ENHANCED ACTIVITY OF *AGROTIS SEGETUM* (HUF.)(LEPIDOPTERA: NOCTUIDAE) GRANULOVIRUS BY BORIC ACID ADDITIVE IN THE LABORATORY AND SEMI – FIELD CONDITION

MAGDA KHATTAB

Plant Protection Research Institute, ARC, Dokki, Giza, Egypt

ABSTRACT

The enhancement of *Agrotis segetum* granulovirus (*Agse*GV) activity using the boric acid additive was investigated. The bioassay results revealed that the virus suspension contained 0.5, 1, 3 and 5% boric acid increased the mortality rate of *A. ipsilon* 2 nd (5- day old) larvae as compared with the virus alone. This rate of mortality increased with increasing concentration of boric acid added to the virus. The LC_{50} value of the 2 nd - instar larvae was reduced 10 fold by 3% boric acid + GV. Furthermore, the time required for *Agse*GV to kill larvae was significantly reduced by boric acid. The boric acid alone did not cause larval mortality at the concentration 0.5 or 1%. The light microscope examination showed that the peritrophic membrane (PM) of *A.ipsilon* exposed to boric acid alone, or the combined treatment, was markedly disrupted. Obvious ruptures on the outer surfaces of the PM were observed, which potentially facilitated the passage of virions through the PM. Under the semi- field conditions, the reduction of cutworm damage to cotton plants was 62.96% in case of virus alone and reached up to 96% in the boric acid + *Agse*GV treatment. In conclusion, the combination of boric acid with the baculovirus (*Agse*GV) may improve the activity of virus formulation.

INTRODUCTION

Cutworms can be very wasteful feeders, destroying far more plant seedlings than they consume and are also very polyphagous (Bourner *et al.*, 1992). Two lepidopteran species of the subfamily Noctuinae, *Agrotis ipsilon* (Hufnagel) and *Agrotis segetum* Schiff. are the most destructive among cutworm species.

In Egypt, cutworms attack about 50 plant species, *e.g.*, maize, faba bean, wheat, cotton, berseem, soybean, tomato, potato, cantaloupe, cucumber and many other vegetable plant species. The larvae cause considerable damage to both winter and spring crop seedlings (El-Hemaesy 1969, Amin and Abdin, 1997 and El-Malki *et al.*, 1998).

Four types of viruses, a nucleopolyhedrovirus (NPV), a granulovirus (GV), a cytoplasmic polyhedrosis virus (CPV), and a densovirus were isolated from the cutworms larvae: *A. segetum* CPV (*Agse*CPV), Ding and Cai (1983); *A. segetum* NPV (*Agse*NPV) Sherlock, 1983; *A.segetum* GV (*Agse* GV), Tsia and Ding, 1982, *A. ipsilon* NPV (*Agip*NPV), Boughton *et al.*, 1999 and Santharam and Kumaraswami, 1984.

Several authors reported the successful use of *A.segetum* GV (*Agse*GV) for controlling *A. segetum* and the closely related *A.ipsilon e.g.*, in Pakistan (Shah *et al.*, 1979), Denmark (Zethner, 1980) and Spain (Caballero *et al.*, 1990, 1991) in tobacco, root crops and maize, respectively. *Agse*GV was also registered as 'Virin-OS', a wettable powder, in the former Soviet Union for use against *A.segetum* in cotton (Lipa, 1991).

Laboratory and greenhouse experiments have shown that *Agse*NPV had considerable potential as a control agent for *A.segetum* and *A.ipsilon* (Oliveria and Entwistle, 1990 and Khattab *et al.*, 2004).

In greenhouse trials, spray and bait formulations of *Agip*NPV significantly reduced feeding damage to corn seedlings caused by third instar *A.ipsilon* larvae (Boughton *et al.*, 2001 and Prater *et al.*, 2006).

Several substances such as, fluorescent brighteners, (Shapiro and Hamm, 1999; El- Salamouny *et al.*, 2001), neem (Khattab *et al.*, 2004) and Lecithin (Song *et al.*, 2000) have been tested to increase virulence of entomopathogens.

Boric acid, also called orthoboric acid (borax and boron-containing salts) is a low-toxicity mineral with insecticidal, fungicidal, and herbicidal properties (1). It does not evaporate or volatilize into the air. It exists in the form of colorless crystals or a white powder and dissolves in water. It has the chemical formula H_3BO_3 or B (OH) $_3$ (2). Boric acid has an established history of use for the control of ants and cockroaches (Hayes and Laws, 1991). The first use of borates as insecticides was in 1922. Boric acid exists in many commercial products for insect control (4). As an insecticide, boric acid acts as a stomach poison for ants, cockroaches, silverfish and termites, and as an abrasive to the insects exoskeleton and causes death three to ten days post treatment (3). Also, kills roaches, fireants, palmetto bugs, ticks, bedbugs, fleas, boxeder, bugs, carpet beetles, centipedes, crickets, earwigs, grasshoppers, millipedes, scorpions, slugs water bugs and many more creepy crawly insects (4).This compound has been demonstrated to potentiate the activity of several baculoviruses (Shapiro and Bell, 1982; Morris *et al.*, 1995). The degree of potentiation increases with the concentration of the acid. For example the LC_{50} of an NPV of *Anticarsia gemmatalis* (Hübner) (*Ag*NPV) was reduced by a factor of approximately five fold in the presence of

0.045% boric acid. The lethal time (LT_{50}) was also reduced (Morales *et al.*, 1997). Similar results have been reported for the NPV's of *Lymantria dispar* (L.) and *Spodoptera litura* (F.) when mixed with 0.5-1% boric acid (Shapiro and Bell, 1982; Chaudhari, 1992; *Heliothis armigera* (Hüb.) and Bijjur *et al.*, 1991). Boric acid may be used either in a bait formulation containing a feed attractant or as a dry powder (1). Incorporation of boric acid into baculovirus formulations is an attractive proposition as it is inexpensive and has a low mammalian toxicity.

The present study was aimed to evaluate the role of boric acid additive in the enhancement of *Agse*GV activity for the control of *A.ipsilon* larvae.

MATERIAL AND METHODS

All laboratory procedures were performed at 26 ± 2°C, and 75 ± 5% RH. To determine the degree of enhancement provided by different concentrations of boric acid added to *Agse*GV, a laboratory bioassay based on the technique described by Cisneros *et al.*, 2002 was performed.

1. Insect colony, virus strain and boric acid

Insects: A disease-free culture of *Agrotis ipsilon* larvae was obtained from a laboratory colony. A semi - synthetic diet was necessary for handling large numbers of the test insect, as well as standardizing experiments. The semi-synthetic diet described by Shorey and Hale (1965) was used, except for the exclusion of formaldehyde from diet ingredients.

Virus production: Granulovirus *A. segetum* GV (*Agse*GV) was used in the present study. The source of the virus is from: BBA (Institute for Biological Control, Federal Biological Research Center, Darmstadt, Germany). *Agse*GV was propagated in the 3rd instar larvae of *A.ipsilon* orally which inoculated with virus, then maintained on the semi-synthetic diet until death. Virus-killed larvae were collected and occlusion bodies (OB's) were purified according to Khattab (2003). The OB's were resuspended in Tris buffer and checked by spectrophotometer Du-70 through 450 nm wavelength. One OD 450 = 4.48 x 10^{10} capsule/ml and 1 ml at OD 450= 0.125 mg capsule/ ml (Chang and Tanada, 1978). The virus suspension was stocked in Tris buffer at-20°C.

Additive: The commercial boric acid ($Na_2B_4O_7$. $10H_2O$) with a molecular weight of (381.36), was obtained as a powder and diluted to different concentrations (w/vol) using distilled water.

2. Bioassay

Laboratory bioassay was performed using-the diet surface contamination technique described by Cisneros *et al.* (2002). A special bioassay plates (L1CEFA. Bad-Salzuflen, Germany) was used. The plate (measuring 14 x 7 x 2 cm) contains 50 cells (144m^2 each), was filled with artificial diet (50 ml) to form a 5 mm thick layer. When solidified, a 2.0 ml volume of virus suspension with or without boric acid was spread over the diet surface (9313 mm^2) using finnpipette. Each bioassay test involved different concentrations of virus and/or additive estimated to result in LC_{50} of larval mortality. One 2nd instar (5-day old) *A.ipsilon* larva was placed on each cell then the plates were covered with tissue paper and 14 x 7 cm glass plate was fixed with rubber bands. All treatments were incubated at 26 ± 2°C. Mortality due to virus or additive treatment was recorded daily and up to 14 days. The larval mortality caused by untreated diet (control) was determined. All treatments included 2.5% wetter- sticker (Triton X -100) to reduce clumping of OB's.

To determine the LC_{50} of *Agse*GV, three concentrations of virus were suspended in Tris buffer pH 8. Fifty 2nd instar (5-day-old) larvae pooled out from the laboratory culture were used for each concentration. A similar number of control larvae were placed in a plate containing 2.5% Triton X -100 and diet alone.

The above bioassay procedure was repeated using a commercial boric acid preparation. The following five concentrations were tested: 0.5, 1, 3, 4 and 5% in a solution of 2.5% Triton X-100. This experiment was repeated two times.

The effect of boric acid on the LC_{50} value of virus, was evaluated using 2nd instar larvae (50 larvae/ treatment) exposed to three different concentrations of *Agse*GV ($4.39x10^6$, $2.19x10^5$ and $4.39x10^4$ capsules/ mm^2 diet) mixed with 0, 0.5, 1, 3 and 5% boric acid. Similar numbers of larvae were treated with distilled water to serve as a control treatment. This experiment was repeated two times.

3. Semi-field application tests

Seeds of the cotton variety (Giza 86) were seeded in pots (12.5 cm diameter X 12.5 cm length) and 30 pots were used for each treatment. 2nd instar (5 day-old) larvae were introduced into the pot (one larva per plant) when the plants had 3 leaves.

Wheat bran was used as bait at the rate: 50 gm + the LC_{50} of *Agse*GV mixed with the LC_{50} of boric acid. After 2hr. the formulated bran bait was applied manually to the infested pots near the cotton seedling. Control pots were treated with bran mixed with water only. Samples of *A. ipsilon* were collected from the pots on the three

successive intervals: 24 h., 4 days and 11 days. after treatment, in order to compare between levels of infection in the virus alone and virus+ additive treatments.

Evaluation of damage: Each cotton plant was examined for cutworm damage (either to the plants or leaves) caused by the released larvae. At the end of the experiment (after 11 days), the soil in each pot was examined for surviving larvae.

Light microscope examination: Tissues from both infected and control larvae were dissected, fixed in 3 glutaraldehyde in 0.1M cacodylate buffer (pH 7.3)-0.25M sucrose for 3h, postfixed in 1% osmium tetroxide for 1 hr, dehydrated through an ethanolprophylene oxide series, and embedded in Epon- Araldite resin. Thin sections were stained and examined with the light microscope at the Research Institute of Ophthalomology.

Statistical analysis: Data of bioassay results were subjected to probit analysis using the method described by Ehab Mostafa Bakr (5). The relative potencies of the treatments were calculated according to the changes in LC_{50} value.

RESULTS AND DISCUSSION

The concentration-mortality response of boric acid bioassayed against *A.ipsilon* 2^{nd} instar (5-day-old) larvae was 0, 0, 4.44, 20 and 28.88% for the tested additive concentrations 0.5, 1, 3, 4 and 5%, respectively. The LC_{50} estimated by 6.31 and the slope of the regression line was 4.88 (Table 1).

Also, the concentration-mortality response of *Agse*GV bioassayed against *A.ipsilon* 2^{nd} instar larvae was 51.02, 44.00 and 20.00% for the tested virus concentrations $4.39x10^6$, $2.19x10^5$ and $4.39x10^4$ capsule/mm^2 diet respectively. The LC_{50} value was $3.72x10^5$ capsule/mm^2 diet. The slope of the regression line was 0.883.

Table (1) shows that, the rate of larval mortality increased directly with the increase of boric acid concentration from 0.5% to 5 % as compared with the virus alone treatment.

The LC_{50} value for the virus alone treatment ($3.72x10^5$ capsule/mm^2 diet) decreased to $2.92x10^5$, $1.5x10^5$ and $3.71x10^4$ capsule/mm^2 diet at the tested boric acid concentrations 0.5, 1 and 3%, respectively. The boric acid alone did not cause mortality at the concentrations 0.5 and 1%. Increasing boric acid concentration to 5% in the combined treatment increased mortality to 100, 100 and 81.63% compared with the virus alone treatment which caused 51.02, 44.00 and 20.00% mortality with the tested three *Agse*GV concentrations, respectively (Table 1).

TABLE (I)

Percentage mortality due to addition of boric acid to *Agrotis segetum* GV (*Agse* GV) tested against *Agrotis ipsilon* 2nd instar larvae.

Agse GV Capsule/mm² diet	Mortality %								
	GV alone	0.5% Boric acid		1% Boric acid		3% Boric acid		5% Boric acid	
		alone	+ virus	alone	virus+	alone	+ virus	alone	+ virus
4.39×10^6	(49) 51.02	-	(40) 55.00	-	(45) 77.77	4.44	(42) 97.61	28.88	(49) 100
2.19×10^5	(50) 44.00	-	(50) 46.00	-	(49) 59.18	-	(46) 91.30	-	(48) 100
4.39×10^4	(50) 20.00	-	(49) 30.61	-	(49) 36.73	-	(47) 55.31	-	(49)81.63
LC_{50}	3.72×10^5	-	2.92×10^5	-	1×10^5	-	3.71×10^4	-	---
Slope	0.883	-	0.624	-	1.041	-	1.803	-	---
Potency	---	-	1.27	-	3.72	-	10.02	-	---

The rates of enhancements (potency) were 1.27, 3.72 and 10.02 fold, for the respective concentrations (0.5, 1 and 3%). The slope values were 0.624, 1.04 and 1.803 for the three boric acid concentrations. No virus mortality was recorded in case of control larvae.

A positive relationship between boric acid concentration and the potency of nucelopolyhedrovirus pathogenicity has been previously established. Shapiro and Bell (1982) reported a 2- fold reduction in the LC_{50} of *Lymantria dispar* NPV in the presence of 0.5% boric acid and a 7-to 11-fold decrease in the presence of 1% boric acid. Similarly, Morales *et al.* (1997) detected a 5- fold reduction in the LC_{50} of the NPV of *Anticarsia gemmatalis* in the presence of 0.045% boric acid incorporated into the diet.

The low rate of potentiation observed in the present study may be related to the duration of exposure and the dose of boric acid consumed by *A.ipsilon* larvae. Also, *A.ipsilon* is an alternative host to *Agse*GV, the LC_{50} value of the first instar was estimated by 1.94×10^7 capsule/ml diet (Khattab, 2003). Moreover, the effect of boric acid alone against *A.ipsilon* 2nd instar larvae was detected at the higher concentrations (4 and 5%) which caused 20 and 28.88% mortality, respectively.

On the contrary, Cisneros *et al.* (2002) reported no evidence of direct boric acid toxicity toward *Spodoptera frugiperda* larvae at 4, 5 and 6%. Shapiro and Bell (1982) reported 25-100% mortality of *L.dispar* larvae exposed to 2.5-10% boric

acid. This again may be related to differences in species susceptibility to boric acid.

In the present study, the mixture of boric acid + *Agse*GV decreased the mean time to cause 50% larval mortality (LT_{50}) (Table 2). At the highest GV concentration ($4.39x10^6$ capsule/mm^2 diet), the LT_{50} value for the virus alone treatment was 10.47 days, which decreased to 8.85, 5.02and 5.22 days with the boric acid concentrations 0.5, 1 and 3%, respectively.

In case of the concentration $2.19x10^5$ capsule/mm^2 diet, the LT_{50} value for the virus alone treatment was 11.09 days, and decreased to 7.54 and 5.90 days with the boric acid concentrations 1 and 3%, respectively.

Also, with the lowest concentration, $4.39x10^4$ capsule/mm^2 diet, the estimated LT_{50} value for the virus-alone treatment was 12.62 days, decreased to 9.82, 7.90 and 6.47 days with the boric acid concentrations 1, 3 and 5% (Table 2).

TABLE (I)

Lethal median time (LT_{50}) values of *Agrotis segetum* GV (*Agse* GV) combined with different concentrations of boric acid tested against *Agrotis ipsilon* 2^{nd} instar larvae.

Agse GV Capsule/mm^2 diet	**LT_{50} values (days)**								
	Boric acid conc. (%) ± virus								
	Virus alone	0.5% Boric acid		1% Boric acid		3% Boric acid		5% Boric acid	
		alone	+ virus	alone	+ virus	alone	+ virus	alone	+ virus
4.39×10^6	10.47	---	8.85	---	5.02	---	5.22	16.88	--- *
2.19×10^5	11.09	---	17.39	---	7.54	---	5.90	---	--- *
4.39×10^4	12.62	---	18.74	---	9.82	---	7.90	---	6.47

* 100 % mortality was attained.

The LT_{50} for virus + 0.5% boric acid was not significantly different from that of virus alone at the highest concentration. In contrast, the LT_{50} value for the boric acid alone at 5% was 16.88 days. However, the obtained mortality from (GV + 5%) reached 100, 100 and 77.55% after 7 days post treatment with the three GV concentrations, respectively.

In conclusion, the estimated LT_{50} values for *Agse*GV alone decreased with the gradual increase of boric acid concentrations (Table 2).

Similarly, the LT_{50} was reduced when boric acid was added to NPV. Shaprio and Bell (1982) reported a LT_{50} of 20.5 days for *L.dispar* NPV alone compared to 16.2

days for the NPV + boric acid. Also, the LT_{50} of the *Heliothis armigera* NPV was 194.5 hours compared to 136.8 hours for NPV + boric acid (0.5%) (Chundurwar *et al.*, 1990). The LT_{50} of *A.gemmatalis* NPV was reduced from 11.3 to 7.2 days when boric acid was added at 0.045 g/ml of diet + 1.215 PIB/ml NPV (Morales *et al.*, 1997). The LT_{50} was not significantly different from that of *Spodoptera frugiperda* NPV alone or mixed with 0.5 or 1% boric acid (Cisneros *et al.*, 2002).

In the present work, the average weight of larvae was decreased to 0.419, 0.417, 0.105 and 0.014 g/larva by increasing boric acid concentrations 0.5, 1, 3 and 5% compared to 0.422 or 0.478 g/ larva for the untreated control and virus alone treatments, respectively. No significant difference was found in the larval weight between *Agse*GV alone or that mixed with boric acid at 0.5 or 1% with highest GV concentration (Table 3). Thus, one advantage of addition of boric acid at low concentration to virus formulation is the increase of virus produced and released into the environment. However, decrease in body weight was observed at the higher dose levels of boric acid. In this respect, reduction in larval weight was observed in neem + *Agse*GV tested against *A.ipsilon*, thus, virus produced and released into environment is less (Cook *et al.*, 1996 and Khattab *et al.*, 2004).

TABLE (III)

Average weight of *Agrotis ipsilon* survivals after virus treatments of 2nd instar larvae (5 – day old) with or without boric acid.

Treatments	Weight ** of 30 larvae (in gram)	Average weight of larvae (in gram)
Control	4.22 (10 L.)	0.422
0.5 % *	4.19 (10 L.)	0.419
1.00 % *	4.17 (10 L.)	0.417
3.00 % *	1.05 (10 L.)	0.105
5.00 % *	0.13 (9 L.)	0.014
Virus – alone	14.35	0.478
Virus + 0.5 %	15.57	0.519
Virus + 1.00 %	13.28	0.442
Virus + 3.00 %	5.67 (15 L.)	0.378

* Boric acid concentration.

** All survivals were weighed 14 days after treatment.

In the present study, the light microscope examination showed that the peritrophic matrices (PMs) of *A.ipsilon* larvae treated with boric acid alone, or with *Agse*GV, were markedly disrupted. Obvious ruptures on the outer surfaces of the PM were observed. It seems that, boric acid inhibited PM formation in *A. ipsilon*.

This inhibition increased larval susceptibility to virus infection. Continuous inhibition resulted in retarded larval development and mortality (Fig. 1).

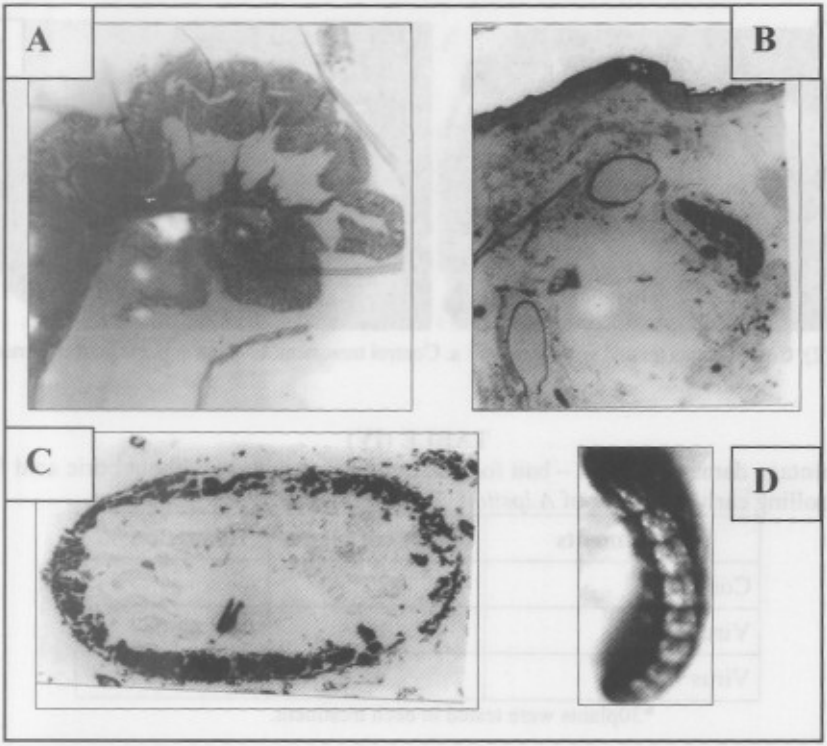

Fig. (1): Cross section of *Agrotis ipsilon* larvae: A. Healthy larvae of *A. ipsilon,* showing normal cell size and structure (100X). B. *A. ipsilon* larvae after dosing with *Agse* GV + boric acid, most cells lysing and body cavity full of lysed tissues (100X). C. *A. ipsilon* larvae after dosing with boric acid alone, the mid gut showing dissociation (100X). D. *A. ipsilon* healthy larva.

Boric acid also potentiates the activity of baculoviruses and is active by ingestion (Govidarajan *et al.*, 1976; Morris *et al.*, 1995). Cisneros *et al.* (2002) assumed that boric acid affects the conditions in the insect's gut, possibly by altering the integrity or permeability of the peritrophic membrane or the cells of the gut epiderms. Alternatively, the toxicity of boric acid may cause physiological stress in the insect, increasing its susceptibility to virus infection (Shapiro and Bell, 1982).

The role of boric acid in enhancing *Agse*GV infectivity was also demonstrated in semi-field studies using bran bait formulation. The mixture of LC_{50} *Agse* GV+ LC_{50} boric acid ($3.72x10^5$ capsule/mm^2 + 6.3%) in bran bait formulation resulted in decreasing the number of cut plants by *A. ipsilon* larvae compared to virus alone or untreated control treatments. The reduction of cutworm damage was 62.96 % for the virus alone treatment and reached to 96 % in boric acid + *Agse* GV (Table 4 & Fig. 2).

This bran bait formulation protected the virus from the adverse effect (UV irradiation) for several days. *A.ipsilon* larvae were often observed feeding directly on the bait.

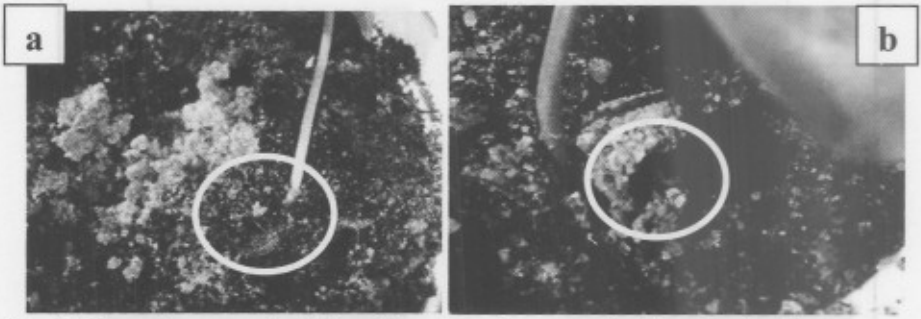

Fig. (2): Cotton plants treated with bran bait: a. Control treatment. b. Virus + Boric acid treatment.

TABLE (IV)

Percentage damage in virus – bait formulation treated with or without boric acid for controlling early 2nd instar of *A.ipsilon* (Semi-field test).

Treatments	No. of cut plants	Reduction %
Control	27	---
Virus-alone	10	62.96
Virus + boric	1	96.00

*30plants were tested in each treatment.

The reduction of cutworm damages in tobacco plots varied between 72 and 100% when *Agse*GV was used against 2nd instar larvae of *A.ipsilon* (Shah *et al.*, 1979). Also, *Agse*GV reduced the natural occurrence of cutworms (*A.ipsilon* and *A.segetum*) damage by 64.82% in tobacco, 85% in Okra, 77% in potato and 78% in sugar beet (Zethner *et al.*, 1987). In field trials, more than 90% and 50% mortality among *A.segetum* on carrots and beets, respectively, were recorded with the *Agse*NPV (Oliveria and Entwistle, 1990). Boruner *et al.* (1992) found that, infection rates in *A. segetum* 2 nd instar larvae after treatment with *Agse*NPV and *Agse*GV were 87.5 or 91% for NPV and 12.5 or 55% for GV in spray or bait treatment, respectively, in maize. Mixed inocula of *Agse*NPV and *Agse*GV gave intermediate results on maize and beet root (Bourner *et al.*, 1994). Sprayed suspensions of *Agip*NPV ($5 \times 10^8 - 6 \times 10^9$ OB's/m^2) resulted in 75 to>93% lethal infection of third or fourth instars in field plots to control *A. ipsilon* in turfgrass (Prater et al., 2006).

There have been very few field studies to evaluate virus + boric acid formulations. In India, Bujjur *et al.* (1991) reported a 4 fold improvement in controlling *Helicoverpa armigera* (Hübner) on sunflower treated with *Ha*NPV +

0.5% boric acid. Also, Chundurwar *et al.* (1990) reported improved control of *H.armigera* on chickpea treated with *Ha*NPV + 0.5% boric acid up to 4 fold.

The mixture of *Spodoptera frugiperda* NPV + 1% boric acid used in the field studies (an queous spray or the granular formulation) resulted in 15-25% higher prevalence of viral infection than that observed in insects exposed to virus alone. This is assumed to be due to loss of viral inocula by UV-inactivation and plant growth duration (Cisneros *et al.*, 2002). Moreover, the same authors, reported that, application of boric acid in aqueous sprays designed to give maximal coverage of maize plants caused no significant reduction in the abundance of insect natural enemies or other nontarget insects, suggesting that boric acid is compatible for baculovirus formulation at all concentrations tested. Besides, the cost of including 1% boric acid in the granule formulation (48 cents/ha) is cheaper than using an optical brightener, which is about 20 times more expensive than boric acid.

Consequently, results obtained by these authors, and those presented in the present work show that boric acid, at very low concentrations, may be used in viral formulations to increase their virulence and speed of kill.

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