often lead to the introduction of high nutrient loads, hazardous chemicals and pathogens causing diseases. In countries like India, shrimp culture practices and aquaculture waste have magnified the adverse situation (Mitra, 1998; Mitra and Zaman, 2015). In a regional level, like Sundarbans deltaic ecosystem (located at the tail end of the mighty River Ganga), a phenomenon like erosion with the subsequent washing of the top soil from the intertidal mudflats of mangroves also contributes a considerable amount of nutrients in the adjacent aquatic ecosystem. The adverse public health, environmental, socio-economic, food quality, security and aesthetic impacts from sewage contamination in coastal areas are well documented (Luger and Brown, 1999; Tyrrel, 1999; Danulat *et al*., 2002; WHO, 2003). Apart from these causes, erosion of river-banks due to tidal surges also conveys nitrate, phosphate and silicate in the estuarine water (Mitra *et al*., 2009), which may have a far reaching effect on the environment. However, very few studies have focused on the trend of nutrient load in the estuarine waters based on the past long-term data bank. The present paper is a road map towards this direction in the frame work of Indian Sundarbans.

2. Materials and Method

2.1. Study area

The mangrove dominated Indian Sundarbans in the lower Gangetic delta region at the apex of Bay of Bengal is inhabited by some 4.2 million populations. Two major estuaries in this delta complex are Hooghly (in the western sector) and Matla (in the central sector). There is

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a multitude of industries located on the western bank of the Hooghly estuary. Apart from this, a large number of tourism units and shrimp culture farms are also located adjacent to the Hooghly estuary. A number of studies are available on the salinity profile, surface water temperature, and pH of the area (Mitra, 1998; Mitra and Bhattacharya, 1999; Mitra, 2000; Majumder *et al.*, 2002; Panja *et al*., 2003; Banerjee *et al*., 2005; Mitra and Banerjee, 2005; Mitra *et al.*, 2011; Zaman and Mitra, 2014; Mitra and Zaman, 2015; Ray Chaudhuri *et al.*, 2015; Trivedi *et al.*, 2015a; Trivedi *et al.*, 2015b, Chaudhuri *et al.*, 1994). However, very few studies have addressed the long-term variation of the nutrient level in the estuarine waters, although the area is presently experiencing a population growth, industrial activities, mushrooming of shrimp farms, a growth of tourism units and establishment of fish landing stations (Mitra, 2013). Apart from these primary sources of nutrients, the churning action of bed substratum due to wave action and currents conveys silica to the overlying aquatic phase.

2.2. Sample collection

Sampling of surface water was done during the high tide from three stations namely Diamond Harbour, Namkhana (in the Hooghly estuary) and Ajmalmari (in the Matla estuary) (Table 1). Sample collection was carried out during May (premonsoon), September (monsoon) and December (postmonsoon) for a period of 31 years (1984-2014).

Table 1. Location of sampling stations

Stations	Geographical locations	
	Latitude	Longitude
Diamond Harbour	22°11'4.2"N	88°11'22.2"E
Namkhana	21°45'53.7"N	88°13'51.5"E
Ajmalmari	21°49'42.9"N	88°37'13.7"E

2.3. Nutrient analysis

Surface water samples collected for nutrient analysis were filtered through a 0.45 μm polycarbonate filters (Millipore 47 mm diameter) and then deep frozen for further analysis in the laboratory. The standard spectrophotometric method of Strickland and Parsons (1972) was adopted to determine the nutrient concentrations in surface water. Nitrate was analyzed by reducing it to nitrite by means of passing the sample with ammonium chloride buffer through a glass column packed with amalgamated cadmium filings and finally treating the solution with sulphanilamide. The resultant diazonium ion was coupled with N – 1 - napthyl ethylene diamine to give an intensely pink azo dye. Estimation of the phosphate was carried out by treating an aliquot of the sample with an acidic molybdate reagent containing ascorbic acid and a small proportion of potassium antimony tartarate. The dissolved silicate was analyzed by treating the sample with acidic molybdate reagent. The resultant silico-molybdic acid was reduced to molybdenum blue complex by ascorbic acid and incorporating oxalic acid prevented formation of similar blue complex by phosphate.

2.4. Statistical analysis

ANOVA was performed in order to analyze whether the selected nutrients vary significantly between years and stations ($p < 0.01$).

3. Results

We noted the significant spatio-temporal variations of nitrate, phosphate and silicate in the study region. Also, the sudden rise of the nutrient level during premonsoon, 2009 is attributed to super-cyclone, AILA that contributes nutrients through the massive erosion of river banks, washing of top soil of intertidal mudflats along the estuaries and churning of the river bed.

3.1. Dissolved Nitrate

At Diamond Harbour, the dissolved nitrate concentration ranged from 20.84 ppm (during premonsoon in 1984) to 48.15 ppm (during premonsoon in 2009). At Namkhana, the dissolved nitrate concentration ranged from 18.90 ppm (during premonsoon in 1984) to 43.44 ppm (during premonsoon in 2009). In the case of Ajmalmari, the values ranged between 9.44 ppm (during premonsoon in 1984) to 24.89 ppm (during premonsoon in 2009).

In both Diamond Harbour and Namkhana of the Hooghly estuarine system, the nitrate increased 9.49 μg at l^{-1}/ decade and 10.57 μg at l^{-1}/ decade, respectively. In Ajmalmari, located in the Matla estuarine complex, the increase was relatively low (5.98 μg at l^{-1}/ decade).

3.2. Dissolved Phosphate

At Diamond Harbour, the dissolved phosphate concentration ranged from 1.54 ppm (during premonsoon in 1984) to 6.99 ppm (during premonsoon in 2009). At Namkhana, the dissolved phosphate concentration ranged from 0.98 ppm (during premonsoon in 1984) to 6.05 ppm (during premonsoon in 2009). Ajmalmari, the station in the Matla estuary, exhibited a phosphate value from 0.56 ppm (during premonsoon in 1984) to 2.96 ppm (during premonsoon in 2009). In both Diamond Harbour and Namkhana of the Hooghly estuarine system, the phosphate increased 1.96 μg at l^{-1}/ decade and 2.11 μg at l^{-1}/ decade, respectively. In Ajmalmari, located in the Matla estuarine complex, the rate of the increase was 0.92 μg at l^{-1}/ decade.

3.3. Dissolved Silicate

At Diamond Harbour, the dissolved silicate concentration ranged from 101.83 ppm (during premonsoon in 1984) to 242.78 ppm (during premonsoon in 2009). At Namkhana, it ranged from 44.68 ppm (during premonsoon in 1984) to 211.49 ppm (during premonsoon in 2009). In the case of Ajmalmari, the silicate ranged from 31.43 ppm (during premonsoon in 1984) to 111.99 ppm (during premonsoon in 2009). In both Diamond Harbour and Namkhana of the Hooghly estuarine system, the silicate increased 64.57 μg at l^{-1}/ decade and 54.56 μg at l^{-1}/ decade, respectively. The rate of the increase was 31.18 μg at l^{-1}/ decade in Ajmalmari, which is relatively

low compared to the stations selected in the Hooghly estuary.

The temporal variations of the selected nutrients during 1984-2014 are shown in Figures. 1- 9.

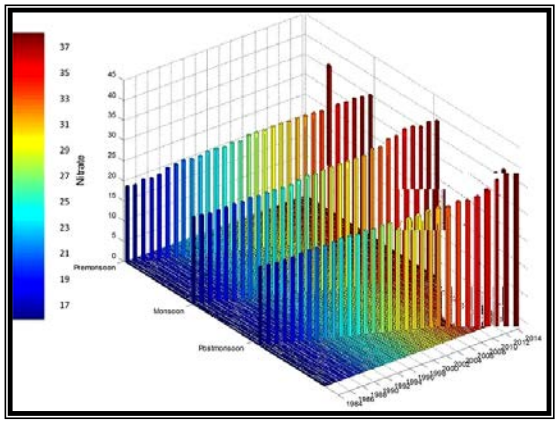

Figure 1. Dissolved Nitrate (in µg at l^{-1}) in Diamond Harbour during 1984 – 2014

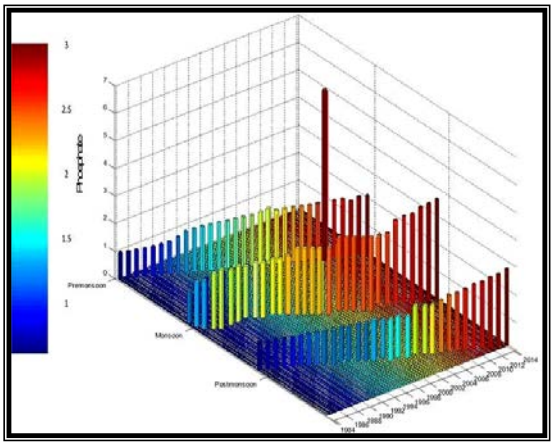

Figure 2. Dissolved Phosphate (in µg at l^{-1}) in Diamond Harbour during 1984 – 2014

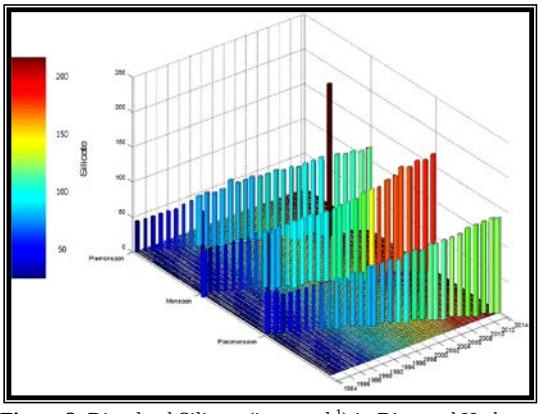

Figure 3. Dissolved Silicate (in µg at l^{-1}) in Diamond Harbour during 1984 – 2014

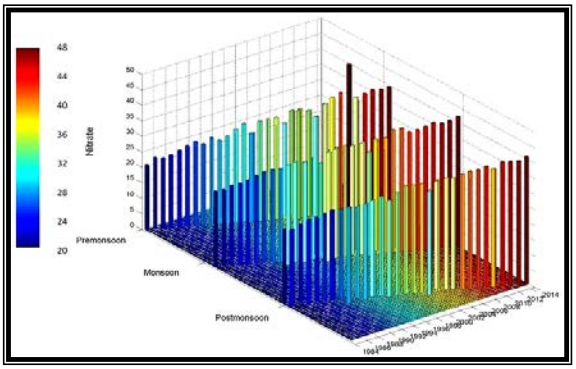

Figure 4. Dissolved Nitrate (in µg at l^{-1}) in Namkhana during 1984 – 2014

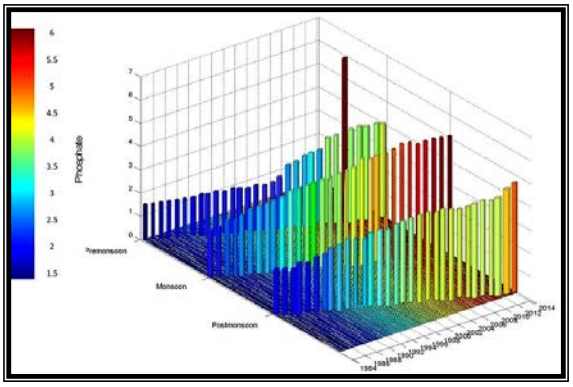

Figure 5. Dissolved Phosphate (in µg at l^{-1}) in Namkhana during 1984 – 2014

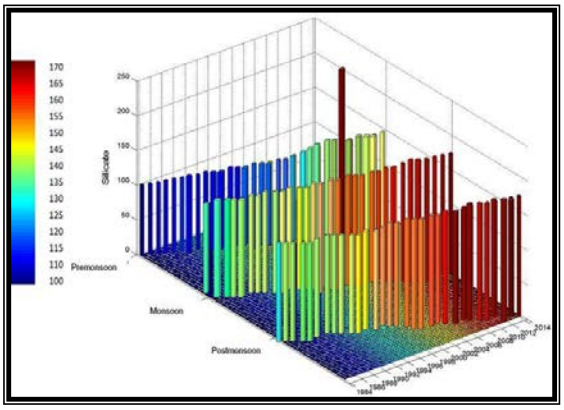

Figure 6. Dissolved Silicate (in µg at l^{-1}) in Namkhana during 1984 – 2014

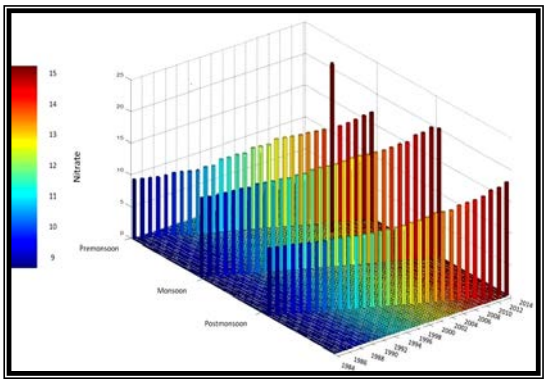

Figure 7. Dissolved Nitrate (in µg at l^{-1}) in Ajmalmari during 1984 – 2014

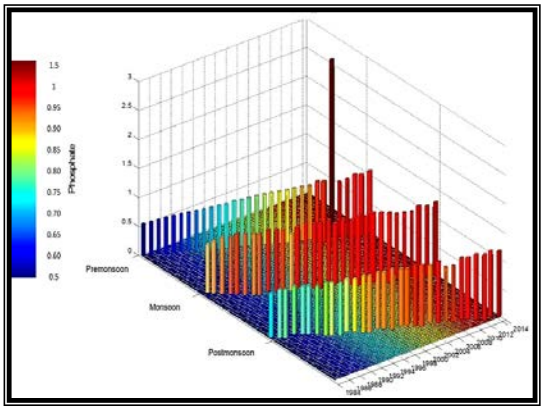

Figure 8. Dissolved Phosphate (in µg at l^{-1}) in Ajmalmari during 1984 – 2014

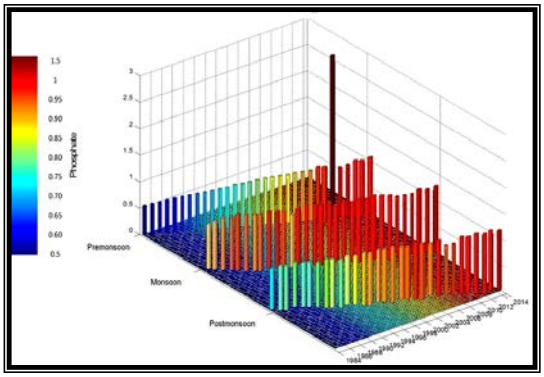

Figure 9. Dissolved Silicate (in µg at l^{-1}) in Ajmalmari during 1984 – 2014

4. Discussion

The enrichment of the aquatic system by nutrients has both a natural and an anthropogenic origin. The main sources of nutrient input in the present study area are run-off from the adjacent landmasses (Mitra, 2013), erosion and leaching (Mitra *et al.*, 2009), sewage from cities and industrial wastewater (Mitra and Choudhury, 1993; Mitra, 1998; Bhattacharyya *et al.*, 2013), untreated sewage disposal from shrimp farms and tourism units (Mitra, 2013; Zaman and Mitra, 2014; Mitra and Zaman,2015). The atmospheric deposition of nitrogen (from combustion gases) can also be important for Hooghly estuarine system and its surrounding area as multifarious industries are concentrated in this estuarine region. The effects of nitrogen on marine and estuarine systems, the pathways for nitrogen transport between land and aquatic habitats, and the positive correlation between nitrogen and primary production and often secondary production (i.e., fishery yields) have been widely reviewed (Hecky and Kilham, 1988; Howarth, 1988; Rabalais, 2002).

Most of the phosphorus pollution in the present study area comes from the households and industries including phosphorus-based detergents which are widely used in thickly populated cities of Kolkata, Howrah and Haldia industrial complex adjacent to the Hooghly estuary.

Silicate, being an important ingredient of bed material and soil substratum, originates due to erosion, churning action of the water that amplifies during tropical cyclone, which is common in the present geographical locale.

The ratio of nitrogen and phosphorus plays a regulatory role in the phytoplankton diversity spectrum of brackishwater. It determines which of the two elements will be the limiting factor and, consequently, which one has to be controlled in order to arrest the bloom condition (Table 2).

Table 2. Nitrogen / Phosphorus ratios (expressed in weight) for various limiting conditions in freshwater and estuarine/coastal water

Type of waterbody	N-limiting Ratio N/P	Intermediate Ratio N/P	P-limiting Ratio N/P
Freshwater	≤ 4.5	4.5-6	> 6
Estuarine / coastal water	≤ 5	5-10	≥ 10

Source: WHO (2003)

Large marine areas frequently have nitrogen as the limiting nutrient, especially in summer. Intermediate areas, such as river plumes, are often phosphorus-limited during spring, but may turn to silica or nitrogen limitation in summer. When phosphorus is the limiting factor, a phosphate concentration of 0.01 mg l^{-1} is enough to support plankton and concentrations from 0.03 to 0.1 mg l^{-1} or higher will be likely to promote blooms.

In coastal areas, the growth and proliferation of diatoms are promoted by the presence of silica. When the silica concentration is low, diatoms cannot develop. Then, other opportunistic toxic algal species, which are no longer submitted to competition, can grow and form blooms. Species from the genus *Phaeocystis* and several dinoflagellates *(Prorocentrum, Dinophysis, Gymnodynium)* are known to proliferate under such conditions.

In the present study, the N: P ratios in all the selected stations and seasons are greater than 10 (except monsoon season in Namkhana) (Table 3). This implies that the aquatic phase of the present study area is P limiting (WHO, 2003). A high N: P ratio normally increases the standing stock of dinoflagellates and diatoms.

Significant variations in the level of the dissolved nitrate, phosphate and silicate between years and between stations were observed ($p < 0.01$), which reveals the impact of season and anthropogenic pressure in the present study area (Table 4).

Table 3. Avarage Nitrogen / Phosphorus ratios in different seasons in the three sampling stations

Stations / Seasons	**N/P ratio**		
	Premonsoon	**Monsoon**	**Postmonsoon**
Diamond Harbour	15.53	12.60	17.04
Namkhana	12.26	8.85	10.18
Ajmalmari	14.55	15.39	14.18

Table 4. ANOVA result showing temporal and spatial variations of dissolved nutrients (nitrate, phosphate and silicate)

		Variables	F_{cal}	F_{crit}
Dissolved nitrate	Premonsoon	Between stations	876.8915	3.150411
		Between years	21.43769	1.649141
	Monsoon	Between stations	709.1798	3.150411
		Between years	15.52743	1.649141
	Postmonsoon	Between stations	846.2506	3.150411
		Between years	13.00163	1.649141
Dissolved phosphate	Premonsoon	Between stations	92.25571	3.150411
		Between years	8.000291	1.649141
	Monsoon	Between stations	268.6471	3.150411
		Between years	4.05633	1.649141
	Postmonsoon	Between stations	337.6358	3.150411
		Between years	4.725808	1.649141
Dissolved silicate	Premonsoon	Between stations	517.3935	3.150411
		Between years	21.15371	1.649141
	Monsoon	Between stations	426.0496	3.150411
		Between years	7.435455	1.649141
	Postmonsoon	Between stations	1061.878	3.150411
		Between years	11.78569	1.649141

5. Conclusion

The core findings of the present study are listed here:

1. The main sources of nutrients in the present study area are primarily anthropogenic in nature, although natural disasters (like super cyclone AILA) resulted in a sudden rise in the nutrient level during premonsoon, 2009.
2. The concentrations of nutrients exhibit a gradual increasing trend, which is an evidence of the rising anthropogenic pressure in and around the mangrove dominated Indian Sundarbans.
3. The situation seems to be alarming in terms of nutrient enrichment if a proper management/control measure is not adopted. The policymakers must foster appropriate actions with a true partnership with the private sector. The regulations should also be put in place to force the polluters (from urban areas, industries, shrimp farms and tourism units) to pay the principal cost and also to foster willingness to pay among polluters through the provision of better and efficient services. This should ensure operational sustainability of the services.

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Ethyl Methane Sulphonate Induced Desynaptic Variants in Ajwain (*Trachyspermum ammi* (L.) Sprague)

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Abstract

Synaptic mutants are the subject of a special concern to cytologists and geneticists as they hold the variability in their recombination pattern. Seeds of *Trachyspermum ammi* (L.) Sprague (ajwain) were treated with Ethyl Methane Sulphonate (EMS) solution at 3 different concentrations *viz*. 0.1%, 0.3% and 0.5% for 5 h, prepared in potassium phosphate buffer with 7.0 pH. During meiotic observation of 0.3% concentration of EMS treated population of ajwain, the Pollen Mother Cells (PMCs) of the two plants showed a desynaptic behavior with a high frequency of a univalent formation along with a few bivalents at diakinesis/metaphase I. Apparently, EMS might be involved in the formation of substandard synaptic proteins which reduced the chiasma formation, suggesting that EMS has the ability to generate the male sterile lines. Along with the univalent formation, other various chromosomal aberrations were also analyzed in the subsequent stages of meiotic division *viz*. laggards, bridges, unequal separation, and disturbed polarity with abnormal tetrads. Thus, both the desynaptic plants displayed a high incidence of pollen sterility. The present investigation made an attempt to understand the meiotic behavior of desynapsis and its consequences on the pollen fertility of the ajwain crop.

Keywords: Synapsis, Ajwain, Desynaptic plants, Ethyl methane sulphonate, Recombination, Chiasma.

1. Introduction

Meiosis is a highly organized and conserved process, which facilitates the reduction of the chromosome number of all sexually reproducing organisms and ensures the accuracy of their genetic balance. It constitutes an array of different coordinated events. Although all of them play an essential role but synapsis during the zygotene stage of prophase I is one of the most significant events in meiosis. During this process, the homologous chromosomes recognize each other by their telomeres and synapse intimately along their length. After pairing, recombination is initiated by meiosis specific Double Strand Breaks (DSB) (Kleckner, 1996). DSB and the subsequent genetic exchange precede the formation of the Synaptonemal Complex (SC) (Hawley and Arbel, 1993). The pairing and SC formation in homologous chromosomes are essential for their consequent orderly segregation during the anaphase of the first meiotic division. However, any deviation from this normal incident results into two types of mutations (for example, asynaptic and desynaptic mutation), which affects the pairing of homologous chromosomes and chiasma formation. Mutations that partially or completely prevent homologous chromosome pairing are classified as asynaptic mutants, while those that cause the premature separation of homologous chromosomes are classified as desynaptic (Cai and Makaroff, 2001).

Ajwain (*Trachyspermum ammi* (L.) Sprague) is an annual herb of a Mediterranean origin and is used as a traditional ayurvedic medicine in India. Thymol is the main ajwain essential oil constituent and may be yielded from 35% - 60% (Ishikawa *et al.*, 2001; Zershinas *et al*., 2014). The non-thymol fraction (thymene) contains Para-cymene, γ-terpinene, α-pinene, β-pinene, α-terpinene, Styrene, δ-3-carene, β-phyllanderene, terpinene-4-ol and Carvacrol (Mohagheghzadeh *et al*., 2007; Ranjan *et al*., 2012). The phenols, thymol and carvacrol are responsible for the antiseptic, antitussive, and expectorant properties. Although a huge amount of work has been done related to the antimicrobial activity of ajwain (Khan *et al.*, 2010; Singh *et al*., 2004; Mood *et al.*, 2014), but there is much to explore in the area of cytogenetics.

The factors influencing the nuclear male sterile mutants (desynaptic mutants) having a reduced amount of chiasma formation are valuable resources in the field of cytogenetics, hold a variability in their recombination pattern resulted aneuploid formation. The objective of this study was to describe the meiotic behavior of induced desynapsis, and to evaluate the induced pollen sterility,

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and to investigate the unexplored aspect, especially in the case of ajwain.

2. Materials and Method

2.1. Procurement of seeds

Healthy and fresh seeds of *T. ammi* var.AA-1 were collected from National Research Centre for Seed Spices, Ajmer, Rajasthan, India.

2.2. Seed treatment

Seeds were treated with EMS solution at 3 different concentrations *viz.* 0.1%, 0.3% and 0.5% for 5 h, prepared in potassium phosphate buffer with 7.0 pH with a constant shaking and a thorough washing with tap water. Three replicates were given for each treatment along with their respective controls. After that, seeds were sown to raise the M_1 generation.

2.3. Meiotic analysis

At the time of flowering, young floral buds were fixed in ethanol and glacial acetic acid (3:1) solution for 24 h, after which they were transferred to 70% ethyl alcohol and stored at 40ºC. The meiotic slides were prepared using anther squash technique with 2% acetocarmine (Fürste, 1962). Slides were analyzed and suitable cells were photographed under a Nikon research photomicroscope. The pollen fertility was estimated using a glyceroacetocarmine (Marks, 1954). Fertile pollen grains were recorded with stained cytoplasm with whereas undersized and unstained pollen grains without nuclei were considered sterile.

2.4. Statistical analysis

For all measurements, data collected were subjected to analysis of variance (ANOVA) performed with SPSS 16.0. A pair wise comparison of means was made using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ significance level.

3. Results

Five plants were cytologically analyzed out of which the Pollen Mother Cells (PMCs) of two plants showed a desynaptic chromosomal behavior. The two plants from 0.3% concentration of EMS treated population were morphologically normal and showed normal flowering. The seed formation in these plants was negligible. The cytological studies of these plants revealed aberrant meiotic behavior. In control plants, 9 bivalents were observed frequently, at diakinesis and metaphase I (Figures 1A and 1B, respectively) followed by normal anaphase I (9:9 separation). Due to some environmental factors, the PMCs of control also showed univalents, but this occurred in a very low frequency (0.55 % in diakinesis and 0.36 % at metaphase I). While in sterile plants, only few bivalents and a high frequency of univalents were recorded. In the desynaptic mutants, univalents were predominantly present at diakinesis and metaphase I stages, although most of the univalents were observed in the stages of diakinesis. The maximum chromosomal configuration *i.e.* 0-2(II)+14-18(I) in E-1 plant, was 26.09 % at diakinesis while 5.81% at metaphase I. However, in E-2 it was 12.85 % at diakinesis while 4.83 % at metaphase I (as mentioned in Table 1). In E-2, chromosome configuration of 6-8(II)+2-6(I) were found to be predominant (28.25 % at diakinesis and 5.05 % at metaphase I) as compared to E-1 (15.33 % at diakinesis and 4.15 % at metaphase I).

In the desynaptics, the univalents were not found independently but they belonged to the same pair and were preferably found in close proximity to each other. In plant E-1, a high frequency of univalent was reported (64.69 %) as compared to E-2 (56.32 %) at diakinesis/ metaphase I (Table 2). Hence, E-1 showed a high frequency of pollen sterility than E-2 (Table 2). The univalents and bivalents varied considerably from PMC to PMC in both the desynaptics. Consequently, both the desynaptic plants (E-1 and E-2) fitted into the category of medium-strong type.

Figure 2 shows the comparative frequencies of univalents, bivalents and pollen fertility of control and two desynaptic plants. At metaphase I, bivalents and univalents were arranged at the equatorial plate but the chromosome segregation was not perfect. As a consequence of univalent formation, various other chromosomal aberrations were observed in the succeeding stages of meiotic division (Table 2). At anaphase I, unequal separation of chromosomes (Figure 1I) was observed frequently in E-1 while in E-2 it was scarcely reported. A part from causing unequal separation, laggards, bridges (Figure 1J), late separation, disturbed polarity were also observed. The bivalents had 1 or 2 chiasmata, delay in chiasma terminalisation which promoted the occurrence of laggards at anaphase I. Simultaneously, the second meiotic division in desynaptic mutants also showed abnormal meiotic divisions as in the anaphase I. More commonly dyads (Figure 1L), triads were also observed. Laggards were also reported in anaphase II (Figure 1K).

A variable number of microspore formations at tetrad stage were observed from 3-6 microspores. In normal plants, quartet of cells were observed. In plant E-1 very few PMCs showed a tetrad of homosized spores, which is a characteristic of normal plants. These types of various aberrations lead to pollen sterility in both the plants (88.38% in E-1 whereas 83.79% in E-2, Table 2).

Table 1. Chromosomal configurations at diakinesis/metaphase I in two induced desynaptic plants of ajwain (*T. ammi* (L.) Sprague) var. AA-1

Plant No.	Stages	Total no. of PMCs observed	Chromosomal Configurations (Mean±S.E.*)			
			9II	6-8(II)+2-6(I)	3-5(II)+8-12(I)	0-2(II)+14-18(I)
Control	Diakinesis	379	68.65±2.35	0.55±0.01	-	-
	Metaphase I	120	21.49±3.40	0.36±0.18	-	-
E-1	Diakinesis	413	2.25±0.14	15.33±0.66	14.97±0.34	26.09±0.29
	Metaphase I	162	0.86±0.16	4.15±0.43	6.95±0.18	5.81±0.32
E-2	Diakinesis	402	1.96±0.12	28.25±0.33	15.04±0.37	12.85±0.71
	Metaphase I	157	0.71±0.17	5.05±0.52	6.60±0.48	4.83±0.22

*S.E. - Standard Error, II- Bivalents, I- Univalents.

Table 2. Frequencies of univalents and bivalents along with anaphasic abnormalities and pollen sterility in two induced desynaptic plants in ajwain (*T. ammi* (L.) Sprague) var. AA-1

Plant no.	Frequencies (%Mean±S.E.)		Anaphasic Abnormalities (Mean±S.E.)				Pollen Sterility (Mean±S.E.)
	Bivalents	Univalents	Unequal seperations (8:10/7:11)	Laggards	Bridges	Others	
Control	98.18±0.79	1.67±0.92	-	-	-	-	2.43±0.45
E-1	35.30±1.20	64.69±1.20	9.93±0.40	5.21±0.19	4.69±0.20	3.99±0.07	88.38±0.16
E-2	43.52±1.69	56.32±1.54	7.49±0.33	5.80±0.29	5.75±0.38	5.26±0.21	83.79±1.08

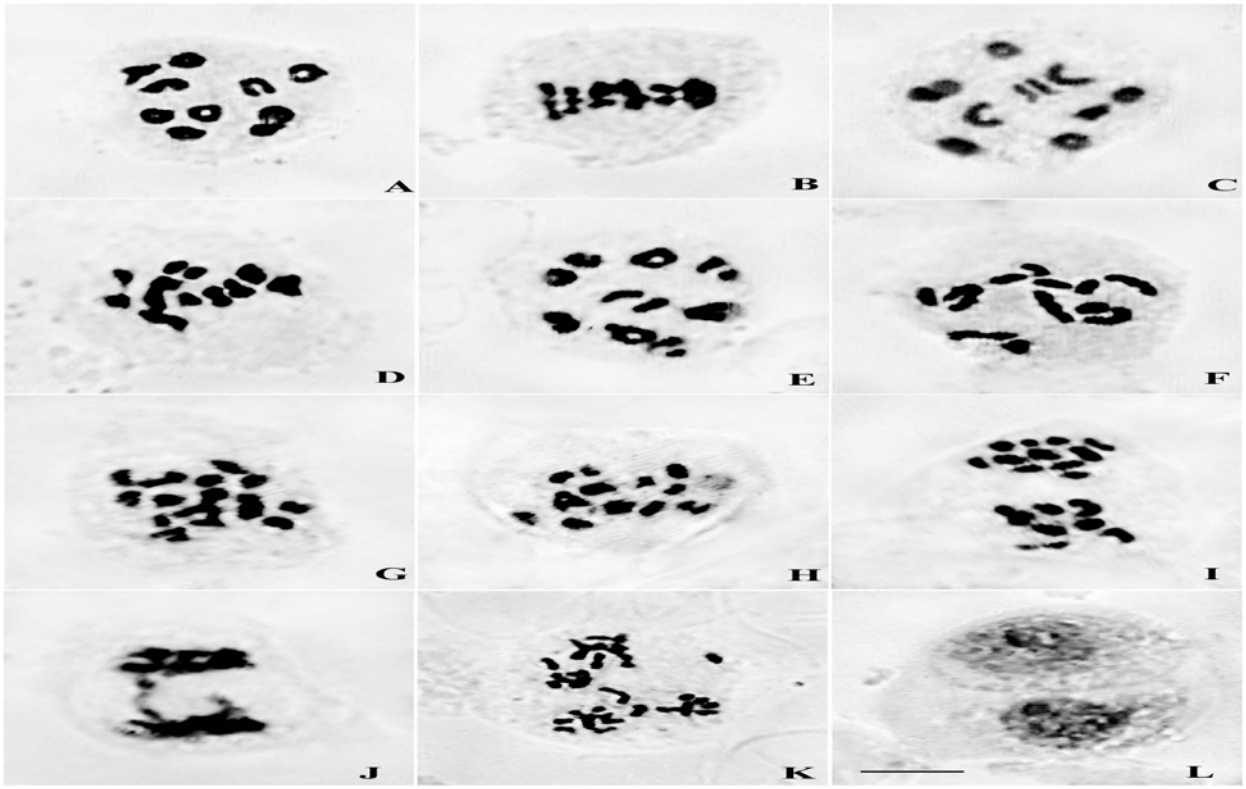

Figure 1. The normal Ajwain chromosome complement; 2n=18. **A**.Normal Diakinesis (9 II), **B.** Normal metaphase I , **C.** Diakinesis (8II+ 2I), **D.** Metaphase I (7II+ 4I), **E.** Diakinesis (6II+ 6I), **F.** Diakinesis (5II+ 8I), **G.** Diakinesis (3II+ 12I), **H.** Diakinesis (3II+ 12I), **I.** Anaphase I (7:11 separation), **J.** Bridge at anaphase I, **K.** Laggards at anaphase II, **L.** Dyad. Scale bar: 1cm.=4.28µm

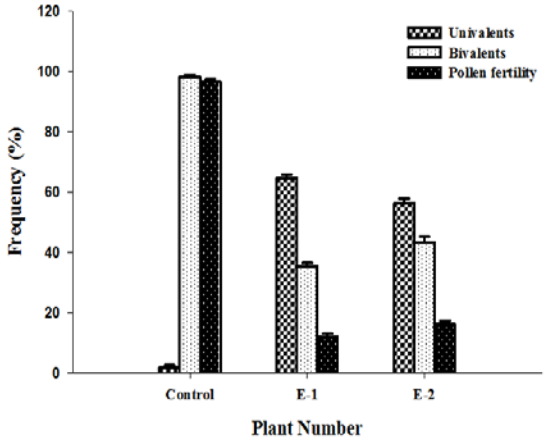

Figure 2. Comparative account of univalents, bivalents and pollen fertility of control and tow desynaptic plants of ajwain (*T. ammi* (L.) Sprague)

4. Discussion

The cytogenetical study based on M_1 generation of ajwain introduces more or less sterile individuals indicating that the mutation affects the process of microsporogenesis. Peirson *et al*. (1996) suggested that the mutation may disrupt the early stage of meiosis and results in the formation of aberrant microsporocytes and megasporocytes. Research on desynaptic mutants continues in many economically important plants but primarily used as a source of trisomics for genetic analysis (Jackson *et al*., 2002).

Desynaptic mutants are classified into three types according to chromosome dissociation into weak, medium-strong and complete (Prakken, 1943). In the present case, both the desynaptic plants (*i.e.*, E-1 and E-2) obtained are of a medium-strong type, as a number of cells possess one or two or rarely up to four bivalents and more than five bivalents, respectively, at stages of diakinesis and metaphase I (Table 1). According to Pierson *et al.* (1997), in desynapsis, the bivalents as well as univalents congregate at metaphase plate. The two plants (*i.e*., E-1 and E-2) obtained during the present investigation are in accordance with the findings.

In the present case, anomalous pairing of homologous chromosomes (Figure 1C to 1H) was induced by EMS, which is a potent mutagen creating the base pairing mistakes. However, the exact mechanism of EMS in the formation of univalents has not been known yet, but according to Naseem and Kumar (2013), EMS that might have acted on some genes was responsible for synapsis and chiasma formation, and resulted in early chiasmata dissociation.

The PMCs of both the mutant plants showed few bivalents with a high incidence of univalents. Various researchers gave many explanations regarding desynapsis stating that the univalent formation is linked with mutation at the gene level. The recombination modifier genes (*rec* genes) play an important role in the formation of viable cross over product. According to Simchen and Stamberg (1969), these genes are also defined as coarse control. The coarse control system has numerous genes that rigidly control the progression of meiotic events (Ji *et al*., 1999). A mutation in this gene system might lead to a failure of chiasma formation and recombination (Simchen and Stamberg, 1969). Mutations that alter the chiasma development and formation are more numerous than the other types of meiotic mutants, and they provide a source of variability in the otherwise conserved *rec* alteration system (Kaul and Murthy, 1985). The commencement of the mutation in these genes may lead to the non-disjunction and can generate aneuploids. Earlier workers, like Sharma and Reinbergs (1974) and Gottschalk and Klein (1976), also proposed that the recessive homozygous condition of *ds* gene might cause chiasma to dissociate early leading to desynapsis, *i.e.*, the formation of univalents.

Thus, from all these statements, it can be concluded that there is a large number of genes ingeniously involved in the formation of univalents which reflect the complexity of the genetic control system of chiasma formation and chiasma maintenance (Kitada and Omura, 1983). Cohesin is the protein which is able to hold the homologous chromosomes together. Some sort of mutations in these protein encoding genes might also be the reason of univalent formation (Figure 1C-1H and Table 2) which resulted in the premature separation of sister chromatids. Defects in synapsis not only result in recombination and chiasmata deficiency but might also hinder the establishment of sister chromatid cohesion necessary for proper chromosome segregation (Bickel and Orr-Weaver, 1996).

Consequently, due to the formation of univalents, the following meiotic stages were also asymmetrical like laggards (Figure 1K), unequal separation (Figure 1I) and bridges (Figure 1J) at anaphase I (Table 2). Klein (1970) stated that pairing and chromosome breakage are essential for crossing over to occur in the synaptic mutant during which “U” type reunions occur between sister chromatids leading to bridge and fragments instead of “X” type reunions leading to crossing over. The unequal distribution of chromosomes at anaphase attributed to different sized microsporocyte formation and ploidy levels that do not generally develop into viable pollen grains. Alternatively, a laggard develops into the micronuclei at telophase I/II.

5. Conclusion

From the present study, it can be concluded that desynapsis emerges due to the mutated genes. Such individuals are favourable material for the cytogenetic analysis based on chiasma formation and crossing over and provide the genetic information about the male sterility and aneuploid lines and to generate the anueploids in higher plants.

Although all the control and treated plants were grown in same environmental conditions, only E-1 and E-2 showed a high incidence of univalent formation and pollen sterility. Due to the formation of univalents, some other meiotic abnormalities, *viz*. laggards, bridges, unequal separation, triads, dyads, etc., were also reported. Decisively, the present study clearly elucidates that both plants are of medium-strong type of desynaptic mutants.

The study also suggests that EMS has the ability to generate the male sterile lines and can be used for the development of aneuploids in ajwain. Further related studies are required to support these results and to explore our knowledge related to the univalent formation and its consequences for their resourceful utilization in breeding programs of higher plants.

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Jordan Journal of Biological Sciences **Short Communication**

Freshwater Snails of Tabuk Region, Northern Saudi Arabia

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Abstract

Two freshwater snail species (*Melanoides tuberculata* and *Melanopsis buccinoidea*) belonging to two families (Thiaridae and Melanopsidae), respectively, were recorded for the first time from six localities in Tabuk region, northern Saudi Arabia.

Keywords: Freshwater Snails, Gastropods, Tabuk region, Saudi Arabia.

1. Introduction

Despite that Saudi Arabia is considered one of the driest countries in the world, comprehensive studies on the Saudi Arabian freshwater snails were conducted (Brown and Wright, 1980; Neubert, 1998). A total of 29 species were recorded covering the entire Arabian Peninsula, with the highest diversity in Yemen. These studies covered the eastern, western, south western regions of Saudi Arabia.

Studies on the freshwater snails of the northern regions of Saudi Arabia are scarce. A recent study on the freshwater snails in the northern region of Saudi Arabia was conducted by Amr and Alshammari (2012), with a single record of one species, *Bulinus beccari*, in Hail region. Studies of the freshwater snails in Tabuk region, northern Saudi Arabia, are unavailable. The aim of this study is to document the freshwater snail fauna in the Tabuk region.

2. Materials and Methods

2.1. Study Area

Tabuk is a province of Saudi Arabia, located along the north-west coast of the country, facing Egypt across the Red Sea. The boundaries of Tabuk region extend from the Saudi-Jordanian borders in the north to the north of Medina Al Munawwarah Province, and from the Red Sea on the west to the Hufa depression in the east. It lies at the junction of Hejaz mountain range and the plains of the north in the basin of sedimentary area (Figure 1a).

Seventy-five freshwater localities, representing several aquatic habitats (springs, artificial lakes, swamps, and streams, ponds and irrigation canals), were visited between 2012 and 2014;six sites only had records of *Melanoides tuberculata* and *Melanopsis buccinoidea* (Table 1, Figure 1b). Coordinates for the six sites were recorded using Eten smarts Global Position System. Physico-chemical parameters for the freshwater locations were measured: air and water temperature, dissolved oxygen (mg/l), total dissolved salts (ppt), and pH (Table 2). Samples were collected manually, labeled and preserved in plastic containers containing 70% ethanol.

Table 1. Localities from which materials were collected.

Locality	N	E
TayyebEsm	28 33 591"	34 48 089"
Magna	28 23 787"	34 45 063"
Geyal	28 09 736"	35 02 482"
Deba	27 41 123 "	35 29 096 "
Aldeesah	27 38 021"	36 31 284 "
Umluj	25 04 215"	37 20 982"

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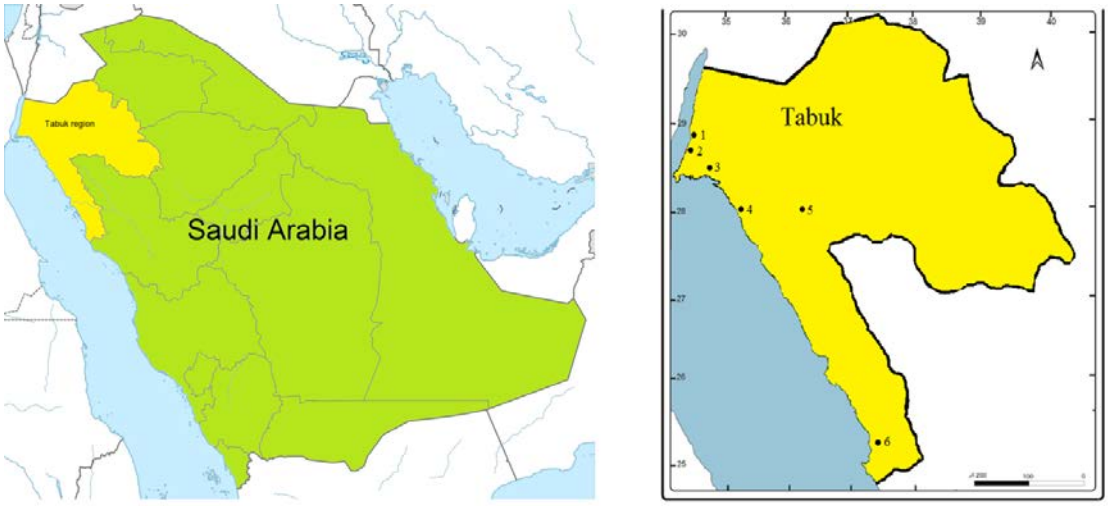

Figure 1. (A) Location of Tabuk region in Saudi Arabia (B) Freshwater localities that showed records in Tabuk region

Table 2. The physico-chemical parameters of different sites measured during sampling.

Site No.	Site name	Maximum depth (cm)	Temperature		Conductivity (µs)	Total Dissolved salts (ppt)	Dissolved oxygen (mg/l)	pH	Transparency (cm)
			Air	Water					
1	TayyebEsm (Permanent stream inside the mountain with a length about 1Km).	30	25	21	5420	3.85	6.99	8	Clear
2	Magna (Moosa springs).	50	25	28	2450	1.37	6.01	8	Clear
3	Geyal (Artificial lake in a farm filled with ground water for irrigation).	200	26	31	5910	3.64	6.15	8	very clear
4	Deba (Pond in a farm at Al-moweeleh)	150	26	22	12780	9.06	9.40	8	very clear
5	Aldeesah (Running water from the mountain)	-	23	23	487	0.262	NR	8	very clear
6	Umluj (A pond at a farm)	100	30	29	4050	2.86	4.88	8	Clear

3. Results

A total of 220 freshwater snail individuals were collected from the study area. Snails were identified as *Melanoides tuberculata* (Family Thiaridae) and *Melanopsis buccinoidea* (Family Melanopsidae).

3.1. Family Thiaridae

Appendix A. *Melanoides tuberculata* (Müller, 1774)

Appendix B. Description: Shells vary in size, the larger ones reaching nearly 50 mm in length and containing up to 15 whorls. The shell is imperforate, and has moderately rounded whorls, which are separated by moderately impressed sutures. The shell surface is sculptured with transverse ribs and spiral ridges and grooves. The shell is light horn or somewhat darker in color, with reddish-brown color patches. The anterior shell aperture is evenly curved (Figure 2a).

Distribution: Distributed in artificial lakes, ponds, springs and streams in Aldeesah, Geyal, Deba, Umluj, and in Moosa springs on Magna near Gulf of Aqaba (Figure 3).

3.2. Family Melanopsidae

Appendix C. *Melanopsis buccinoidea* (Olivier, 1801)

Description: The shell is turreted and broad conical;it has a shiny black color, commonly reaches a length of 20 mm and has flat and smooth whorls (Figure 2b).

Distribution: Distributed in the springs and streams near the Gulf of Aqaba, recorded in TayyebEsm and Moosa springs in Magna (Figure 3).

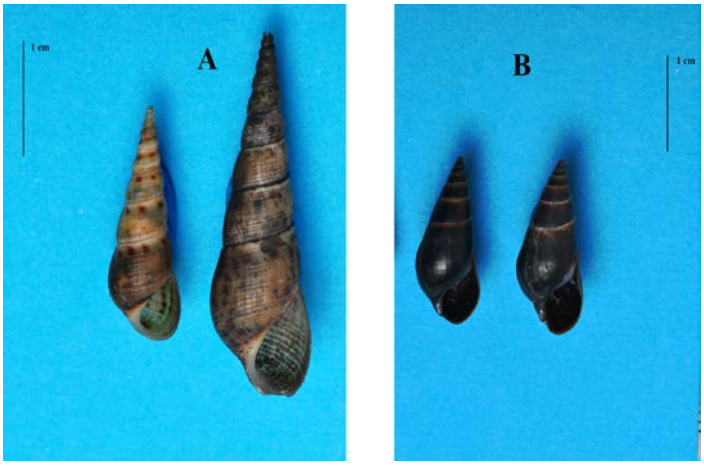

Figure 2. Shells of (A) *Melanoides tuberculata* and (B) *Melanopsis buccinoidea* from Tabuk region**.**

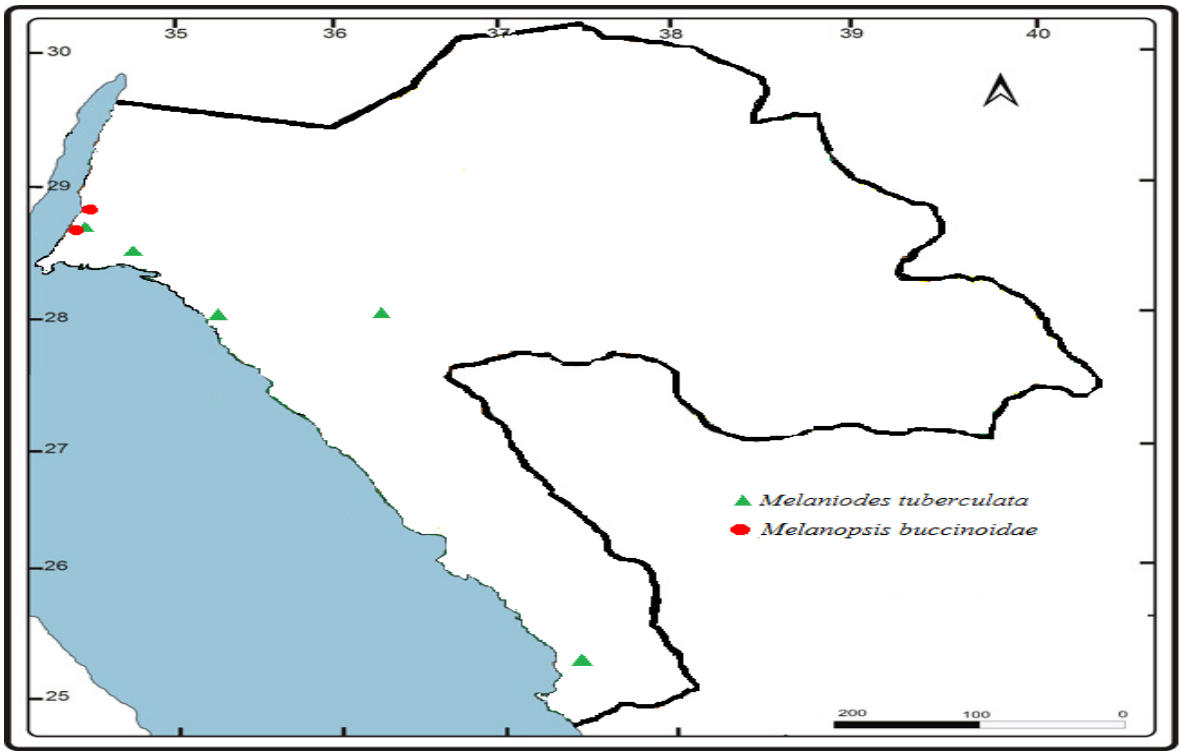

Figure 2. Distribution of *Melaniodes tuberculata* and *Melanopsis buccinoidae* in Tabuk region.

4. Discussion

The most recent comprehensive study on the freshwater snails of Saudi Arabia and Arabian Peninsula (Neubert, 1998) recorded a total of 29 freshwater snail species distributed over the Arabian Peninsula; the localities of the recorded species were distributed in the central province, eastern province, southern Hejaz, and Asser Mountains in the South west of Saudi Arabia (Neubert, 1998; Bin Dajem, 2009). On the other hand, the freshwater malacofauna of the northern regions of Saudi Arabia is not well-known;the most recent study on the freshwater snails in the northern regions of Saudi Arabia was conducted by Amr and Alshammari (2012), recording one freshwater snail of the genus *Bulinus* in Hail region.

During the present study, two species (*M. tuberculata* and *M. buccinoidea*) were recorded for the first time in Tabuk region. *Melanoides tuberculata* is known to carry several trematode parasites which are dangerous to humans. Pinto and de Melo (2011) compiled a checklist of 37 species of trematode parasites from the *M. tuberculata* which is considered as the first intermediate host, eleven of these trematodes were parasites for human.

Melanoides tuberculata was more abundant and was recovered from five sites, compared with two sites for *M. buccinoidea.* According to Neubert (1998),*M. tuberculata* is the most common freshwater snail in the Arabian Peninsula.

Melanopsis buccinoidea is the most common smooth shelled *Melanopsis* species in the Levant (Heller*et al*., 2005; Amr *et al*., 2014). *Melanopsis praemorsa* is considered to be a superspecies by Glaubrecht (1993), whereas, if we follow Brown (1994) who based his conclusion on older articles, then there exists only a single *Melanopsis* species (*M. praemorsa*) with a disjunct range along the Mediterranean-Black Sea. Ever since, this view has been abandoned (e.g. Heller *et al*., 2005) for the

Levantine species and concluded that *M. praemorsa* does not occur in the Mediterranean region. According to the view of Heller *et al*. (2005), *M. praemorsa* does not occur in Saudi Arabia; therefore, the *Melanopsis* species, recorded by Neubert (1998), is not for *M. praemorsa*; rather, it is for *M. buccinoidea*.

The recording of the two freshwater snails *Melanoides tuberculata* and *Melanopsis buccinoidea* in Tabuk region will pave the way for future taxonomical ecological studies.

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