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SIMULTANEOUS EFFECT OF CERTAIN BOTANICALS ACTIVITY ON THE RELATIVE PATHOGENCITY OF (*Agse*GV) ON *AGROTIS IPSILON*

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Abstract

In the present study, the combination of neem oil extract with ***AgseGV*** tested against *A. ipsilon* neonate larvae enhanced the activity of GV, measured by the decrease of the LC_{50} value from 3.59 x 10^7 in the virus alone- treatment to 7.11 x 10^6 capsule/ml diet in the treatment of virus and neem at 10ppm (5.0567 fold). Present results also proved that the combination of neem oil with *AgscGV* decreased the LT_{50} value about 1.938 fold; from 14.136 days in the virus alone- treatment to 7.295 days at virus concentration 3.85 x 10^7 capsule/mi diet **+** neem (10ppm). The effect of neem on larval weight in present results was evident among the post-treatment survival *A. ipsilon* larvae. The observed reduction in larval weight (from 0.292676 gm / larva in the control treatment and 0.243376 in the virus alone- treatment to 0.003486 gm/larva in the combined treatment) is probably due to the act of neem as anti-feeding. The neem mode of action is explained by its effect on the digestive enzyme activity and the biochemical composition in the mid gut. One disadvantage of addition of Azadirachtin to viral formulation is less virus produced and released into the environment

Introduction

Neem seed extract obtained from the neem tree *Azadirachta indica* A. Juss is a demonstrated anti-feeding, insecticide, and insect growth regulator against many insect species including lepidopterans (Schmutterer, 1995*).* Neem extract in combination with the Lyd*1NPV* on artificial diet was evaluated, the extract decreased the time required for viral kill of the larvae but did not decrease the concentration of virus (LC_{50}) required for kill (Shapiro *et at.,* 1994). The combination affected larval development (weight gain and molting) and survival, larvae died significantly faster compared with larvae, which consumed only Azadirachtin or virus. The additions of Azadirachtin to viral formulations also result in fewer viruses released into the environment because the affected larvae are smaller (Cook *et at.* 1996). When Neemazal-T was included in the virus mixtures *(Sp1*NPV*,* SpexNPV, *AucaMNPV & AgseNPV)* a dose dependent increase in potency. The effect was greatest for S. *littoralis* NPV, in which the effectiveness of the virus were, increased 2.9-fold relative to normal NPV inoculum's (El-Salamouny *et at.* 1997). Neem seed kernel extract (NSKE) at *2.5 %* enhanced the activity of NPV at 102 PJB against *H. armigera* on cotton leaves (Murugan and Jeyabalan, 1998). The larval weight and growth rate were significantly reduced in the S. *litura* NPV *(Sp1iNPV)* -neem combination (Rabindra *et at.* 1997). When azadirachtin (1.0 ppm) was combined with *H. armigera* NPV (1.5 x 104 PIB's/ml), the LT_{50} of 4th instar was reduced to 96 h. The larval duration was extended to 23 days (Kumar and Murugan, 1998). *S .litura* N7PV with 1 % neem oil, NSKE and *5 %* neem-cake extract increased the efficacy of NPV and enhanced the virus mortality from 31.1 % to 81.5, 81.5 and 57.6 %, respectively. Neem oil and NSKE (0.10-1.0 %) reduced the LC_{50} of NPV by 1.06-1.43 fold and 1.03-1.33 fold, respectively (Baskaran *et at.* 1999). None of the combinations of *H. virescens* NPV and azadirachtin tested against neonate or 2nd instar larvae of *H. virescens* significantly increased mortality compared to the better of the respective single agents (Koppenhofer and Kaya, 2000). In field studies for the control of *P. gossypiella,* NSKE (6 %) with *Heliothis* NPV 500 larval equivalents/ha was the most effective treatment (Sarode *et at.,* 1995,). Another field trials showed that application of the *Heticoverpa* NPV at 500 LE/ha plus the neem extract at 6 % gave the maximum reduction in larval numbers of H. *armigera* (79.8 and *65.2 %, 7* and 14 days after spraying, respectively) (Sarode *et at.* 1995, Sarode *et al,* 1996 and Sarode *et al*. 1997). There was no enhancement of viral activity when the Azadirachtin was added to the *L. dispar* NPV formulation (Cook *et al.,* 1997).

The goal of the present study is to measure the enhancement of the combination of neem oil extract with ***AgseGV*** against *A. ipsilon* neonate larvae.

Material and Methods

1-Test Insects

Laboratory colonies of the black cutworm (BCW), *Agrotis ipsilon* (Hufnagel) were established. Semi-synthetic diet was necessary for handling large numbers of the test insects, 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.

2- Virus

A multiply embedded granulovirus isolates, *A. segetum* GV *(AgseGV)* was used in the present investigation. The used virus was obtained from BBA (Institute for Biological Control, Federal Biological Research Center, Darmstadt, Germany).

3- Neemazal-T

The commercial extract (Neemazal-T, Trifolio, Germany), which is an extract from the neem tree *Azadirachta indica* (A-Juss) was obtained as a seed oil with an active material of 1 % Azadirachtin, and tested.

4-Methods

Mass rearing of insects: A disease free culture of each test insect species was successfully maintained in a temperature-controlled insectary at ca 26±2^{0}C throughout the duration of study.

Adults and Oviposition. Newly emerged adults were kept every 20 pairs of moths in a cylindrical mating cage (26 cm in diameter and 32 cm in length) for oviposition. The cage was lined from the inner side with tissue papers in the case of cutworms; otherwise two strips of muslin pad were inserted from the upper rim to hang down in the cage, in order to serve as both resting and oviposition sites for females. Each cage was provided with a piece of cotton wool in a small plastic cup containing 10 % sugar solution that was replenished daily. The tissue papers containing eggs were daily removed and labeled.

Eggs. Newly deposited eggs were sterilized by formalin vapor *5* % for *5* minutes. The eggs were placed in plastic container (5.5 cm height and 7.5 cm diameter), and a small piece of wetted cotton wool was placed in the bottom of the container to facilitate egg hatch.

Larvae. The neonate larvae were kept individually in clean plastic cups (4 cm height and 4.5 cm diameter). A small piece of tissue paper, was placed in the cup and renewed when.2.1.4-

Pupae. At the end of larval stage, the formed pupae were collected daily from the rearing pots, and sterilized by washing in 10 % Clorox (sodium hypo-chlorite solution), then washed twice with the tap water. About 50 pupae were kept in plastic cylindrical cages (23 cm length and 14 cm diameter) until adult emergence.

5-Virus Propagation

The diet in plastic plates (LICFFA, Bad-Salzuflefl, Germany, 14 x 7 x 2 cm) was surface-treated with virus (3.85 x 10^{10} capsule/ml.) by an atomizer. After drying, 50 test larvae per plate were allowed to feed for 24 h., after which were transferred individually into another plate of artificial diet and checked daily. Dead larvae were collected in glass tubes and frozen at -20^{0}C until purification.

6-Virus Purification

Dead larvae in distilled water at 4^{0}C were blended and the resulting suspension was filtered through several layers of muslin cloth. The suspension was centrifuged at 1000 rpm for 5 mm. and the pellet was discarded. The obtained supernatant was centrifuged twice at 4000 rpm for 20 mm. After clarification of the filtrate suspension, the GV virus was pelleted from the supernatant by centrifugation at 10000 rpm for 30 mm. using Beckman J2-2 1 MIE centrifuge, rotor 20 JA. The pellet was re-suspended in Tris buffer (50 mm, pH 7.8) deposited on 30-70 % (w/w) continuous sucrose gradient and centrifuged at 11000 rpm for 20 mm. using Beckman L7-65 ultracentrifuge, rotor SW 28. The band containing granules was drawn-off with a Pasteur pipette, re-suspended in Tris (50 mm, pH 7.8), and centrifuged at 10000 rpm for 30 mm. using Beckman J2-21 MIE centrifuge, rotor 20 JA (Khamiss, 1997). The pelleted highly purified granules were then re-suspended in Tris buffer and checked by spectrophotometer DU-70 through 450 nm wavelengths. 1 OD 450 = 1.48 x 1010 capsule/mi and 1 ml at 1 OD 450 = 0.125 mg capsule/ml (Chang and Tanada**,** 1978). The viral suspension was stocked in Tris under -20^{0}C.

7-Preparation of Virus Inocula

Serial dilutions of the stock virus suspension were made in tris-buffer pH 8, in addition to 2.5 % Teepol as wetting agent. The tested additives were used in diluting virus suspensions.

8-Preparation of Additives

The Neemazal-T was further diluted in distilled water and tested at five concentrations of 1, 5, 10, 20 and 50ppm.

9- Bioassay

Standardization was based on the number of polyhedral inclusion bodies (PIB's)/ml of aqueous suspension. Insects were maintained at 26^{0}C and bioassay tests were performed using the semi-synthetic diet.

Diet incorporation bioassay: Five ml of virus (with or without additives), resuspended, from each treated sample, were mixed with 45 ml of semi-synthetic diet at a diet temperature below 40^{0}C. The contaminated diet was then distributed into special bioassay plates (LICEFA, Bad-Salzuflen, Germany). The plate measuring 14 x 7 x 2 cm, contains 50 cells. One newly hatched larva was placed into each cell. The plates were covered with tissue paper and 14 x 7 cm glass plate and fixed with rubber bands. All treatments were incubated at 26"C, for 16 days. Mortality due to virus infection was recorded every two days and up to 16 days. The larval mortality caused by untreated virus (control) was determined.

10-Statistical Analysis

The data of bioassay results were subjected to probit analysis using the method described by Finney (1971). The relative potencies of the treatments were calculated according to the changes in LC50value.

Results

Neemazal-T Additive

A preliminary bioassay test was conducted to determine the sub lethal concentrations of Neemazal-T tested alone against neonate larvae of *A. ipsilon* (Table 1 and Fig. 1). The results showed the antimoulting and antifeedant effects of Neemazal-T, particularly at the high concentrations. In case of incorporation of the Neemazal-T with the diet, the rate of concentrations: 1, 5, 10, 20 and 50ppm, respectively. Five different Neemazal-T concentrations (0, 1, 5, 10, 20 and 50ppm) were tested as additives to *AgseGV* (Table 2 and Fig. 2). The obtained LC_{50} values were 3.59 x 10^7, 3.32 x 10^7, 1.51 x $10^7$and 7.11 x 10^6 capsules/ml diet for the tested *Spl*NPV alone treatment that with Neemazal-T additive at concentrations of 1, 5 and 10ppm, respectively (Table 2 and Fig. 2). The potency values were 1.081, 2.379 and 5.057 fold, at the respective concentrations of Neemazal-T. The LT_{50} values for the neem alone treatment at concentrations of 10, 20 and 50ppm were 53.2 16, 9.393 and 3.2 19 days, respectively (Table3). At the highest GV concentration (3.85 x 10^8 capsules/ml diet), the LT_{50} value for the virus-alone treatment was 5.44 1 days, while was 7.548, 7.106, 6.836 and 6.512 days with neem additive at concentrations 1, 5, 10 and 20ppm, respectively. In case of the concentration (1 x 10^8 capsules/nil diet), the LT_{50} value for the virus-alone treatment was 10.158 days, that was decreased to 11.706, 10.424, 7.888, 7.380 and 2.083 days with neem additive at concentrations 1, 5, 10, 20 and 50ppm, respectively. Also, with the lowest concentration (3.85 x 10^7 capsules/ml diet), the estimated LT_{50} value for the virus-alone treatment was 14.136 days, which was decreased to 13.147, 10.565, 7.295, 7.188 and 2.870 days with all abovementioned tested neem additive concentrations, respectively.

In conclusion, the estimated LT_{50} values for the virus-alone treatment were decreased with the gradual increase of neem additive concentrations. However, there was no regular trend coincided with increasing neem concentrations in the case of the highest virus concentration (3.85 x 10^8 capsules/mi diet) (Table 3).

It has been observed that *A. ipsilon* larvae treated with Neemazal-T were smaller in size than those of the control treatment. Data presented in Table (4) showed that the average weight of larvae in either the untreated control or virus-alone treatment was 0.292676 or 0.243376 gram which was decreased by the addition of Neemazal-T to 0.209522, 0.91822 and 0.003486 gram for the respective serial concentrations 1, 5 and 10ppm. From previous study, Neem seed kernel extract is demonstrated as antifeedant, insecticide and insect growth regulator (Schmutterer, 1995).

Discussion

Neem Additive

Neem seed kernel extract (NSKE) obtained from the neem tree *Azadirachta indica* A. Juss (Meliaceae) is a demonstrated antifeedant, insecticide and insect growth regulator against many insect species including lepidopterans (Schmutterer, 1995). Evaluation of the combined efficacy of baculoviruses with neem extract as an enhancing additive to NPV virulence was previously reported.

In the present study, the combination of neem oil extract with ***AgseGV*** tested against *A. ipsilon* neonate larvae enhanced the activity of GV, measured by the decrease of the LC_{50} value from 3.59 x 10^7 in the virus alone- treatment to 7.11 x 10^6 capsule/ml diet in the treatment of virus and neem at 10ppm (5.0567 fold).

This result agrees with that of El-Salamouny *et al.* (1997). They found that the effect of Neemazal-T increased the infectivity of *Sp1i*NPV by 2.9 fold compared to the virus alone- treatment.

The activity of NPV tested against *H. armigera* larvae was enhanced by the addition of NSKIE (neem seed kernel extract) at 2.5 % (Fiurugan and Jcyabalan, 1998). The combination of neem oil at different concentrations (0.10-1.00 %) reduced the

LC_{50} of S. *litura*-NPV by 1.06 to 1.43 fold, respectively (Baskaran *et al.,* 1999). In field trials, application of NPV ± neern extract gave the maximum reduction in larval numbers of both *P. gossypiella* or *H. armigera* on different crops (Sarode *et al.,* 1995,a,b, 1996 & 1997).

Present results also proved that the combination of neem oil with *AgscGV* decreased the LT_{50} value about 1.938 fold; from 14.136 days in the virus alone- treatment to 7.295 days at virus concentration 3.85 x 10^7 capsule/ml diet + neem (10ppm). The same result was observed with *Ld*NPV or S. *litura* NPV (Shapiro et al. 1994 and Baskaran et al*,* 1999**).**

The effect of neem on larval weight in present results was evident among the post-treatment survival *A. ipsilon* larvae. The observed reduction in larval weight (from 0.292676 gm / larva in the control treatment and 0.243376 in the virus alone-treatment to 0.003486 gm/larva in the combined treatment) is probably due to the act of neem as antifeedent. This effect of neem is well documented (Shapiro et al*,* 1994; Cook et al*.,* 1996; Cook et al*,* 1997 and Rabindra et al*.,* 1997)

The neem mode of action is explained by its effect on the digestive enzyme activity and the biochemical composition in the midgut. One disadvantage of addition of azadirachtin to viral formulation is fewer viruses produced and released into the environment (Cook et al**,** 1996).

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Table 1. Rate of mortality among *Agrotis ipsilon* neonate larvae treated with different concentrations of Neemazal-T.

Neemazal-T Concentrations (ppm)	**No. Of infected larvae**	**No. Of tested larvae**	**M (%)**
1	0	49	(0.00)
5	2	50	(4.00)
10	10	42	(23.80)
20	47	47	(100.0)
50	49	49	(100.0)
LC50			11.463 ppm
Slope			6.707

Table 2. Effect of Neemazal-T as an additive to *Agrotis segetum* GV *(AgseGV)* against *Agrotis ipsilon* neonate larvae.

***AgseGV* (capsule/ ml diet)**	***AgseGV*-alone**	**+ Neem conc. (ppm)**				
		+1	**+5**	**+10**	**+20**	**+50**
3.85 $X10^6$	18.60 (8/43)	16.00 (8/50)	20.83 (12/so)	25 (21/45)	100 (41/41)	100 (45/45)
1.00 X 10^7	26.00 (13/50)	30.00 (15/50)	45.38 (13/39)	71.87 (3 1/40)	100 (34/34)	100 (42/42)
3.85X 10^7	54.16 (26/48)	58.00 (29/50)	75.00 (25/37)	87.50 (36/40)	100 (33/33)	100 (40/40)
1.00 X 10^8	64.00 (32/50)	62.00 (3 1/50)	77.08 (33/44)	87.50 (46/50)	100 (42/42)	100 (50/50)
3.85 X 10^8	85.71 (47/49)	88.00 (44/50)	91.66 (33/37)	100.0 (42/42)	100.0 (30/30)	100.0 (50/50)
LC50	3.59x 10^7	3.32x10^7	1.51x10^7	7.11x10^6	--	--
Slope	0.988	1.023	1.071	1.309	--	--
Potency	--	1.081	2.379	5.057	--	--

Between brackets is no. of dead-larvae no. of tested
Mortality % is corrected (against neem alone treatment) by Abbott's formula (Abbott, 1925)

Table 3. Lethal median time (LT50*)* values of AgseGV combined with different concentrations of Neemazal-T, *Agrotis ipsilon* neonate larvae.

***AgseGV* (capsule/ ml diet)**	**LT value days**					
	***AgseGV*-alone**	**+ Neem conc. (ppm)**				
		+1	**+5**	**+10**	**+20**	**+50**
Control (neem)	0.000	0.000	0.000	*53.216*	9.393	*3.219*
3.85 x 10^7	14.136	13.147	10.565	*7.295*	7.188	2.870
1.00x 10^8	10.158	11.706	10.424	7.888	7.380	2.083
3.85x10^8	5.441	7.548	7.106	6.836	*6.512*	0.000

Table 4. Average weight of *A. ipsilon* neonate exposed to *Ags*GV and Neemazal-T.

Treatment		Average weight of larvae (gm)
Control		**0.2926**
Neem	1ppm	0.1769
Neem	5ppm	0.1082
Neem	10ppm	0.0035
Virus	Alone	0.2433
Virus	+ 1ppm	0.2095
Virus	+ 5ppm	0.0918
Virus	+ 10ppm	0.0034

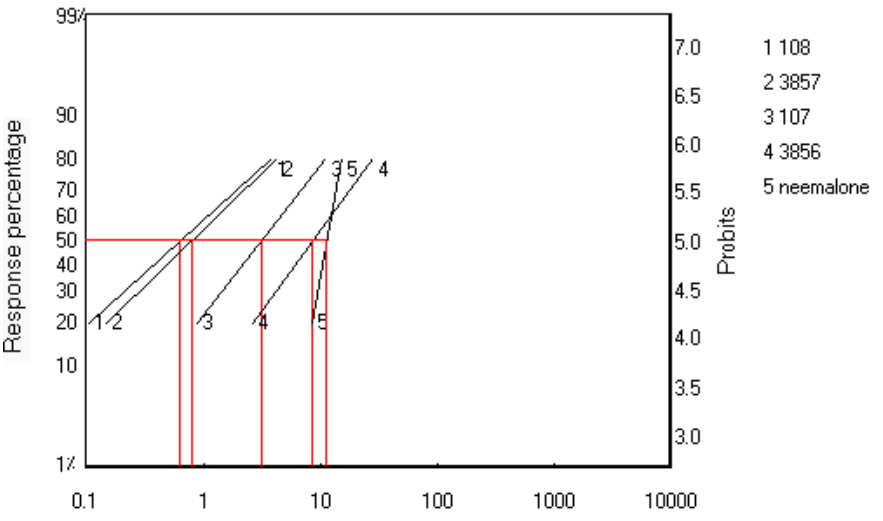

Figure 1. Concentration-mortality response of *Agse* GV, with or without Neemazal-T additive, bioassay against *Agrotis ipsilon* neonate larvae.

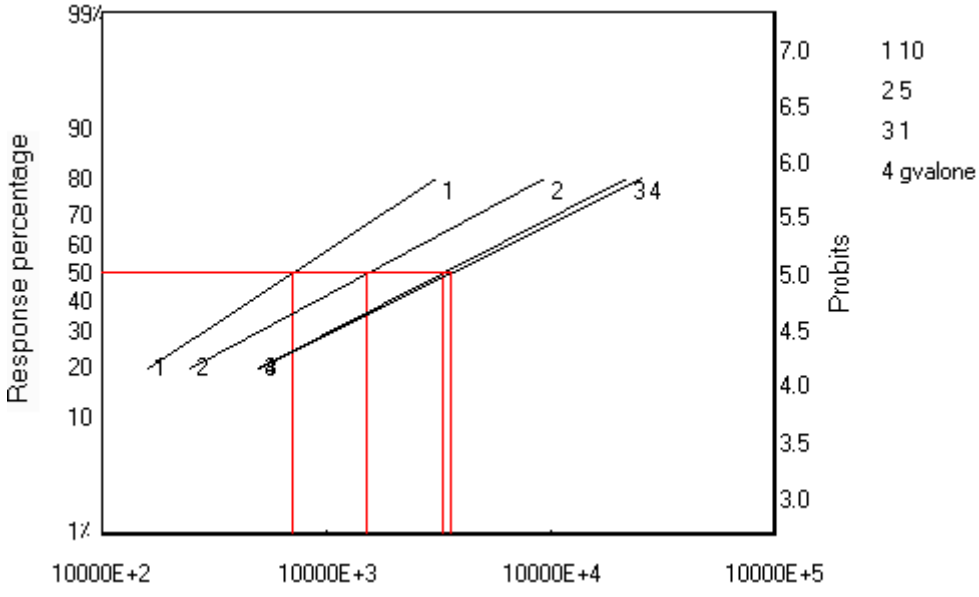

Figure 2. Concentration-mortality response of *Agse* GV, with or without Neemazal-T additive, bioassay against *Agrotis ipsilon* neonate larvae.