# Immunological and Histopathological studies on Schistocerca gregaria fed on plants and infected with Bacillus thuringiensis

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# Immunological and Histopathological studies on grasshopper *Schistocerca gregaria* fed on four different plants and infected with *Bacillus thuringiensis*

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## Abstract

The desert locust, *Schistocerca gregaria*, is the most dangerous herbivore species of locust.  The present study has been conducted to investigate the effect of dietary changes on differential and total haemocyte counts and midgut histopathologiocal changes of *S. gregari*a and their susceptibility to *Bacillus thuringiensis israelensis (Bti)*. Four types of haemocytes were characterized; prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs) and oenocytoids (OEs). In *Bt*i-injected locusts, the percentage of PRs decreased in insects fed on grass and quietly disappeared insects fed on sorghum, sesban and clover. The percentage of PLs increased, while the percentage of GRs decreased in all tested insects fed on sorghum, sesban, grass and clover. The percentages of oenocytoids (OEs) increased in all treatments except in insect fed on sesaban compared with control.  Results indicated that insects fed on clover had the highest number of haemocytes, followed by those fed on sorghum and sesban, while those fed on grass had the lowest number. The THCs of *Bti*-injected adults significantly decreased in all insects fed on all experimental plants. Certain pathological symptoms in the midgut of *Bti* treated adults  were observed after 48 hr post injection, such as (1) disorganisation, disintegration and vacuolization of epithelial cells, (2) lysis of the basement membrane and surrounding muscles and (3) detachment of microvilli from epithelial cells. The most deformed group to *Bti* was grass fed locust. Subsequently, the host plant could be a major factor that affect on the response of herbivorous insects to pathogens.

## Introduction

The desert locust, *S.  gregaria* Forskal, is the most dangerous herbivore species of locust. Specialist and generalist insect herbivore species often differ in how they respond to host plant traits and these responses can include weakened or strengthened immune responses to pathogens

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**(Lampert 2012).** Host plant quality affects herbivorous insect performance and consequently their susceptibility to natural enemies. Recently, it has been hypothesized that the immune function of herbivorous insects can be altered by their host plant, thus generating variation in their susceptibility to entomopathogens **(Vogelweith et al., 2011).** When phytophagous insects are reared on host plants with differing nutritional qualities and defence chemicals, it is expected that the baseline defence response would also differ. Invasion by a pathogen can induce the cellular and humoral defence response to higher levels **(Gillespie et al., 1997)**. Poor nutritional quality may result in less investment in immune factors resulting in lower resistance to pathogens or cause physiological changes that may influence the entry of pathogens via the mid-gut or the cuticle **(Shikano *et al.*, 2010)**. **Plymale *et al.* 2008** showed that diet alters the structure of the peritrophic matrix (PM) in Lepidoptera larvae, a change that can have a profound influence on pathogen infection. Also hemocytes have a key role in the cellular component of immune defense **(Strand 2008)**.

Fewer studies have addressed the influence of plant quality on insect susceptibility more indirectly either before pathogen exposure or after infection. Accurate methods to measure immune response in the presence and absence of pathogens are necessary to determine whether susceptibility to these natural enemies is reduced or increased by host plant traits **(Lampert 2012).**

Based on the success of *Bacillus thuringiensis israelensis* as a bio-control agent in the Egyptian fields against Lepidoptera and Diptera. The present study deals with the interactions among herbivorous insect (*S. gregaria*), host plant, and pathogen (*Bti*). We studied two categories: i) Differential and total haemocyte counts ii) Midgut histopathologiocal changes. The expected results may lead to understanding of the physiology of infected insects, and hence encourage the using of *Bt* agents this dangerous pest.

**Materials and Methods:**

**Insect rearing:**

The desert locust, *S. gregaria* (Forskal), was obtained from Plant Protection Research Institute, Agricultural Research Center, Egypt, reared for several generations and had shown no infectious diseases. Locusts were reared according to the method that was previously described by **Huxham *et al.* (1989).** Insects were divided into four groups; two groups were reared on

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sorghum (*Sorghum bicolor*) or sesban (*Sesbania sesban*) in summer, while in winter the other two groups were reared on grass (*Andropogon citratus*) or clover (*Trifolium alexandrinum*).

**Bacterial pathogen:**

    *Bacillus thuringiensis israelensis* (1600 IU/mg, wettable powder) was produced from the Agricultural Genetic Engineering Research Institute (AGERI) at the Ministry of Agriculture, Egypt.

**Injection technique:**

    Four groups of *S. gregaria* adults (2-4 days) fed on sorghum, sesban, grass or clover were injected with *Bti* $LD_{20}$ ($3.1 \times 10^4$, $1.37 \times 10^{5}$, $1 \times 10^5$, and $1.1 \times 10^5$ for grass, clover, sorghum and sesban respectively according to **Barakat *et. al.* (2013).** 10 μl were injected into each insect. Control insects were injected only with equivalent volumes of sterile distilled water.

**Differential haemocyte counts (DHCs):**

    After 48 hrs of *Bti* injection the haemolymph samples were withdrawn from the coxal corium according to **Hoffmann (1980)**. Haemocytes (blood films) were stained by Giemsa stain and examined by light microscopy. The haemocytes are classified according to the classification scheme of **Hoffmann *et al.* (1969).**

    Various haemocytes were differentially counted by examining approximately 100 cells per slide. 10 slides prepared from 10 locusts / count. The percentages of haemocyte types were calculated by the formula:

$$\% = \frac{\text{Number of each haemocyte type} \times 100}{\text{Total number of haemocytes examined}}$$

**Total haemocyte counts (THCs):**

    The oozed haemolymph was collected directly into Thoma- white blood cell diluting pipette to the mark 0.5. Diluting solution (NaCl- 4.65 g, KCl- 0.15 g, CaCl2- 0.11 g, crystal violet- 0.05 g and acetic acid- 1.25 ml/liter distilled water) was taken up to the mark 11 on the pipette (dilution is 20). The mixture was dispensed to both chambers of the counting slide (the chamber depth is 1.0 mm) and the total number of cells were counted according to the formula of **Jones (1962):**

$$\frac{\text{Haemocytes in 1 mm squares} \times \text{dilution} \times \text{depth of chamber}}{\text{Number of 1mm squares counted}}$$

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**Midgut histological analysis:**

Desert locust adults (2-4 days) fed on four experimental plants in normal state or injected with $LD_{20}$ *Bti* were dissected from the ventral side to extract the midguts then washed rapidly in saline solution 0.9% NaCl (Merck), and then intensely immersed in distilled water. histological analysis were carried out according to Disbrey and Rick (1977). The samples were fixed in Bouin's solution. Dehydration of samples was carried out through putting the samples in ascending series of ethyl alcohol (70%, 80%, 90% and 100%) and embedded in paraffin, the samples were sliced in 5 and 10 µm in thickness sections and stained with hematoxylin-eosin stain. Morphological changes of the midgut cell structure and organization were analyzed and compared to the tissues taken from the control group.

**Statistical analysis:**

Data of the experiments were analyzed by using the statistical computer application origin program, Version 7.0. Analysis of one-way ANOVA was made on the data obtained, and Student's *t*-test was used for testing the significance differences. The level of significance for each experiment was set at $P < 0.05$ or $P < 0.01$.

**Results:**

**Differential haemocyte counts (DHCs):**

The differential haemocyte counts of adults (2-4 days) fed on four different experimental plants varied considerably as shown in table Table (1).

The percentages of different haemocyte types of adults injected with $LC_{20}$ *Bti* were presented in the same table. The percentage of Prohaemocytes(PRs) decreased in treated insects fed on grass (3.20 %) compared to the control (14.50 %). In treated insects fed on sorghum, sesban and clover, PRs disappeared. The results also recorded that the highest number of these Plasmatocytes (PLs) was in treated insects fed on clover (75.10%) than all treatments , normal and control insects. The lowest number of PLs was recorded in treated insects fed on grass (65.9%) than other treatments. Granulocytes (GRs) percent decreased when treated insects fed on sorghum, sesban, grass or clover compared with control insects. The percentages of Oenocytoids (OEs) increased in all treatments except in insect fed on sesaban compared with control.

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**Total haemocyte counts:**

Figure (1) showed the effects of $LC_{20}$ of *Bti* on $THC_S$ of *S. gregaria* adults fed on experimental plants at 48 hr post-injection. The THC values showed a significant decrease (P < 0.05) with all treated adults fed on clover, sorghum or sesban (9413±71.10, 9260 ± 76.103, or 9225±96.91 cells/mm$^3$, respectively) compared with controls (9625±45.49, 9580±42.95 and 9805± 43.75 cells/mm$^3$, respectively) except insect fed on grass (8975±39.58 cells/mm$^3$) which showed highly significance decrease at P < 0.01 compared with control (9425±80.02 cells/mm$^3$).

**Effect of experimental plants and *Bti* on midgut structure:**

Figures (2-5A) describe the anterior part of the normal midgut of the desert locust adults fed on grass, sorghum, sesban, or clover, respectively. The midgut consists of 4 main parts: a longitudinal muscles layer followed by circular muscles (Mu), a basal membrane (BM), an epithelial layer (Ep) and a single uniform layer of cells with apical microvilli (Mi). The epithelium is composed of columnar cells, with a great number of small undifferentiated groups of regenerative cells (RC) scattered along the basal portion of the epithelium. A single uniform layer of cells with apical microvillus constitutes the absorbing part which is protected by a thin peritrophic membrane. Insects fed on grass (Fig.2A) showed more delicate microvilli than insects fed on sorghum, sesban or clover (Fig. 3-5A) which were not different from each other.

Histopathological changes in the midgut of *S. gregaria* adults fed on experimental plants and treated with $LC_{20}$ of *Bti* at 48 hrs post injection revealed certain pathological symptom. Adult injected with *Bti* and fed on grass were the most susceptible group and showed obvious changes in the mid-gut. Epithelial cells appeared to be completely disorganised and disintegrated downwardly. Some of these cells were sloughed in the gut lumen and destroyed in some instances. The basement membrane and surrounding muscles seemed to be destroyed. Vaculation appeared in regenerative cells (Fig. 3B).

According to pathological symptoms in insects fed on sorghum, there was fading of cellular boundaries and slight vaculation (Fig. 4B). Some areas of epithelial cells disintegrated and sloughed in the gut lumen . The basal part of epithelial cells were disintegrated and separated from the muscle. Injected adult and fed on sesban showed cell disorganization and lysis in some areas cell boundaries disappeared(Fig. 5B). Regarding to infected adults fed on clover, detachment of microvilli from epithelial cells and degeneration of basement membrane appeared

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in some areas epithelial cells disorganized, slightly vaculated and cell boundaries disappeared(fig. 6B).


**Discussion:**

Alteration of herbivorous insect immunity by the host plant could be of major importance as it should determine the ability of natural enemies to control the herbivorous insect population and therefore the damage to the host plant **(Lampert 2012)**.

The present study demonstrated the impact of four plants with different nutritional value on the immune response of *S. gregaria* to *Bti* infection.  According to **Barakat et al.  (2013),** the tested plants grass, sorghum, sesaban and clover are differed from each other in  the nutritional value.  This variation was directly propotional to the ratio of protein to carbohydrate content of the plants. protein to carbohydrate (P: C) ratio in clover was the highest (1:3.3) followed by sesbsn then sorghum and the lowest ratio was in grass (1:3.7).

Circulating haemocytes have important functions on the immune system, metabolism, and detoxification, and play a crucial role in the defense of xenobiotics or microbial infection (**Zhu *et al.,* 2012**). This work proceeded to the impact of host plant- pathogen interaction on differential and total haemocyte count of adult locust before and after injection with *Bti*. Four types of haemocytes were found in locust fed on each experimental plant (PRs, PLs, GRs and OEs).

The present results investigated that normal locusts fed on clover (high protein diet) had the highest percentage of PLs (55.80%) and the highest percentage of GRs (30. 30%) was found in normal locusts fed on sorghum (high carbohydrate diet) . **Szymaś and Jędruszuk, 2003** stated that GRs cells may act as storage cells in insect. **Idowu and Sonde (2003)** also found that Pls numbers are the most in cassava (high protein diet)  fed grasshopper and are the lowest in acalypha (low protein diet) fed grasshopper while GRs  numbers are the highest in grasshopper fed highest carbohydrate diet. Further,


Following bacterial-injection into the locusts, the PLs increased and the GRs decreased at post-injection in all treatments than control. The increase in PLs was attributed to the release of sessile haemocytes after infection, and the decrease in GRs may be attributed to their involvement in phagocytosis and nodule formation. This concept is supported by the

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observations of **Anandakumar and Michael (2011)** on silk worm larvae infected with *Bti*. **Hillyer *et al*. 2005** shed light on GRs which play an important role to phagocytose bacteria and showed that a reduced GRs population in insects may affect their capacity to clear high infection levels**.** In addition, insects fed with protein might invest more resources in certain types of haemocytes (e.g. Grs or PLs) at the expense of others types **(Alaux *et al.,* 2010)**. Also we couldn't detect PRs in insects fed on sorghum, sesban or clover. **Mori (1979)** suggested that the decrease of PRs after injection may be explained with the transformation of PRs (stem cells) into PLs and GRs which are needed in phagocytosis and nodule formation. From our observation to haemocyte pathological conditions we suggested that toxins secreted by injected bacteria cause lysis of haemocytes or induce programmed cell death.

The low values presented by OEs in infected  and non-infected desert locust in this study was confirmed by **Silva *et al.* (2002)** who reported the absence of quantitative alterations of OE in response to pathogens in the haemocoel of *Anastrepha oblique* larvae. It is known that the OE cells are involved in the production of prophenoloxidase, an enzyme that actively participates in the mechanisms of defense in insects **(Lavine and Strand, 2002)**. However, in the period of infection used, we could not detect alteration in the frequency of the OEs in infected *S. gregaria* adults compared to non-infected adults, suggesting that these cells are not directly involved in the processes of immunological defense against the bacterial infection or that its participation occurs in the posterior stages of immunological responses (**Andrade *et al.,* 2010**).

In this study, we provided experimental evidence for a link between nutrition and haemocyte concentration in desert locust. Locusts fed on high protein diet (clover) had the highest haemocyte count while those fed on low protein diet (grass) had the lowest haemocyte count.  These results suggest that the abundance and diversity of environmental resources can have a direct impact on insect immune system. Higher haemocyte concentrations are expected to be associated with high protein diet and higher resistance to disease. Natural proteinaceous food is an important factor in the function of the cellular system of insect haemolymph where a lack of protein in the diet causes considerable changes in the function and structure of the cellular system of the haemolymph (**Szymaś and Jędruszuk, 2003**). One possibility is that the high food quality stimulates increased proliferation of one or more haemocytes types **(Castillo *et al*., 2006)**.

Generally, the THC is positively correlated with the rate of phagocytosis and nodule formation. The total number of haemocytes in haemolymph is likely to reflect the capability of immune system to deal with pathogens or chemical molecules **(Mo`men 2010).** The present results indicated that THCs decreased significantly after bacterial-injection in all treatments than controls. Similar observations were also reported by **Anandakumar and Michael (2011)** who noticed that about 15.3% of the total haemocytes were decreased after bacterial infection in silk worm larvae when compared to normal worms. They explained the decrease in total THCs infected worm with *Bacillus* was due to the depletion of prohaemocytes, which accounts for most of the lowered total counts compared to normal. **Banville *et al*. (2012)** stated that immune response requires a high degree of resources to be maintained at an optimum level to fight infection. Also authors observed that the higher level of haemocytes and antimicrobial peptide expression in unstarved *Galleria mellonella* larvae leads to significantly greater ability to curtail or eliminate the pathogen than starved insects.

In this study the kind of food that desert locust consumes has a clear impact on the structure of the midgut. We observed thicker microvilli in locusts fed on clover, sesban or sorghum whereas thinner microvilli were found in those fed on grass. Like these differences are observed by **Terra *et al.* (2006)** who stated that the densities of isolated plasma membranes of insect cells depend essentially on their protein contents. **Szymas and Przybył (2007)** noticed that the midgut epithelium of worker bees fed sugar syrup didn't revealed numerous peritrophic membrane but those fed the pollen substitute showed a distinct peritrophic membrane. Further, **Aljedani *et al.* (2010)** found that the tissues of the queen honey bee, throughout various stages, were more advanced than that of the worker honey bee due to the kind of food that bee consumes which has a clear impact on the structure of the midgut.

Many enteric pathogens have evolved specific strategies such as production of toxins that physically disrupt cells of the gut epithelium **(Kaur and Ganguly, 2003)**. The results of our study demonstrated that injection of *Bti* in desert locust adult lead to damage in the midgut. The experimental plants consumed prior to challenge with bacterial injection has been shown to modify susceptibility of desert locust to disease. The well-nourished (clover, sesban and sorghum) insects decreased performance of *Bti* than fed grass by less deformation in midgut structure. This confirmed by **Plymale *et al.* (2008)** who stated that *Heliothis virescens* larvae fed on cotton had more robust peritrophic membrane than those fed on artificial diet. Moreover, the

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gut epithelium and its associated microorganisms provide an important barrier that protects animals from the external environment. This barrier serves both to prevent invasion by potential pathogens and limit the elicitation of host responses to the resident microbiota (**Artis, 2008**). Dysfunction of this barrier, which can occur as a result of alterations of the normal gut ecology, impairment of host immune defenses, or physical disruption of intestinal epithelia, may lead to pathological states (**Stecher and Hardt, 2008)**.

. Finally, we can concluded that, the host plants with high nutritional quality; clover, surgham or sesaban; have a higher immune potential than those with poor quality (grass). As the results showed that, the normal or control and *Bti* infected adults had considerable variation in the differential and total haemocyt counts according the host plant. Furthermore, the host plant has clear impact on the structure of the midgut and its response to the pathogen. Subsequently, the host plant could be a major factor that affect on the response of herbivorous insects to pathogens.

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hemolymph physiology of the cutworm, *Spodoptera litura*. Journal of Insect Science, 12 (27): 1-13.

**Table 1: Differential haemocyte counts (DHCs) of normal and LC$_{20}$ *Bti* infected *S. gregaria* adults (2-4 days old) fed on experimental plants at 48 hr post-injection.**

| Experimental plants | Haemocyte type %[•] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Prohaemocytes | | | Plasmatocytes | | | Granulocytes | | | Oenocytoids | | |
| | Normal | Control | Treated | Normal | Control | Treated | Normal | Control | Treated | Normal | Control | Treated |
| **Sorghum** | 14.80 | 13.90 | 00.00 | 51.10 | 50.60 | 69.00 | 30.30 | 31.00 | 25.90 | 3.80 | 4.50 | 5.10 |
| **Sesban** | 13.60 | 15.90 | 00.00 | 52.70 | 52.20 | 70.50 | 27.50 | 27.30 | 25.50 | 5.20 | 4.60 | 4.00 |
| **Grass** | 15.80 | 14.50 | 3.20 | 52.20 | 53.80 | 65.90 | 26.70 | 25.90 | 24.00 | 5.30 | 5.80 | 6.90 |
| **Clover** | 12.90 | 15.40 | 00.00 | 55.80 | 56.40 | 75.10 | 29.60 | 30.80 | 22.80 | 1.70 | 1.50 | 2.10 |

●n = 10   replicates

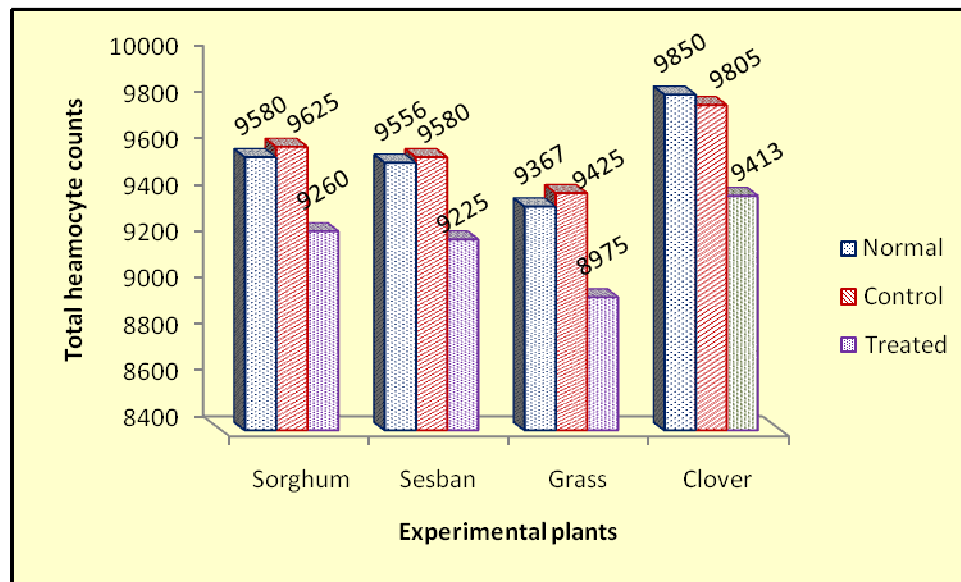Normal adults: un-injected, control: water-injected, treated: bacteria-injected.

Fig. 1: Effects of $LC_{20}$ of Bti on total haemocyte counts of *S. gregaria* adults (2-4 days old) fed on experimental plants at 48 hr post-injection.
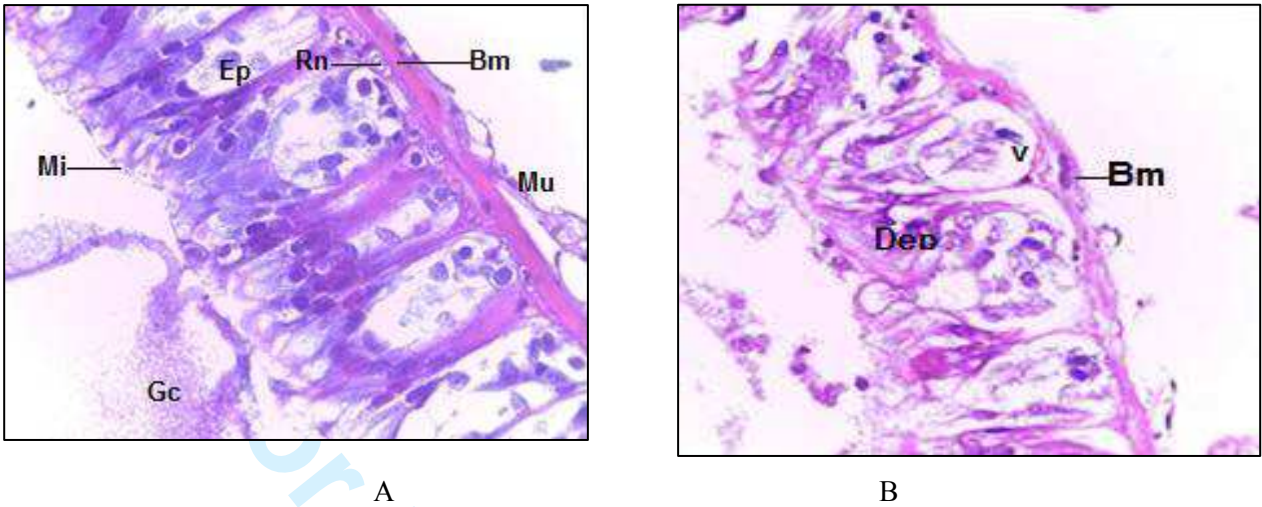
Fig. 2: Transversal section in normal (A) and infected Bti  (B) *S. gregaria* adult (2-4 days old) midgut   fed on grass   (X- 400).
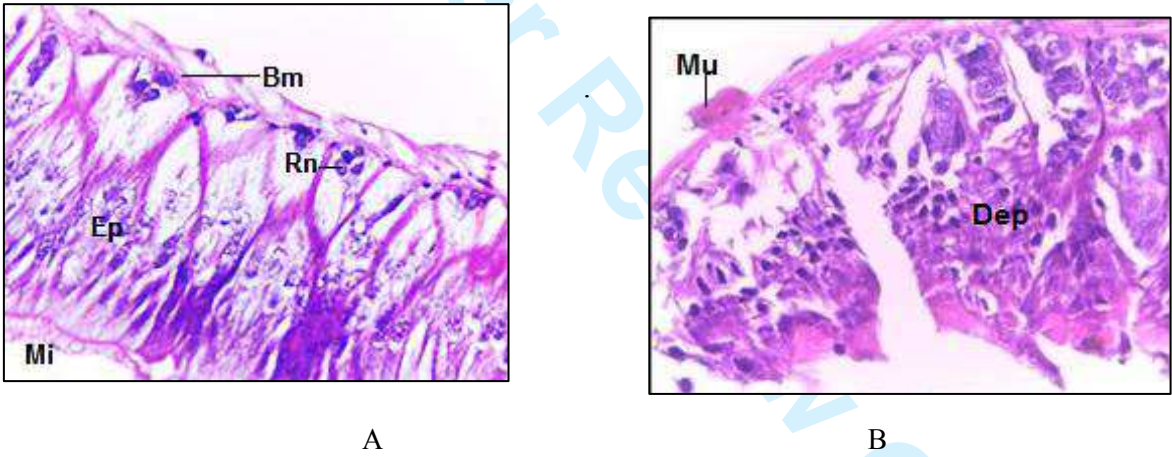


Fig. 3: Transversal section in normal (A) and infected Bti  (B) *S. gregaria* adult (2-4 days old) midgut   fed on sorghum   (X- 400).
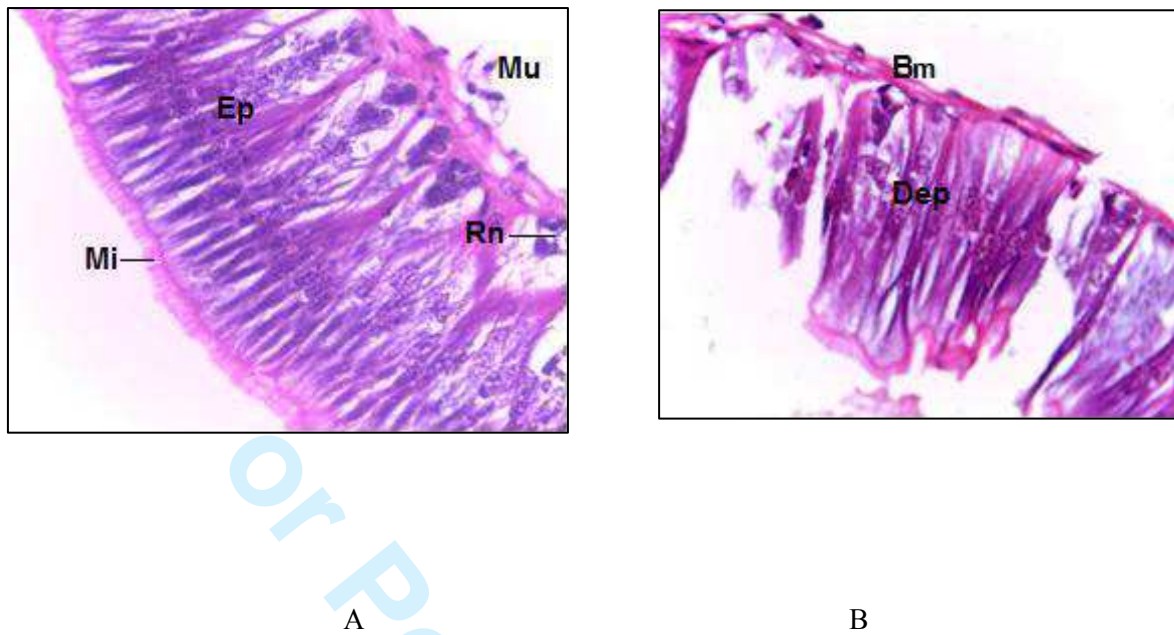
A                                                    B

Fig. 4: Transversal section in normal (A) and infected Bti (B) *S. gregaria* adult (2-4 days old) midgut   fed on sesaban   (X- 400).



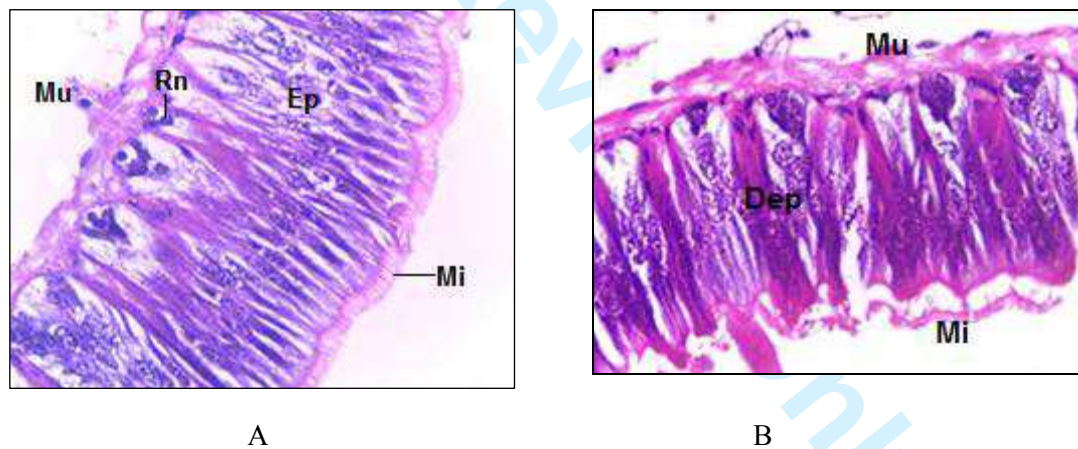A                                                    B

Fig. 5: Transversal section in normal (A) and infected Bti  (B) *S. gregaria* adult (2-4 days old) midgut   fed on clover   (X- 400).