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HISTOCHEMICAL EFFECTS OF SOME BIOLOGICAL AGENTS ON *CULEX PIPIENS* LARVAE

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Abstract

The histochemical effects of the lethal concentration that kills 50% of larvae (LC50) of three biological agents, abamectin, *Bacillus thuringiensis* and spinosad on the carbohydrates (polysaccharides), proteins, nucleic acids and lipids content of the midgut and fat bodies of *Culex pipiens* 2[nd] instar larvae were studied. The results showed that the three tested compounds reduced the carbohydrates (polysaccharides), proteins, RNA synthesis and lipids content after 72 hours of treatment where abamectin was the most effective followed by *Bacillus thuringiensis* then spinosad.

Key words: Egypt, Culex *pipienslarvae,* Abamectin, *Bacillus thuringiensis,* Spinosad

Introduction

Culex (C.) pipiens is the mosquito vector of human filarial and viral diseases. In Egypt, *C. pipiens* is vector of *W. bancrofti* (Gad *et al,* 1996), Rift Valley fever virus (Darwish and Hoogstraal, 1981) and Western Nile virus (El Bahnasawy *et al*, 2013).

Many problems such as vector resistance, toxic hazards to man and environmental pollution have been encountered in control of *C. pipiens* by chemical insecticides (Krishnamoorthy *et al,* 2002). So, the use of biological agents as natural products are being encouraged either alone or in integrated control programmes against mosquito vectors (Cetin *et al,* 2005). Among the most promising biological agents for mosquito control are abamectin (ivermectin), *Bacillus (B) thuringiensis* and spinosad.

Abamectin is a natural compound comprised of a mixture of avermectin B1a and B1b produced by fermentation of soil bacteria actinomycete *Streptamyces avermitilis* (Brewer *et al,* 2004). It was used as antiparasitic drug especially in the treatment of filariasis as it showed a microfilaricidal activity (Richards *et al,* 2005) and interrupted the uptake and development of microfilaria in the vector (Ramaiah *et al,* 2003). Also, it has been reported as a potent insecticide against many arthropods of medical and veterinary importance and in agriculture field (Clark *et al,* 1995). Abamectin causes increase in the larval and adult mortality as well as decrease in female fecundity and egg hatching of mosquitoes such as *Cule*x (Nasr *et al,* 1996; Buss *et al,* 2002), *Anopheles* (Foley *et al,* 2000; Richards *et al,* 2005), *Aedes aegypti* and *Aedes albopictus* (Focks *et al,* 1991; Gardner *et al,* 1993), house fly (Scott *et al,* 1991; Assar, 2004a), stable fly and horn fly (Miller *et al,* 1986; De Macedo *et al,* 2005), tsetse fly (Langley and Roe, 1984) and black fly (Chandre and Hougard, 1999) as well as ticks (Van Der Merwe *et al,* 2005).

Bacillus thuringiensis is an aerobic, Gram-positive spore-forming bacterium commonly found in the soil, producing parasporal crystal (Cry) proteins with insecticidal activity against a wide range of pests. Its strains, mainly *B.t.* H-14, were utilized as biological control of mosquitoes (Merdan *et al,* 1991; Aidara-kane *et al,* 1998; Lonc *et al,* 2003; Zayed and Bream, 2004), myiasis producing larvae (Morsy and Mazyad, 2000), black flies (Cavados *et al,* 2004) and house flies (Shakoori *et al,* 1999; Kutasi *et al,* 2000; Labib and Rady, 2001). They are widely used in the Egyptian agriculture particularly in rice fields, the major breeding places for mosquitoes (Merdan and Labib, 2003).

B. thuringiensis strains produce two major classes of insecticidal toxins, Beta- exotoxin (Sebesta *et al,* 1981; Maciejewska *et al,* 1988) and other major toxins mainly endotoxins or crystal proteins (Hafez, 2000; Kamauchi *et al,* 2003). Beside, its larvicidal activities extended to pupae and adults causing pupal mortality, and adults deformities with reduced female fecundity (Ignoffo and Gregory, 1972; Zayed and Bream, 2004).

Spinosad is a natural insecticide derived as a fermentation product from the soil actinomycete *Saccharopolyspora spinosa* that displays the efficacy of a synthetic insecticide. It is toxic by ingestion and contact and has a unique mode of action on the insect nervous system (Mayes *et al,* 2003). It has a high toxicity on lepidopteran larvae and some Diptera, Coleoptera, Thysanoptera and Hymenoptera insects but with less to zero activity on others and low toxicity to beneficial insects, mammals and wildlife (Tirry *et al,* 2005). Several studies showed its' highly effective against mosquitoes; as *Culex* (Cetin *et al,* 2005; Romi *et al,* 2006), *Anopheles* (Bond *et al,* 2004) and *Aedes* (Darriet and Corbel, 2006), as well as tsetse flies (De Deken *et al,* 2004) and significant acaricidal effect (Villanueva and Walgenbach, 2006) and as pediculicides, especially in lice-resistant permethrin (Mougabure Cueto *et al,* 2006).

Histochemical study on insects is one of the most specific and interesting types of investigation. The histochemistry of insect midgut and fat bodies received very little attention. Little information is available concerning the histochemical effects of abamectin, *B. thuringiensis* and spinosad against different types of insects, but no information about their histochemical effects on *C. pipiens* larvae.

This study aimed to evaluate the histochemical effects of abamectin, *B. thuringiensis* and spinosad on carbohydrates (polysaccharides), proteins, lipids and nucleic acids of the mid gut and fat bodies of *Culex pipiens* larvae.

Materials and Methods

Culex pipiens used was obtained from the laboratory bred strain at Research Institute of Medical Entomology, Dokki, Egypt. The colony was maintained under laboratory conditions of 27±2ºC & 75±2 RH% (El-Bokl and Moawad, 1996). The second instar larvae were collected for bioassay tests. Different concentrations of biological agents (0.001, 0.01, 0.1, 1 &10 ppm) were tested. Each experiment was replicated 3 times and in each, 25 larvae were put in plastic cup with 100ml tap water and treated with the biological agents. Control experiments were carried out using water. A mixture of ground dried bread and Brewer's yeast pellets (3:1) were added daily as food. Dead larvae were daily removed and recorded for 72 hours. LC_{50} were determined 72 hours post-treatment (Finney, 1971). The 2^{nd} instar larvae of *C. pipiens* were treated with LC_{50} of abamectin (0.0018 ppm), *B. thuringiensis* (10.0 ppm) and spinosad (0.0016).

Compounds: 1- Abamectin (Avermectin B1 MK-936 11M12; 1.8% wt/vol emulsified concentrate, 2- *Bacillus thuringiensis* (32000 IU/mg, 9.4% active ingredient and 90.6% inert ingredient (carrier), and 3- Spinosad (24%) active ingredient in Tracer Naturatyle insect control, the first product within naturatyle class of insect control products to be marked worldwide by Dow Elanco. All were kindly obtained from the Egyptian Plant Protection Research Institute.

Histochemical studies: 72 hours larvae post-treatment with LC_{50} of compounds, some larvae were dissected in Ringer's saline solution. Parts of the mid gut and fat bodies were quickly removed and put in appropriate fixatives as Carnoy's fluid for proteins, nucleic acid and polysaccharides or formalin for lipids.

Staining: 1- Periodic acid Schiff's technique (PAS) detected polysaccharides, the PAS positive material appears pink or red violet (Hotchkiss, 1948), 2- Mercury bromphenol blue method detected total proteins, which appeared blue in color (Bonhag, 1995), 3- Schiff Feulgen methylene blue reaction detected nucleic acids where DNA stains red to purple while RNA stains blue (Garvin *et al*, 1979), 4- Sudan Black B solution detected lipids, hydrated sections stained in saturated solution of Sudan black B in 70% ethanol for 30 minutes, differentiated in 70% ethanol, washed in distilled water and mounted in glycerin jelly. Lipids acquired a black or dark blue color (McManus, 1946).

Results

The results are in figures (1 to 34).

Discussion

In the present study, the polysaccharide material was observed in the cytoplasm of the midgut (Fig.1) and the fat cells (Fig.5) of normal control larvae as indicated by strong PAS- positive reaction given by these cells as red-violet color, while the nuclei displayed a weak reaction for the polysaccharide material.

All the LC_{50} of the three tested biological agents decreased the polysaccharide content in the midgut and fat body cells to variable degrees. The LC_{50} of abamectin, induced a very weak reaction with PAS in the midgut and fat cells of 2nd instar larvae of *C. pipiens* in comparison with strong reaction in the control larvae (Fig.2 & 6), while *B. thuringiensis* (Fig.3 & 7) and spinosad (Fig.4 & 8) showed weak and moderate reactions with PAS, respectively. The results showed that abamectin was the most effective followed by *B. thuringiensis* and lowest was spinosad on larvae' polysaccharides content of midgut and fat cells.

As to histochemical studies the present results agreed with Hamed *et al*. (1974) who reported that the gut cells of *Anopheles pharoensis* larvae lost most of their carbohydrate content following dieldrin and DDT treatment; Oakley and Kalmus (1987) found that polysaccharide content of 5th instar larvae of *Tribolium confusum* was decreased after treatment with diflubenzuron and benzoyl peroxide; Assar and Emara (1997) reported that dimilin decreased the carbohydrate content in the midgut of *Spodoptera (S) exigua*; Shaurub *et al.* (1998) stated that pyriproxyfen and extracts of *Schinus terebinthiloolius* decreased the carbohydrate content in the 4th instar larvae of *S. littoralis*; Assar (2003) reported that the acetone and water extracts of *Artmisia monosperma*, *Zygophyllum coccineum*, *Lupinus termis* and *Brassica tournefortii* decreased the polysaccharide content in midgut of *S. littoralis* and Assar (2004b) found that insect growth regulators, pyriproxyfen, hexaflumuron and methoxy-fenozide reduced the polysaccharides content in the midgut and fat body cells of *Parasarcophaga aegyptiaca* larvae.

The protein in the midgut and fat body cells of *C. pipiens* larvae was reflected by appearance of a positive affinity to mercury bromphenol blue visualized by the appearance of a bluish colouration. This was illustrated in the normal control midgut cells (Fig.9) and the fat bodies (Fig.13). Total proteins in these sections were pronounced by a great amount of dense blue particles. In the present study, LC_{50} of abamectin induced a very weak reaction with mercury bromphenol blue in the midgut and fat bodies of 2nd instar larvae of *C. pipiens* (Fig.10 &14) in comparison to strong reaction in normal control larvae, that of *B. thuringiensis* (Fig.11&15) and spinosad (Fig.12&16) induced weak and moder-ate reactions, respectively.

Protein substances are essential constituents of the general animal cells and also in the maintenance of different activities. The three biological agents decreased the protein content in the midgut and fat body cells of *C. pipiens* larvae. The most reduction effect was noticed with abamectin, followed by *B. thuringiensis*, then spinosad. These results were in accordance with those reported by Assar (2004a) found that abamectin reduced the protein content in *M. domestica* larvae. The reduction may be due to defects in enzymes responsible for protein synthesis. Besides, Ismail and Fouad (1985) on *Chrysomia albiceps*; Chu *et al.* (2016) on *M. do-*

mestica; Ibargutxi *et al*. (2006) on *Earias insulana*; Assar and Emara (1997) on *S. exigua* and Shaurub *et al*.(1998) on *S. littoralis* found a remarkable reduction in the total protein content after treatment with some insect growth regulators (IGR's). A significant decrease in the total protein content after treatment with some plant extracts was reported by Abou-El-Ela *et al*. (1995) on *M. domestica*; Khalaf (1998) on *Muscina stabulans* and Assar (2003) on *S. littoralis*. But, protein content in larval midgut and fat bodies were increased (Fell *et al*, 1982; Shakoori and Saleem, 1991) and Assar (2004c) who attributed the greater protein synthesis in insecticidal treated larvae to the synthesis of the proteinase needed for insecticide detoxification.

Nucleic acids of midgut and fat body cells: Midgut (Fig.17) and fat body cells (Fig.21) of control larvae stained by Schiff-feulgen methylene blue showed a normal pattern of RNA and DNA. RNA particles appeared as blue granules in cytoplasm and nuclei. The nuclei exhibited a red color indicating their DNA content. The compounds LC_{50} induced a reduction of RNA content in the midgut and fat cells of *C. pipiens* larvae while showed no or little effect on DNA content. Abamectin produced a marked decrease in RNA (very weak) (Fig.18 & 22) followed by *B. thuringiensis* (weak reaction) (Fig.19 & 23) while spinosad showed slight decrease in RNA (moderate reaction) in comparison to the normal control larvae (Fig.20 & 24). The results agreed with Assar (2004a) who found that abamectin reduced synthesis of RNA in *M. domestica* larvae. As to histochemical effect of IGR's on the nucleic acids, Assar and Emara (1997) found that dimilin indu-ced a slight decrease in RNA and high decrease in DNA of *S. exigua* larvae; Shaurab e*t al*. (1998) found that pyriproxyfen reduced synthesis RNA and DNA in ovaries and testes of *S. littoralis* 4^{th} stage larvae; Assar and Abo-Shaeshae (2004) reported that pyriproxyfen and methoxyfenozide reduced the RNA content in midgut and fat cells of *M. domestica*. Assar (2004b) stated that hexaflumuron and methoxyfenzoide induced reduction of RNA content in midgut and fat cells of *Parasarcophaga aegyptica* larvae, pyriproxyfen elicited a mild decrease. DNA did not affect IGR's.

Lipid content of the midgut and the fat body cells: Midgut section (Fig.25) and fat body cells (Fig.29) of control larvae stained by Sudan black B showed black or dark blue colouration, indicating lipid material in cytoplasm of midgut and fat cells (strong reaction), but nuclei showed very few lipid content.

In the present study, LC_{50} reduced lipid content of the midgut and fat cells of *C. pipiens* larvae. Abamectin reduced the lipid content (Fig.26 &30) more than *B. thuringiensis* (Fig.27 & 31) (very weak and weak reactions, respectively), spinosad showed slight effect on lipid content (moderate reaction) in comparison with normal control larvae (Fig.28 & 32). As to histochemical effect of other compounds on midgut and fat cells of larvae lipid content of different insects Assar and Emara (1997) reported lipid decreased in midgut cytoplasm of *S. exigua* larvae treated with LC_{50} of dimilin. Assar (2004b) found no difference in histochemical synthesis of the lipid in midgut and fat cells of *Parasarcophaga aegyptica* larvae treated with IGR's.

In the present, abamectin acts on the mediation neurotransmission by gamma aminobutyric acid (GABA) led to paralysis, and delaying activity, abamectin (avermectin B_1) exhibited the growth regulatory activity (Wright, 1984) and inhibit feeding (Beach and Todd, 1985). Its toxic effect correlated with chloride channel activation on the cell membrane (De Freitas *et al,* 1996). It inhibits ATP- depentant pump, p-glycoprotein that pum-ps drugs out reducing cellular concentration of chemicals. This is found in several invertebrates and provide a defence against environmental xenobiotics, including insecticides (Buss *et al,* 2002) and target specific receptors and ion channels on cell membrane as L glumate receptors (Raymond *et al,* 2005). As regards the mode of action of bacterium *B. thuringiensis*, it inhibits the protein synthesis through interference of DNA- dependent RNA polymerase by structurally mimicking ATP and competing for binding site (Sebesta *et al,* 1981). Beta exotoxin of *B. thuringiensis* caused mortality, problems associated with moulting processes, teratological abnormalities, reduced fecundity (Ignoffo and Gregory, 1972) and feeding deterrent of Lepidopteran larvae (Herbert and Harper, 1987). *B. thuringiensis* produce de-lta-endotoxin crystals causing selective insecticidal activity on larvae. Upon ingestion, crystals are solubilized in midgut lumen and converted into active toxins that bind to receptors present on the microvilli causing serious damage to the epithelial columnar cells (Kamauchi *et al,* 2003; Cavados *et al,* 2004). Spinosad in the present study caused rapid killing of *C. pipiens* larvae with 0.001 ppm than abamectin or *B. thuringiensis*, it showed the least effect on protein, carbohydrates, RNA and lipid contents of larvae midgut and fat body cells. This rapid killing effect is due to a unique mode of action on nervous system causing general activation of nicotinic acetylcholine receptors but by a novel mechanism among all insecticide compounds.It cau-ses widespread hyperactivity in nervous system leading to involuntary muscle contractions and tremors. The insects become prostrated with tremors and after long exposure become paralyzed due to neuromuscular fatigue (Mayes *et al,* 2003; Tirry *et al,* 2005).

Conclusion

Abamectin, *B. thuringiensis* and spinosad caused decrease of carbohydrate, protein, RNA and lipid content in midgut and fat body cells of larvae. Aba-mectin was most effective one followed by *B. thuringiensis*, then spinosad. Polysaccharides and lipids are essential for energy production, glucose and proteins are essential to chitin synthesis, the depletion of these metabolic macromolecules indicates that chitin production must be inhibited. Variation in histochemical effects of them might be due to difference in chemical structures and mode of action. They are promising environmental fried insecticides.

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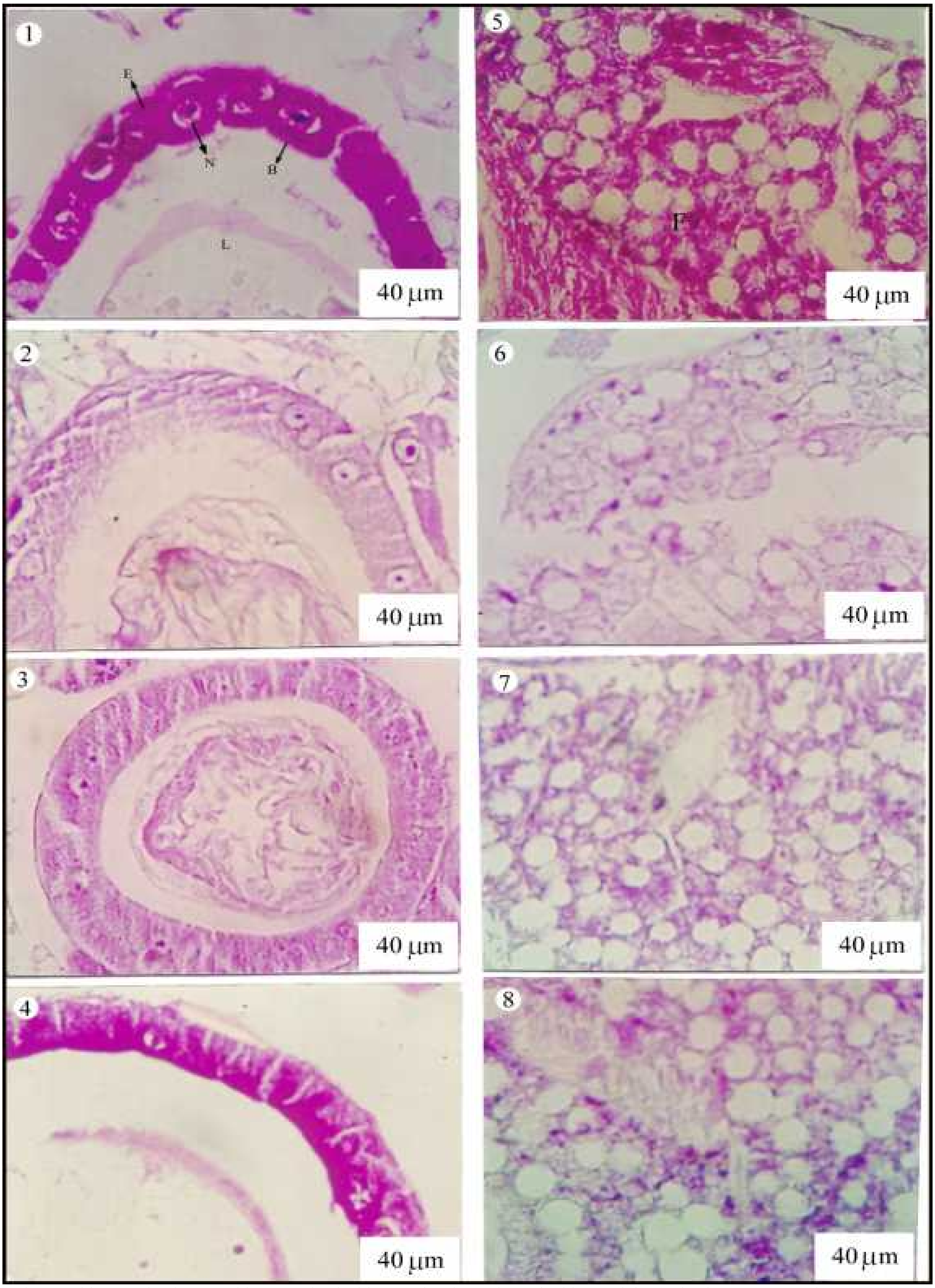

Fig. 1: Midgut section in control larvae stained with PAS showing polysaccharides particles (red color, strong reaction).
Fig. 2: Midgut section in abamectin LC_{50} treated larvae showing very weak reaction with PAS.
Fig. 3: Midgut section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with PAS.
Fig. 4: Midgut section in spinosad LC_{50} treated larvae showing moderate reaction with PAS.
Fig. 5: Fat body section in control larvae stained with PAS showing polysaccrides particles (red color, strong reaction).
Fig. 6: Fat body section in abamectin LC_{50} treated larvae showing very weak reaction with PAS.
Fig. 7: Fat body section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with PAS.
Fig. 8: Fat body section in spinosad LC_{50} treated larvae showing moderate reaction with PAS.

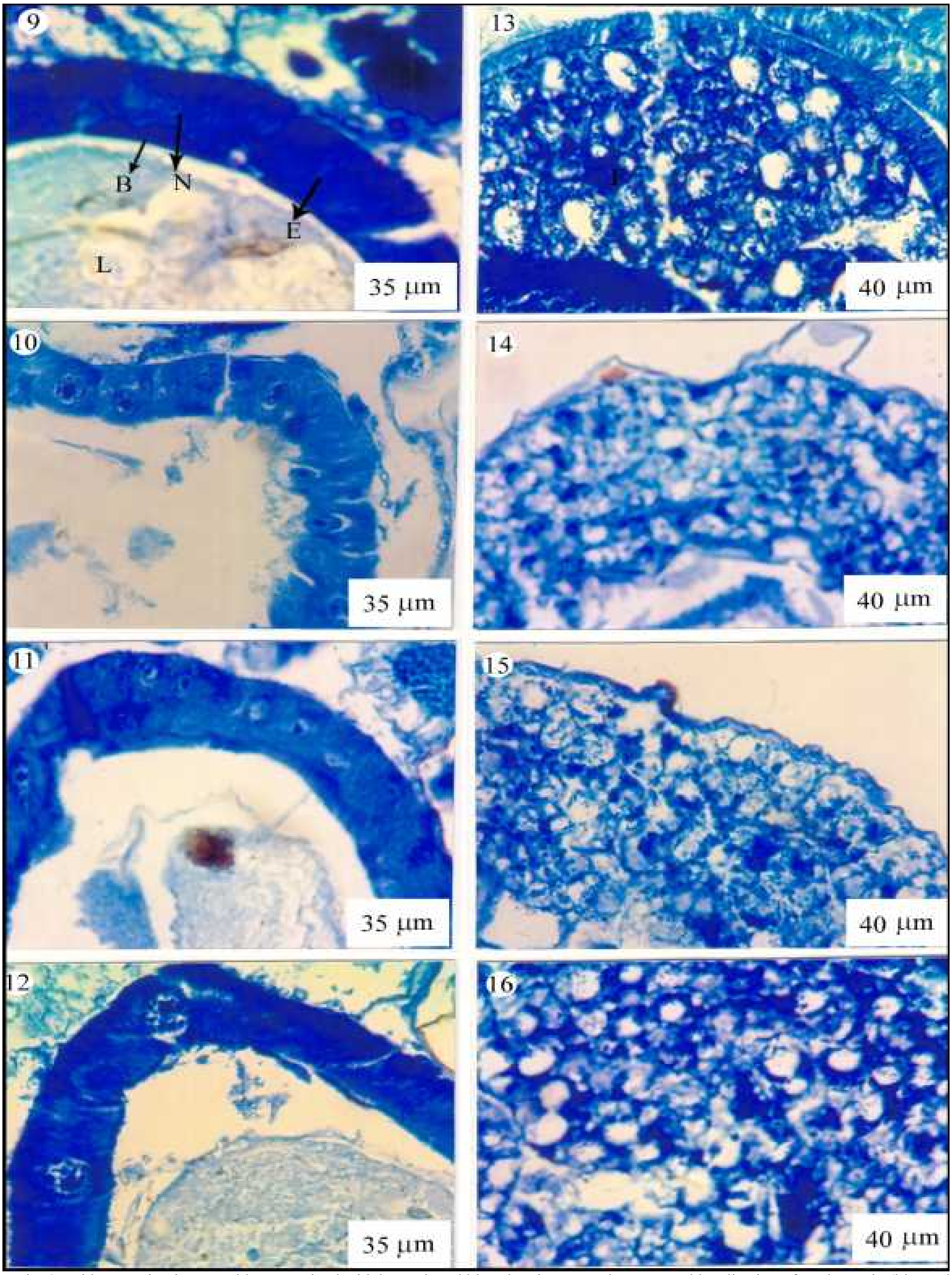

Fig. 9: Midgut section in control larvae stained with bromphenol blue showing normal pattern and localization of total protein (blue color) (strong reaction).
Fig. 10: Midgut section in abamectin LC_{50} treated larvae showing very weak reaction with bromphenol blue.
Fig. 11: Midgut section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with bromphenol blue.
Fig. 12: Midgut section in spinosad LC_{50} treated larvae showing moderate reaction with bromphenol blue.
Fig. 13: Fat body section in control larvae stained with bromphenol blue showing a strong reaction (blue color).
Fig. 14: Fat body section in larvae treated with LC_{50} of abamectin showing very weak reaction with bromphenol blue.
Fig. 15: Fat body section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with bromphenol blue.
Fig. 16: Fat body section in spinosad LC_{50} treated larvae showing moderate reaction with bromphenol blue.

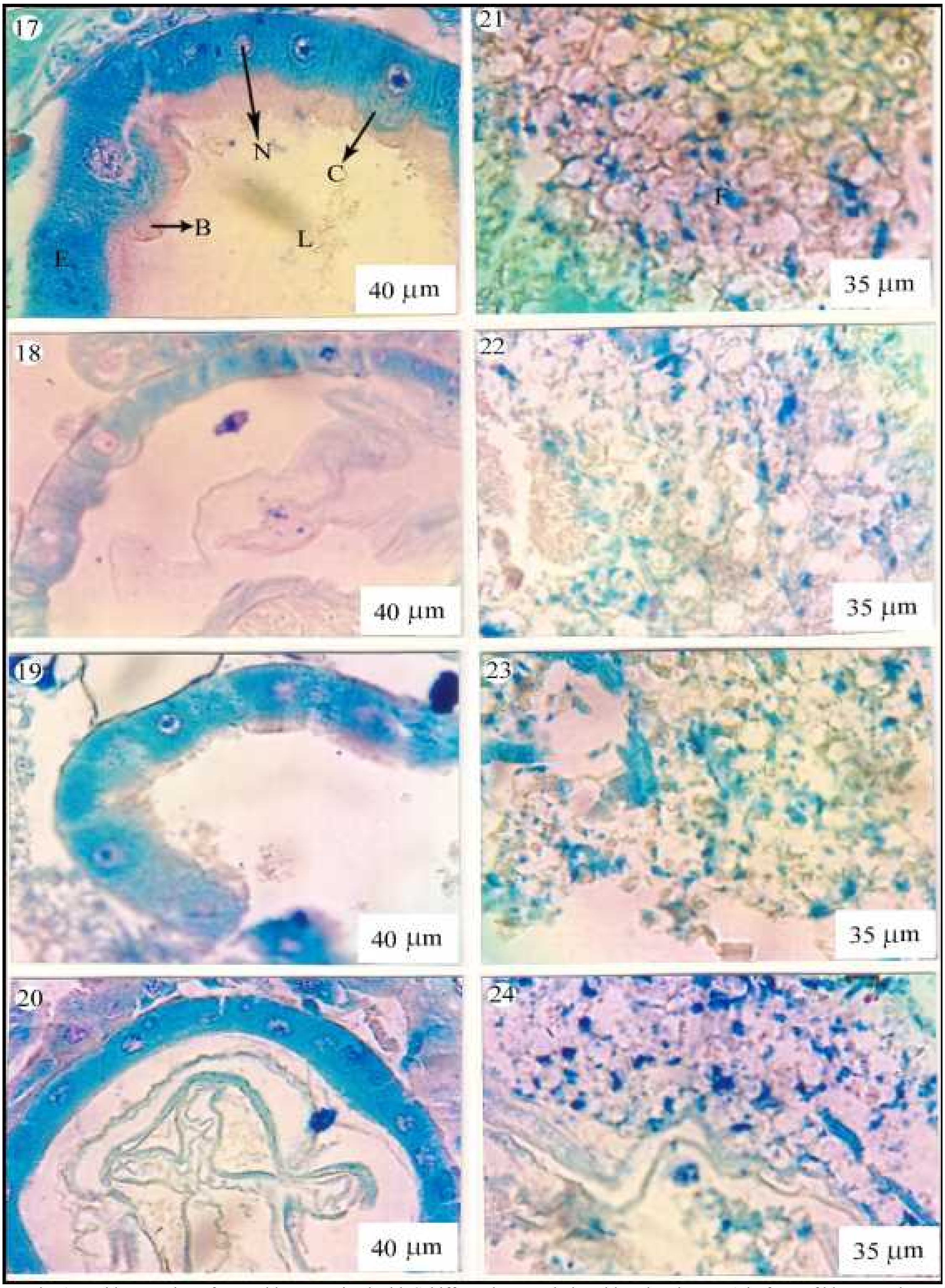

Fig. 17: Midgut section of control larvae stained with Schiff–Feulgen methylene blue showing RNA in form of blue granules and nuclei exhibited a red color indicating DNA content.
Fig. 18: Midgut section in abamectin LC_{50} treated larvae showing marked decrease in RNA (a very weak reaction).
Fig. 19: Midgut section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with Schiff–Feulgen methylene blue.
Fig. 20: Midgut section in spinosad LC_{50} treated larvae showing moderate reaction with Schiff–Feulgen meth-ylene blue.
Fig. 21: Fat body section in larvae control larvae stained with Schiff–Feulgen methylene blue showing strong reaction (RNA, blue) (DNA, red).
Fig. 22: Fat body section in abamectin LC_{50} treated larvae showing a very weak reaction with Schiff–Feulgen methylene blue.
Fig. 23: Fat body section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with Schiff–Feulgen methylene blue.
Fig. 24: Fat body section in spinosad LC_{50} treated larvae showing moderate reaction with Schiff–Feulgen methylene blue.

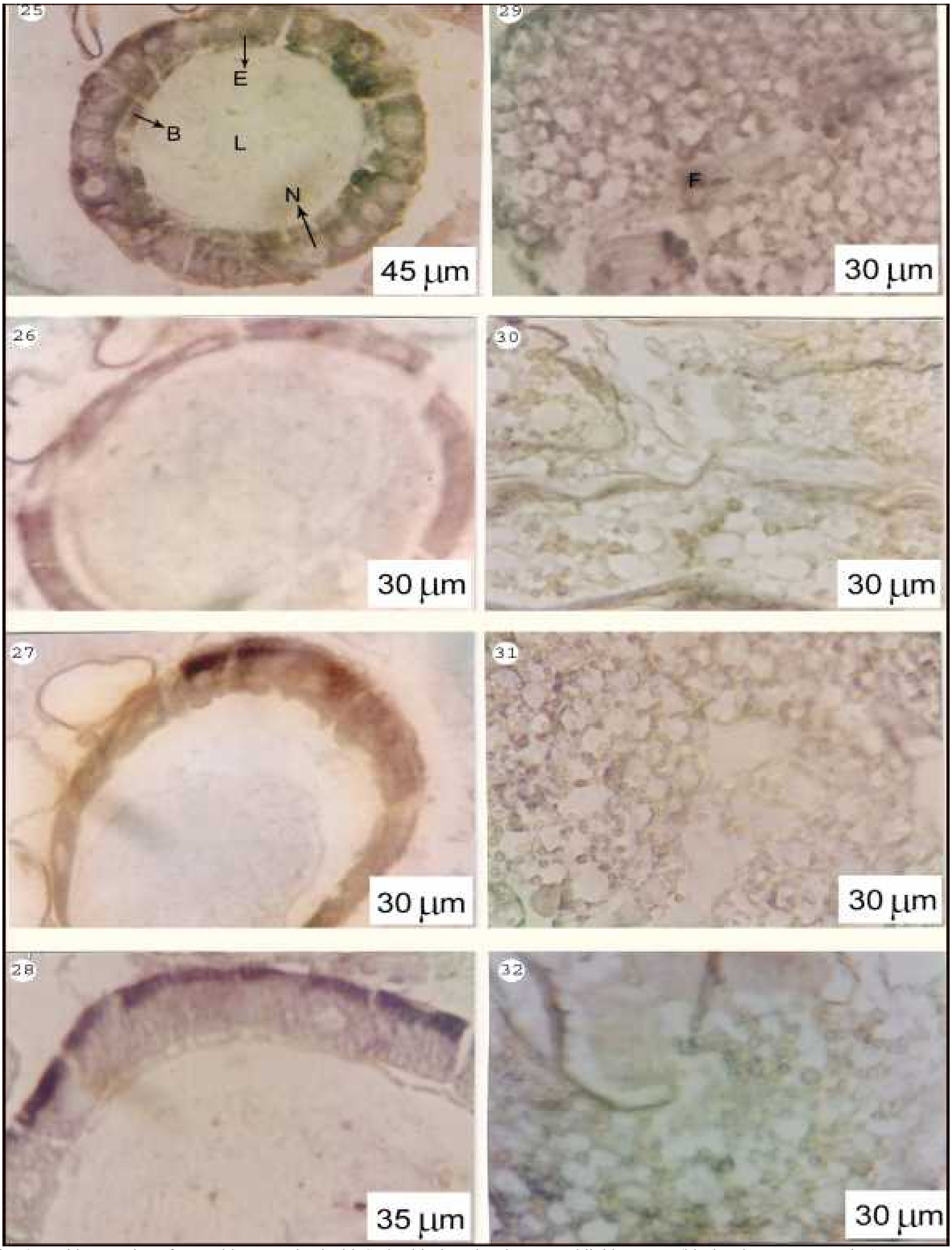

Fig. 25: Midgut section of control larvae stained with Sudan black B showing normal lipids pattern (black color, strong reaction).
Fig. 26: Midgut section in abamectin LC_{50} treated larvae showing marked reduction in lipid (very weak reaction).
Fig. 27: Midgut section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with Sudan black B.
Fig. 28: Midgut section in spinosad LC_{50} treated larvae showing moderate reaction with Sudan black B.
Fig. 29: Fat body section in larvae control stained with Sudan black B showing the normal pattern of lipids (black color, strong reaction).
Fig. 30: Fat body section in abamectin LC_{50} treated larvae showing a very weak reaction with Sudan black B.
Fig. 31: Fat body section in *B. thuringiensis* LC_{50} treated larvae showing weak reaction with Sudan black B.
Fig. 32: Fat body section in spinosad LC_{50} treated larvae showing moderate reaction with Sudan black B.