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Impact of Nuclear Polyhedrosis Virus and Azadirachtin on the Digestive Enzyme Activity and Biochemical Composition of the Gut of *Helicoverpa armigera* Hubner (Insecta: Lepidoptera: Noctuidae)

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ABSTRACT. *The combined treatment of nuclear polyhedrosis virus (NPV) and Azadirachtin (AZA) significantly reduced the digestive enzyme activity and biochemical profiles in the midgut of Helicoverpa armigera Hubner. The Protease activity was reduced to 1.0×10^{-4} mg/min, amylase activity to 3.1×10^{-4} mg/min and lipase activity to 0.4×10^{-4} mg/min at 1×10^{4} PIB/ml NPV + 2.0 ppm AZA treatment. Reduced total amounts of protein (25.07 mg/g), lipid (6.7 mg/g), amino acid (35.15 mg/g), DNA at 96 h post treatment (109 µg/g) and RNA at 96 h post treatment (2.6 µg/g) were observed after the combined treatment of NPV and AZA at 1×10^{4} PIB/ml + 2.0 ppm. Absence of certain amino acids, phospholipids and neutral lipids was observed after the combined treatment when compared with the composition in larvae treated with NPV or AZA alone. The combined treatment of NPV and AZA affected the digestive enzyme activity and decreased the biochemical composition of the gut than the individual treatments.*

INTRODUCTION

There is increasing awareness that environmental problems and widespread pest resistance pose a severe threat to pest management programs based on conventional synthetic insecticides. The development of resistance to organophosphates, DDT and other pesticides has necessitated new approaches to control the serious pest of cotton and other vegetable crops, *Helicoverpa armigera* Hubner (Dutton and Komblas, 1989).

Baculoviruses are potential microbial control agents which can be readily integrated into an Integrated Pest Management program because they pose little threat to beneficial insects and other nontarget organisms (Doller, 1985). Nucleopolyhedrosis virus (NPV) infection begins with the ingestion of the occluded virus, dissolution of the protein matrix of the occlusion body

and release of the enveloped nucleocapsids, which enter and replicate in the cells of mid-gut (Smits, 1997). However, the large doses required for effective control and slow speed of kill (Senthil Kumar, 1998) represents a major impediment to the use of NPV in insect pest management programs. Thus, there is an increasing interest in improving the killing effect of NPV.

Results of studies indicate that botanicals are compatible with, and complementary to, the action of NPV (Murugan *et al.*, 1998a; Senthil Kumar and Murugan, 1998; Murugan *et al.*, in press a). Azadirachtin (AZA) is a highly oxidized limonoid compound belonging to the tetranitriterpenoid class and is the principle active ingredient in neem (Murugan *et al.*, 1998b). AZA treatment causes inhibition of insect growth, larval-pupal malformation, larval mortality and changes in physiology and gut motility (Mordue (Luntz) and Blackwell, 1993; Jeyabalan *et al.*, 1998).

The midgut is that part of the alimentary canal in which the cells secrete digestive enzymes and absorb nutrients and plays an important role in ion transport (Martoja and Ballan-Dufrancis, 1984). Alteration in the biochemical processes in the midgut due to toxic effects of AZA and NPV and the resultant effects on insect growth and development are required.

In the present study, the combined effect of NPV and Azadirachtin on the composition and activity of digestive enzymes in the midgut of *H. armigera* was studied.

MATERIALS AND METHODS

Helicoverpa armigera larvae were collected from cotton fields in and around Coimbatore, India. These larvae were cultured in the laboratory and fed with leaves of *Gossypium hirsutum ad libitum*. NPV inoculum was prepared by differential centrifugation method (Senthil Kumar and Murugan, 1998). 95% pure samples of AZA was obtained from Prof. M.B. Isman, University of British Columbia, Canada and diluted with distilled water to get the desired concentrations (Cook *et al.*, 1996).

Treatments

Fresh cotton leaves were coated by using camlin paint brush with NPV (1×10^3 Polyhedral Inclusion Body [PIB]/ml, 5×10 PIB/ml, 1×10 PIB/ml) and AZA (0.5 ppm, 1.0 ppm, 2.0 ppm) individually and combined,

then air-dried and used for various experiments. Control leaves were treated with water alone. Fourth instar larvae which had been starved for 3 h, were individually fed with the NPV/AZA treated and control leaves. Uneaten leaves were removed every 24 h and replaced with fresh treated or control leaves for 4 days. A minimum of 30 larvae per treatment were used for all the experiments which were each replicated 5 times.

Biochemical Studies

Four days treated larvae were dissected in insect ringer's medium and the mid-gut was carefully removed and analyzed for digestive enzyme activity, protein, carbohydrate, lipid and amino acid composition and qualitative analysis of amino acids, phospholipids and neutral lipids.

Digestive enzymes were extracted using phosphate buffer following the method of Applebaum (1964a, b). Protease activity was estimated by colorimetric method as described by Snell and Snell (1971). Amylase activity was determined by colorimetric method of Bernfeld (1955) as described by Ishaaya and Swiraski (1970). Lipase activity was determined by titremetry as described by Cherry and Grandall (1932). The total protein concentration of the tissues was estimated by colorimetric technique following the method of Lowry *et al.* (1951) and total carbohydrates was estimated by colorimetric technique as described by Dubois *et al.* (1958). Total lipids were extracted by the gravimetric method as described by Folch *et al.* (1957). The nucleic acids were extracted by hot acid extraction technique following the method of Schneider (1957). Deoxyribonucleic acid (DNA) content was estimated by spectro-photometer following the method of Burton (1956) and Ribonucleic acid (RNA) by spectrophotometer using the orcinol reaction outlined by Ceriottic (1955).

The three solvent system as described by Mangold (1984) was used for thin layer chromatographic separation of neutral lipids:

I n - hexane - diethylether - glacial acetic acid (60:40:1; v/v/v)
II n - hexane - diethylether - glacial acetic acid (90:10:1, v/v/v)
III n - hexane - diethylether - glacial acetic acid (60:40:1, v/v/v)

After run. the plates were air-dried and placed in iodine chamber for identification of various fractions. The position of various fractions were identified by comparing with the R_f values from the base of the plate upwards.

For phospholipids the solvent system Chloroform-methanol-7N Ammonia (70:25:5 v/v/v) as described by Abramson and Bleecher (1964) was used. The spots were identified in iodine chamber, after the plates were air-dried. The various fractions were identified by comparing with the R_f values. Qualitative analysis of amino acids was conducted by chromatographic technique as described by Fried and Sherma (1986).

RESULTS AND DISCUSSION

The effect of NPV and AZA on IV instar digestive enzyme activities is shown in Figure 1. The protease activity in control insect was 2.89×10^{-4} mg/min whereas it was 1.72×10^{-4} mg/min at 0.5 ppm AZA treatment and further reduced to 1.22×10^{-4} mg/min after the combined treatment of NPV and AZA at 1×10^{-4} PIB/ml + 2.0 ppm. The lipase activity in control insect was

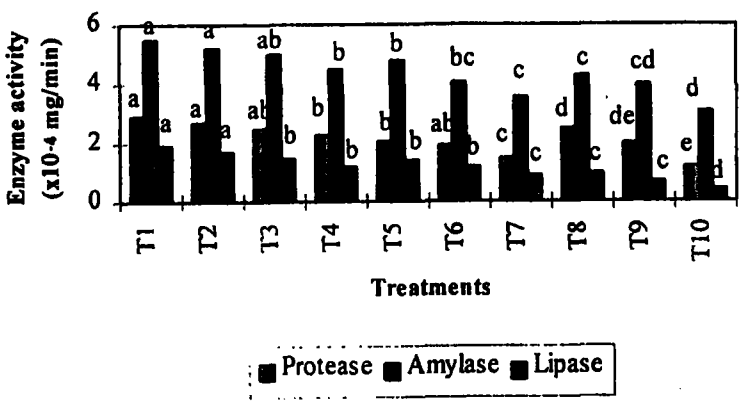

Figure 1. **Digestive enzyme activities of 4th instar *H. armigera* after the treatment of NPV and AZA.**

[Note: T1 - Control; T2 - NPV (1×10^3 Polyhedral Inclusion Body [PIB]/ml); T3 - NPV (5×10^3 PIB/ml); T4 - NPV (1×10^4 PIB/ml); T5 - AZA (0.5 ppm); T6 - AZA (1.0 ppm); T7 - AZA (2.0 ppm); T8 - NPV (1×10^3 PIB/ml) + AZA (0.5 ppm); T9 - NPV (5×10^3 PIB/ml) + AZA (1.0 ppm); T10 - NPV (1×10^4 PIB/ml) + AZA (2.0 ppm)].

1.73×10^{-4} mg/min, whereas 0.58×10^{-4} mg/min in 0.5 ppm AZA treatment and was significantly reduced to 0.37×10^{-4} mg/min in the combined treatment at

1×10^4 PIB/ml NPV + 2.0 ppm AZA. Similarly amylase activity was also reduced drastically in the combined treatment of NPV and AZA. High dose treatment of NPV and AZA were statistically significant when compared to the low doses and control. Significant variations was observed in combined treatment when compared with individual treatments.

In the present study, activities of digestive enzyme were significantly reduced by increasing dose of NPV and AZA treatment. This might be as a result of reduction in nutritional value of the diet (protein) by AZA which in turn may have affected the digestive enzymes (Engelman, 1970). The reduction in the digestive enzyme activity was dose-dependent, *i.e.*, increase in dose caused decrease in the enzyme activity. The decrease in the protein content in NPV diseased *H. armigera* larva is caused by starvation, which derives from a dysfunction of the midgut, since it is known that the protein content of a starved larva decreases markedly (Inagani and Suto, 1955). Differences in the activity of these enzymes might result from the decreased protein, carbohydrate and lipid levels due to the combined treatment of NPV and AZA and stunted growth. AZA treatment lead to starvation which might result from post-ingestive toxic effects on digestive enzyme activities and digestive physiology. Timmins and Reynolds (1992) showed that AZA inhibits secretion of trypsin and subsequent reduced growth due to impaired protein digestion in midgut of *Manduca sexta.*

A significant reduction of total proteins (48.11 mg/g), lipids (53.56 mg/g), carbohydrates (50.82 mg/g) and amino acids (39.44 mg/g) from control was observed when the larvae were treated with the highest combined dose of NPV and AZA (1×10^4 PIB/ml + 2.0 ppm) than in treatments with NPV and AZA alone (Table 1). The individual treatments with NPV and AZA reduced the biochemical composition when compared to controls. The effect was dose dependent. The mean values between the individual and combined treatments were highly significant.

Reduction in some midgut proteins was shown by Watanabe (1970) who separated midgut proteins of *Bombyx mori* (L) infected with cytoplasmic polyhedrosis virus. Lethal doses of NPV in *Spodoptera mauritia acronyctoides* larvae resulted in general reduction of haemolymph proteins in infected larvae (Takel and Tamashiro, 1975). Proteins are an essential component for both virus particles and occlusion bodies. This means that a considerable amount of protein is to be synthesized in NPV-infected cells. Keeley and Vinson (1975) suggested that proteins were used as building materials for virus and polyhedral proteins and that viruses usurp the protein

Table 1. Biochemical composition of midgut of IV instar. *H. armigera* after treatment with NPV and AZA.

Treatment	Total proteins (mg/g)	Total carbohydrates (mg/g)	Total lipids (mg/g)	Total amino acids (mg/g)
Control	89.98^{a}	93.56^{a}	108.10^{a}	78.07^{a}
NPV (PIB/ml)				
1×10^{3}	83.62^{ab}	88.61^{a}	96.21^{ab}	73.31^{ab}
5×10^{3}	74.31^{bc}	80.42^{ab}	89.46^{bc}	66.84^{abc}
1×10^{4}	68.07^{cd}	72.61^{bc}	78.37^{cd}	58.26^{cd}
AZA (ppm)				
0.5	75.34^{ac}	81.38^{ab}	89.98^{bc}	69.33^{abc}
1.0	63.19^{cdc}	69.19^{bc}	77.04^{cd}	59.11^{bcd}
2.0	54.26^{e}	52.48^{dc}	61.93^{ef}	47.86^{dc}
NPV (PIB/ml) + AZA (ppm)				
1×10^{3} + 0.5	71.84^{bcd}	9.26^{ab}	78.60^{cd}	62.09^{bcd}
5×10^{3} + 1.0	58.36^{de}	60.66^{cd}	69.14^{e}	51.15^{cde}
1×10^{4} + 2.0	41.87^{f}	42.74^{e}	54.44^{f}	38.63^{e}

Within a column means followed by same letter (S) are not significantly different at 5% level by DMRT.

synthesis of their host cells and as such are in competition with the host cell for the available substrates.

In the present study, AZA treatment of *H. armigera* also significantly reduced the total protein content. The reduction was perhaps due to the interference of AZA with the hormones regulating protein synthesis as described by Sieber and Rembold (1983). The lower levels of midgut proteins in AZA treated *H. armigera* may also to attributed to less feeding and improper utilization of digested food.

Carbohydrates and lipids were found to be reduced along with proteins in NPV infected *H. armigera*. Lipids serve as a source of metabolic energy as well as an essential structural component of cells. They also play a part in the synthesis of envelopes of virions (Yamamoto and Tanada, 1978).

NPV treated midguts of *H. armigera* larva exhibited reduction in some amino acids when compared to controls. NPV plus AZA treatment resulted in a complete loss of almost all the amino acids. Qualitative profiles of amino acids in the midgut of 4th instar larvae are given in Table 2. 1×10^4 PIB/ml NPV treatment resulted in the absence of almost all amino acids except glycine and tyrosine compared to lower dose of NPV treatment. AZA at 2.0 ppm resulted in absence of glycine, isoleucine, leucine, serine, threonine, phenylalanine, methionine, proline, aspartic acid, glutamic acid and lysine. Combined treatment of NPV and AZA (1×10^4 PIB/ml + 2.0 ppm) resulted in the presence of only glutamic acid and absence of all the other amino acids. Control larvae showed the presence of all the amino acids except aspartic acid. The lower dose combined treatment had an slight additive effect on the amino acid composition. At higher dose of the combined treatment, the additive effect was enhanced and many amino acids were absent.

Table 2. Amino acid composition of 4th instar larva of *H. armigera* after treatment with NPV and AZA.

Amino acids	Control	NPV (PIB/ml)			AZA (ppm)			NPV (PIB/ml) + AZA (ppm)		
		1×10^3	5×10^3	1×10^4	0.5	1.0	2.0	1×10^3 +0.5	5×10^3 +1.0	1×10^4 +2.0
Alanine	+	+	+	-	+	-	-	+	-	-
Glycine	+	+	+	+	+	-	-	+	-	-
Isoleucine	+	+	+	-	-	-	-	-	-	-
Leucine	+	-	-	-	+	+	-	+	-	-
Serine	+	+	+	-	-	+	-	+	-	-
Threonine	+	+	-	-	+	-	-	+	-	-
Valine	+	+	-	-	+	+	-	+	-	-
Phenylalanine	+	+	-	-	-	-	-	+	-	-
Tyrosine	+	+	+	+	+	+	+	+	+	-
Cysteine	+	+	-	-	+	-	+	-	-	-
Cystine	+	+	+	-	+	+	-	+	-	-
Methionine	+	-	-	-	+	-	-	-	-	-
Proline	+	+	+	-	+	+	-	+	-	-
Aspartic acid	+	-	-	-	+	+	-	+	-	-
Glutamic acid	+	+	-	-	+	+	-	+	+	+
Arginine	+	+	+	-	+	-	+	-	-	-
Histidine	+	+	-	-	+	-	-	+	-	-
Lysine	+	+	-	-	-	-	-	-	+	-

Key : + Present - Absent

The observed depletion of amino acids may be as a result of the viral infection. Marked decrease of almost all amino acids was also observed in the midgut epithelium of CPV infected *B. mori* larva (Kawase, 1965).

Neutral lipids in the midgut of 4th instar larvae is shown in Table 3. At the highest dose of NPV (1×10^4 PIB/ml), monoacyl glycerol, triolein, cholesteryl oleate and triacyl glycerol were absent when compared to absence of only triolein in the lower dose of NPV (1×10^3 PIB/ml). At the highest dose of AZA (2.0 ppm), monoacyl glycerol, diacyl glycerol, free cholesterol, cholesteryl oleate and esterified cholesterol were absent. In the highest combined dose of NPV and AZA (1×10^4 PIB/ml + 2.0 ppm), all neutral lipids classes were absent when compared to absence of only triolein at the lowest dose of combined treatment (1×10^3 PIB/ml + 0.5 ppm).

Table 3. Neutral lipids in midgut of 4th instar larva of *H. armigera* after treatment with NPV and AZA.

Amino acids	Control	NPV (PIB/ml)			AZA (ppm)			NPV (PIB/ml) + AZA (ppm)		
		1×10^3	5×10^3	1×10^4	0.5	1.0	2.0	1×10^3 +0.5	5×10^3 +1.0	1×10^4 +2.0
Monoacyl glycerol	+	+	+	-	+	+	-	+	-	-
Diacyl glycerol	+	+	+	+	-	-	-	+	-	-
Free cholesterol	+	+	+	+	+	+	-	+	-	-
Triolein	+	-	-	-	+	+	+	-	-	-
Cholesteryl oleate	+	+	+	-	-	-	-	+	+	-
Triacyl glycerol	+	+	-	-	+	+	+	+	-	-
Esterified cholesterol	+	+	+	+	+	+	-	+	+	-

Key : + Present - Absent

Table 4 shows the phospholipids composition of the midgut of 4th instar larvae. The low dose (1×10^3 PIB/ml) of NPV treatment resulted in absence of sphingomyelin, median treatment (5×10^3 PIB/ml) resulted in absence of sphingomyelin and phosphotidyl ethanolamine, while at the highest dose (1×10^4 PIB/ml), phosphotidyl inositol, phosphotidyl choline and

cardiolipin were absent. The highest AZA (2.0 ppm) treatment resulted in the presence of only phosphotidyl serine. The highest dose of the combined treatment of NPV and AZA resulted in absence of all phospholipids except phosphotidyl ethanolamine. The highest dose combined treatment had an additive effect on phosphotidyl inositol, phosphotidyl serine, sphingomyelin, cardiolipin and phosphatidic acid.

Table 4. Phospholipids in midgut of 4th instar larva of *H. armigera* after treatment with NPV and AZA.

Phospholipids	Control	NPV (PIB/ml)			AZA (ppm)			NPV (PIB/ml) + AZA (ppm)		
		1×10^1	5×10^1	1×10^4	0.5	1.0	2.0	1×10^3 + 0.5	5×10^3 + 1.0	1×10^4 + 2.0
Phosphotidyl inositol	+	+	+	-	+	+	-	+	-	-
Phosphotidyl serine	+	+	+	+	+	+	+	+	-	-
Sphingomyelin	+	-	-	+	+	+	-	+	-	-
Phosphotidyl choline	+	+	+	-	-	-	-	+	-	-
Phosphotidyl ethanolamine	+	+	-	+	+	-	-	-	+	+
Cardiolipin	+	+	+	-	+	-	-	-	-	-
Phosphatidic acid	+	+	+	+	+	+	-	+	-	-

Key : + Present - Absent

AZA treatment significantly altered the amino acids, phospholipids and neutral lipids in the larval midgut suggesting that AZA inhibits the neurophysiological function leading to suppression of protein and lipid metabolism. The NPV infected larval midguts showed the presence of only a few amino acids. Lysine, valine, leucine, isoleucine, phenylalanine, threonine, serine, proline and tyrosine, all important components of the occlusion body proteins, were absent in midguts of infected larvae.

Ishimori and Muto (1951) found a decrease in most free amino acids in *B. mori* haemolymph after infection with grasserie disease. It is probable,

however, that decreased food intake combined with viral synthesis causes a depletion of amino acid reserves in the gut of *H. armigera*. This may indicate that many of the depleted amino acids are, at least partially, derived from the gut for the synthesis is of the viral protein. Presence of glycine after NPV infection may be due to the retarded host protein synthesis giving rise to a surplus of this amino acid (Shapiro and Ignoffo, 1971).

Absence of many phospho and neutral lipid profiles in the midgut following the highest combined treatment of NPV and AZA may be due to physiological stress induced by the progression of the disease as observed in *Apis melifera* infected with NPV (Nelson *et al.*, 1971).

In this study, NPV and AZA treatment during later stages not only reduced the total lipid content but also reduced the number of individual lipids in the midgut. This might lead to structural alterations in the midgut (Yamamoto and Tanada, 1978).

Figure 2 and 3 show the DNA and RNA levels in midgut of 4th instar larvae during 48 and 96 h after treatment. At the highest dose of NPV (1×10^4 PIB/ml) the DNA content at 48 h post infection was 204.6 μg/g, which was

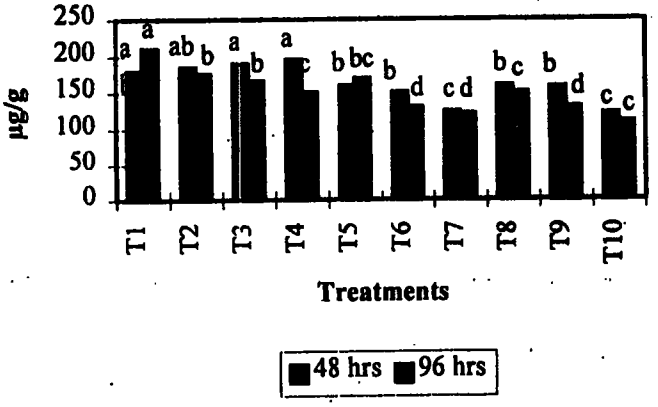

Figure 2. DNA content of midgut of 4th instar *H. armigera* after treatment with NPV and AZA.

[Note: T1 - Control; T2 - NPV (1×10^3 Polyhedral Inclusion Body [PIB]/ml); T3 - NPV (5×10^3 PIB/ml); T4 - NPV (1×10^4 PIB/ml); T5 - AZA (0.5 ppm); T6 - AZA (1.0 ppm); T7 - AZA (2.0 ppm); T8 - NPV (1×10^3 PIB/ml) + AZA (0.5 ppm); T9 - NPV (5×10^3 PIB/ml) + AZA (1.0 ppm); T10 - NPV (1×10^4 PIB/ml) + AZA (2.0 ppm)].

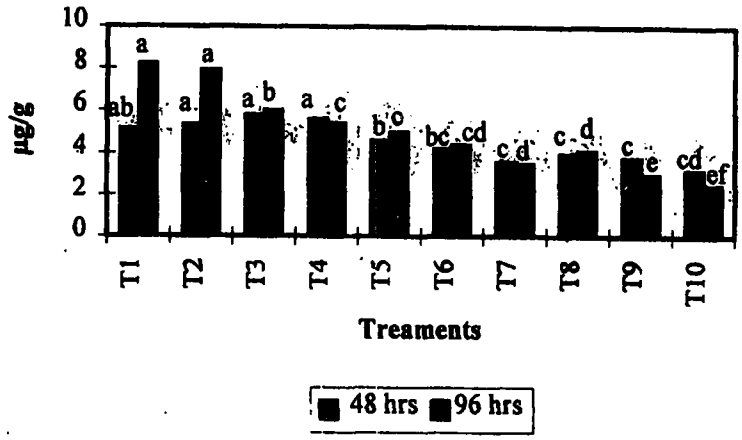

Figure 3. RNA content of midgut of 4th instar *H. armigera* after treatment with NPV and AZA.

[Note: T1 - Control; T2 - NPV (1×10^3 Polyhedral Inclusion Body [PIB]/ml); T3 - NPV (5×10^3 PIB/ml); T4 - NPV (1×10^4 PIB/ml); T5 - AZA (0.5 ppm); T6 - AZA (1.0 ppm); T7 - AZA (2.0 ppm); T8 - NPV (1×10^3 PIB/ml) + AZA (0.5 ppm); T9 - NPV (5×10^3 PIB/ml) + AZA (1.0 ppm); T10 - NPV (1×10^4 PIB/ml) + AZA (2.0 ppm)].

significantly greater than in controls. DNA content in midguts of infected larvae decreased to 134.50 μg/g by 96th h post infection. RNA content also increased by 48 h post infection when compared to controls, but significantly decreased by 96 h post infection.

The DNA and RNA contents of midguts of AZA treated larvae also decreased relative to controls at 48 and 96 h in a dose-dependent manner. The highest combined treatment of NPV and AZA (1×10^4 PIB/ml + 2.0 ppm) resulted in significantly reduced DNA and RNA content at both 48 and 96 h relative to controls. The DNA and RNA levels were statistically significant between the treatments. The decrease in levels was in a dose-dependent manner.

The DNA and RNA content of midgut of NPV treated larvae decreased by 96 h post infection in the present study. During the first 48 h of NPV infection, there is an initial increase in the content of DNA and RNA, as there is an increase in the synthesis due to NPV infection and later these are utilized by the virus itself (Kawase and Hayashi, 1965). The decline of

growth during the disease leads to the relative decrease of the DNA content of the midgut.

AZA strongly decreased DNA and RNA contents in midgut of *H. armigera*. Fritzysche and Cleffman (1987) showed cell proliferation to be inhibited by AZA, with RNA synthesis being strongly affected. Qadri and Narsaiah (1978) have found reduction in DNA and RNA content in AZA treated cockroaches. This might account for the decrease in nucleic acids in AZA treated *H. armigera*.

The combination of AZA and NPV has been shown to significantly increase larval mortality, reduce the time to death and cause reduced food consumption than individual treatments and low doses of AZA were sufficient for the enhancing effect (Senthil Kumar and Murugan, 1998). AZA causes slow necrosis of midgut epithelial cells resulting in a reduction in the number of regenerative cells of the nidi (Nasiruddin and Mordue (Luntz), 1993). Such necrotic epithelial cells with disrupted gut musculatures produce a fragile gut unable to function normally in terms of digestive efficiency and capacity. This might facilitate infection by NPV (Senthil Kumar, 1998). Commercial availability of NPV plus AZA mixtures could greatly enhance the feasibility of NPV use. The virus might have a direct insecticidal effect that would be particularly advantageous, if resistance were to develop to AZA treatment.

CONCLUSIONS

AZA and NPV not only affected the digestive enzyme activity and biochemical contents in midgut considerably, it also significantly lowered the DNA and RNA levels in the midgut. Dose-dependent reduction was observed in biochemical contents of midgut of *H. armigera*. This study reveals that AZA and NPV effectively decreased the essential primary nutrients in the gut necessary for the survival of the insect.

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