Tropical Agricultural Research Vol. 17: 221 - 230 (2005)

Influence of *Pseudomonas fluorescens* and a Nucleopolyhedrovirus on Cotton Bollworm *Helicoverpa armigera* (Hübner)

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ABSTRACT. *Current approaches in crop protection emphasize utilization of microbials with non-toxic modes of action. For cotton, the nucleopolyhedrovirus of Helicoverpa armigera (HaNPV) is advocated against the pest along with chemical pesticides. The plant growth promoting rhizobacteria (PGPR), Pseudomonas fluorescens (P. fluorescens) has been found useful in disease management besides imparting induced systemic resistance. The bacterium has been shown to modulate the disease expression by baculoviruses due to enhanced phenolase activity. We investigated the possibility of integrating HaNPV and P. fluorescens on cotton (LRA 5166) against H. armigera. The HaNPV was applied to leaves (30 DAS), square (45 DAS) and bolls (70 DAS) excised from pot cultured cotton plants previously treated with P. fluorescens as seed treatment (ST) or as foliar application (FA) or combination of both and bioassayed against third instar H. armigera. The biochemical analysis conducted included, phenol, tannin, peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL). The results revealed that the application of P. fluorescens as a ST and FA, either alone or in combination with HaNPV significantly reduced larval and pupal weights of H. armigera that fed on cotton plant parts. Significantly low per cent mortality (60%) was noticed in cotton leaves treated with HaNPV and P. fluorescens through ST + FA than ST alone or FA alone. Enzymes like peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were higher in P. fluorescens treated cotton plants than untreated checks. These phytochemicals and reduction in consumption of HaNPV treated plant parts could be responsible for the significant reduction in NPV induced mortality of H. armigera larvae.*

INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner) is a serious pest of cotton, legumes and vegetables in India and South East Asia (Singh *et al.*, 2002). Increased interest in the use of biopesticides like nucleopolyhedrovirus (NPV) for the control of *H. armigera* has been shown by researchers and end users (Jayaraj, 1985). The pests and diseases management package in cotton integrates several methods, of which, plant growth promoting rhizobacterium (PGPR), *Pseudomonas fluorescens* (*P. fluorescens*) and *H. armigera* NPV (HaNPV) form part. The bacterium is known to induce systemic resistance (ISR) against several insect pest species (Qingwen *et al.*, 1998; Radjacommare *et al.*, 2002) and altered biochemical status in plants (Ramamoorthy *et al.*, 2002). The HaNPV has been shown to perform effectively on susceptible varieties of plants than resistant ones (Rabindra *et al.*, 1992). Phytochemicals have been shown to decrease the effectiveness of the

baculoviruses in general (Felton and Duffey, 1990). The infectivity of HaNPV was influenced by plant surface environment (Rabindra *et al.*, 1994). Hence, in the present investigation, the influence of *P. fluorescens* induced plant defenses in pot cultured cotton plants on the efficacy of nucleopolyhedrovirus of *H. armigera* was studied.

MATERIALS AND METHODS

Sources of materials

The *H. armigera* larvae for the study were obtained from a stable and healthy culture maintained in the Biocontrol Laboratory, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, India. Larvae were reared and maintained on chickpea based semi-synthetic diet and the bio-stages were maintained as outlined by Sathiah (2001). Cotton (LRA 5166) plants were grown in the greenhouse from seeds in a common culture medium enhanced with different treatments (Table 1). HaNPV (CBE 1 isolate) was multiplied *in vivo*, semipurified and strength of viral occlusion bodies was assessed (Sathiah, 2001). The talc based formulation of *P. fluorescens* ($1x10^8$ CFU/g) was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India for the evaluations.

Influence of *P. fluorescens* and HaNPV against *H. armigera* on cotton

The plants (cv: LRA 5166) were tested for integration assays with HaNPV using leaf discs (30 DAS), squares (45 DAS) and bolling stages (70 DAS). Leaf discs (3.3 cm dia) were prepared using a template. Aliquots of 10 µl of the HaNPV at a conc. of 6×10^6 POB/ml in 0.01% teepol were applied to the centre of the leaf discs and smeared uniformly using the blunt end of a glass rod (6mm). Treated discs were air dried and transferred to cheese cups (basal dia 7.5 cm, top dia 8.5 cm, height 6 cm) lined with moistened filter paper. In experiments involving cotton squares and bolls, HaNPV at a conc. of 6×10^6 POB/ml was applied with a hand atomizer and air dried. The treated parts were transferred to disposable cups (6 x 5 cm) at the rate of one per cup. Five, third instar larvae (*ca* 7 days old) of uniform size were released per disc or fruiting part. Each treatment was replicated thrice with 30 larvae per treatment. After 24 hours of feeding, the larvae were transferred to glass vials (5 ml) filled with semi-synthetic diet (lacking formaldehyde) and held at 25 ± 0.5 °C. Developmental parameters and mortality were recorded from 3 to 10 days after treatment.

The data obtained from different experiments were subjected to the analysis of variance and correlations in IRRISTAT ver. 3.1., Biometric unit, IRRI, Philippines and the means were separated using Duncan's new Multiple Range Test.

Biochemical assays

The plant parts were assayed for changes in the activity of phenol (Malick and Singh, 1980) and tannin content (Schanderl, 1970), peroxidase (POD) (Hammerschmidt, Nuckles and Kue, 1982), polyphenol oxidase (PPO) (Mayer *et al.*, 1965) and phenylalanine ammonia lyase (PAL) activity (Dickerson *et al.*, 1984) at leaf (30 DAS), square (45 DAS) and bolling stages (70 DAS) and each treatment was replicated five times. Regression and correlation analysis were carried out on the data.

RESULTS AND DISCUSSION

Influence of *P. fluorescens* and HaNPV against *H. armigera* on cotton

The *P. fluorescens* induced plant resistance in cotton leaf, square and bolls on development parameters of *H. armigera* revealed significant reduction in weight of larvae (leaf: F = 73.68, df = 9, cv = 2.6%; square: F = 129.77, df = 9, cv = 2.2%; boll: F = 129.77, df = 9, cv = 1.7%) and pupae (leaf: F = 39.20, df = 9, cv = 3.0%; square: F = 82.68, df = 9, cv = 2.6%; boll: F = 195.82, df = 9, cv = 1.8%) in seed treatment (ST) + foliar application (FA) of *P. fluorescens* + HaNPV and ST + FA of *P. fluorescens* followed by ST, FA either alone or in combination with HaNPV (Tables 1, 2 and 3). In this evaluation, the leaf area consumption decreased significantly ($P<0.01$) in all treatments involving *P. fluorescens* and its combination with HaNPV (38.67 – 52.67 mm^2) except, application of HaNPV, FA of *P. fluorescens* or combination of both and untreated check (68.33 – 72.00 mm^2) (Table 1).

A positive correlation was obtained between the leaf area consumed and larval weight 7 DAT ($R^2 = 0.9186$; $y = 3.002x + 148.78$; $n = 30$) and weight of surviving pupae ($R^2 = 0.844$, $y = 128.14 + 1.829x$, $n = 30$). Bong and Sikorowski (1991) showed that *P. maltophila* affected the growth of larval instars of *H. zea*, with reduced pupal and adult sizes leading to over 60 per cent reduction in adult emergence. Similarly, the growth rate, consumption rate and digestibility of feed by *H. armigera* were affected when larvae fed on cotton plants treated with *P. gladioli* (Qingwen *et al.*, 1998). Reduction in growth and development of the rice leaf folder by *P. fluorescens* induced systemic resistance was also reported by Radjacommare *et al.* (2002). *H. armigera* is a voracious feeder of economically important plants and has been shown to acquire weight when it feeds on favoured crop plant parts (Dhandapani and Balasubramanian, 1980). Integration of *P. fluorescens* might have rendered the plant parts less favourable to *H. armigera* for feeding even in the susceptible cultivar LRA 5166. The results suggest that *P. fluorescens* stimulated ISR by ST and FA (15 DI) in sequence and the combination with HaNPV interfered with the feeding by test larvae leading to weight reduction (Tables 1, 2 and 3). The percentage of larval population moving to pupation was influenced by HaNPV applied to leaves ($P<0.01$) squares ($P<0.01$) and bolls ($P<0.01$) than by *P. fluorescens* (Tables 1, 2 and 3) indicating that *P. fluorescens* plays only an indirect role in fitness of the population *vis a vis* suitability of feeding substrates and is not a determinant of the survival of the individuals.

Table 1. **Influence of sequential and simultaneous treatment of *P. fluorescens* and HaNPV on cotton (LRA 5166): Growth parameters and mortality of *H. armigera* fed on leaves**

Treatments[$]	Leaf area fed (mm^2)	Larval weight - 7DAT (mg)	Pupal weight (mg)	Pupation (%)	Larval mortality (%)
ST + *Pf*@ 1g/100g (T_1)	52.67^{d}	306.33^{b}	235.33^{c}	96.67^{cf}	0.00
FA of *Pf*@ 1% at 15 DI (T_2)	51.33^{cd}	310.67^{b}	234.67^{c}	98.33^{cf}	0.00
ST (1%) + FA (1%) of *Pf* (T_3)	42.00^{b}	277.33^{a}	199.00^{a}	95.00^{c}	0.00
FA of *Pf*@ 1% on DE (T_4)	70.33^{ef}	374.00^{d}	258.00^{d}	100.00^{f}	0.00
T_1 + HaNPV* (T_5)	49.00^{c}	297.33^{b}	218.00^{b}	33.33^{bc}	65.00^{ab}
T_2 + HaNPV* (T_6)	49.33^{cd}	298.00^{b}	219.00^{b}	36.67^{cd}	63.75^{ab}
T_3 + HaNPV* (T_7)	38.67^{a}	269.00^{a}	190.27^{a}	38.33^{d}	60.00^{b}
T_4 + HaNPV* (T_8)	68.33^{e}	354.00^{e}	246.33^{cd}	31.67^{ab}	68.75^{a}
HaNPV* alone (T_9)	71.67^{ef}	342.67^{e}	241.67^{c}	28.33^{a}	70.00^{a}
Untreated check (T_{10})	72.00^{f}	389.67^{e}	272.90^{e}	100.00^{f}	0.00

* Virus applied @ 6 x 10^6 POB/ml

** In a column means followed by similar letters are not significantly different ($p<0.05$) by DMRT

[$] Mean of three replications, n=30

ST: Seed treatment FA: Foliar application DI: Days interval

DE: Day of experiment *Pf* - *P. fluorescens*

Investigations on the joint action of *P. fluorescens* and HaNPV against *H. armigera* revealed antagonism (Tables 1, 2 and 3). The mortality of *H. armigera* caused by HaNPV was significantly low in ST + FA of *P. fluorescens* than HaNPV without *P. fluorescens* integration (70.00, 77.50 and 81.25%) or HaNPV integrated with *P. fluorescens* by simultaneous application (68.75%, 76.25 and 72.50) or in sequence with *P. fluorescens* as ST alone (65.00, 72.50 and 70.00%) or FA alone (63.75, 70.00 and 67.50%) in leaves ($P<0.05$) squares ($P<0.05$) and bolls ($P<0.05$) respectively. Treatment of *P. fluorescens a priori* (ST+FA) proved to be detrimental for HaNPV performance. The reduction in NPV induced mortality on crop plants in general, have resulted from direct antagonism between leaf characteristics and microbes (Kushner and Harvey, 1962), altered effectiveness of infection barriers such as the biochemical environment of the midgut lumen and the peritrophic membrane (Paschke and Summers, 1975) or physiological stress which inhibited resistance at cellular level (Keating, Hunter and Schultz, 1988). One of the factors in realization of the effectiveness of NPVs in general, is the acquisition of the viral occlusion bodies in lethal doses through *per os* route. In this investigation, HaNPV caused mortality correlated positively with leaf area fed ($R^2 = 0.9615$, $y = 49.989 + 0.28x$, $n = 15$). Rabindra, Sathiah and Jayaraj (1992) reported that plant resistance interfered with uptake of HaNPV in chickpea and there was positive correlation between extent of leaf area fed and HaNPV caused mortality against *H. armigera* on chickpea.

Table 2. Influence of sequential and simultaneous treatment of *P. fluorescens* and HaNPV on cotton (LRA 5166): Growth parameters and mortality of *H. armigera* fed on squares

Treatments[$]	Larval weight -7DAT (mg)	Pupal weight (mg)	Pupation (%)	Larval mortality (%)
ST + *Pf* @ 1g/100g (T_1)	300.67[b]	224.67[b]	98.33[a]	0.00
FA of *Pf* @ 1% at 15 DI (T_2)	303.00[b]	221.33[b]	100.00[a]	0.00
ST (1%) + FA (1%) of *Pf* (T_3)	274.00[a]	192.67[a]	96.67[a]	0.00
FA of *Pf* @ 1% on DE (T_4)	378.00[d]	269.00[cd]	100.00[a]	0.00
T_1 + HaNPV* (T_5)	293.67[b]	215.67[b]	26.67[b]	12.50[ab]
T_2 + HaNPV* (T_6)	292.33[b]	214.00[b]	30.00[b]	10.00[a]
T_3 + HaNPV* (T_7)	265.67[a]	186.67[a]	28.33[b]	07.50[a]
T_4 + HaNPV* (T_8)	355.00[c]	260.00[c]	31.67[b]	06.25[a]
HaNPV* alone (T_9)	361.67[c]	264.00[cd]	21.67[a]	17.50[b]
Untreated check (T_{10})	392.00[e]	273.00[d]	100.00[a]	0.00

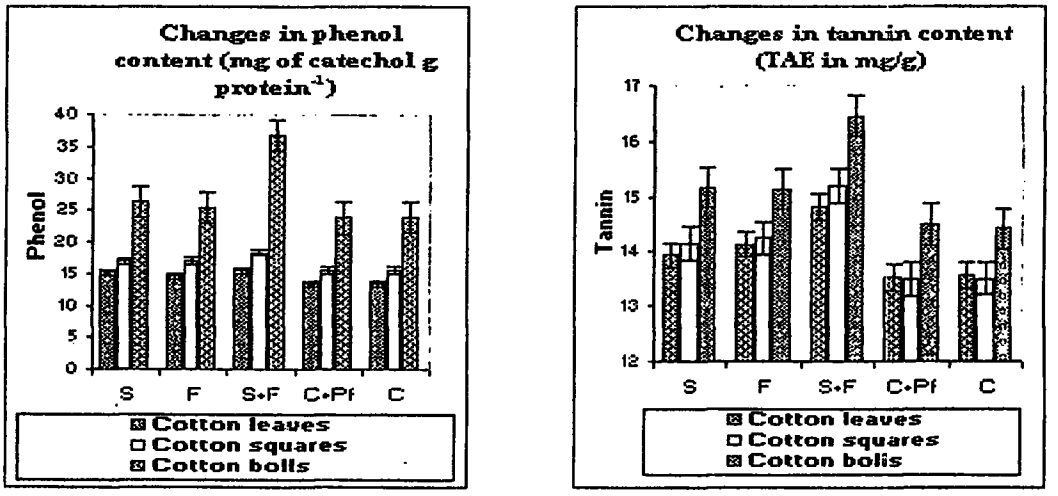
* Virus applied @ 6×10^6 POB/ml

** In a column means followed by similar letters are not significantly different ($p<0.05$) by DMRT

$ Mean of three replications, n= 30

ST: Seed treatment FA : Foliar application DI: Days interval

DE: Day of experiment DAT: Days after treatment *Pf* - *P. fluorescens*



Mortality (%)

S - Seed treatment: F - Foliar Application: S+F - Seed+Foliar treatment:

C+Pf - Foliar application of Pf on the day of experiment: C – Control

Pf - *P. fluorescens*

Fig. 1. Changes in biochemical constituents of *P. fluorescens* treated cotton: Levels of phenol and tannin

Table 3. Influence of sequential and simultaneous treatment of *P. fluorescens* and HaNPV on cotton (LRA 5166): Growth parameters and mortality of *H. armigera* fed on bolls

Treatments[s]	Larval weight - 7 DAT (mg)	Pupal weight (mg)	Pupation (%)	Larval mortality (%)
ST + *Pf*@ 1g/100g (T_1)	290.33[b]	213.00[d]	96.67[de]	0.00
FA of *Pf*@ 1% at 15 DI (T_2)	291.33[b]	209.00[d]	98.33[de]	0.00
ST (1%) + FA (1%) of *Pf* (T_3)	261.67[a]	187.00[b]	93.33[d]	0.00
FA of *Pf*@ 1% on DE (T_4)	378.00[d]	256.67[f]	100.00[e]	0.00
T_1 + HaNPV* (T_5)	282.33[b]	201.67[c]	30.00[b]	70.00[b]
T_2 + HaNPV* (T_6)	283.00[b]	200.67[c]	31.67[b]	67.50[bc]
T_3 + HaNPV* (T_7)	253.67[a]	179.00[a]	36.67[c]	62.50[c]
T_4 + HaNPV* (T_8)	330.00[c]	249.33[e]	26.67[b]	72.50[b]
HaNPV* alone (T_9)	337.33[c]	260.33[ef]	18.33[a]	81.25[a]
Untreated check (T_{10})	390.67[e]	266.33[f]	100.00[e]	0.00

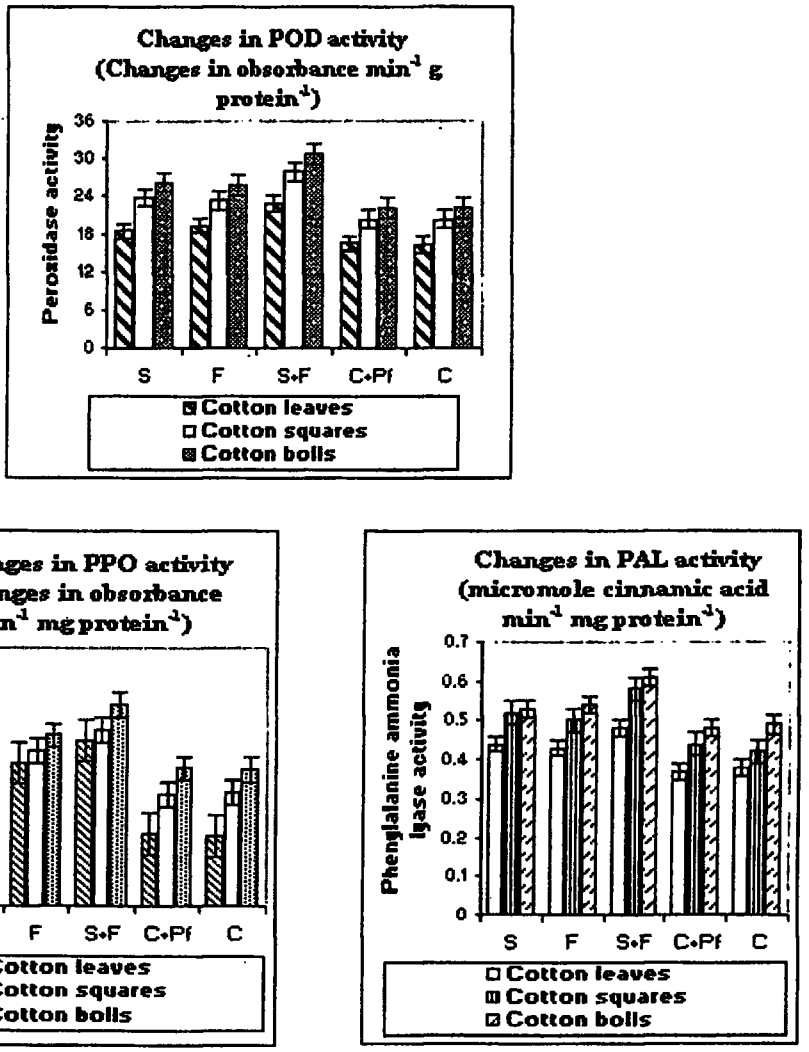
* Virus applied @ 6 x 10^6 POB/ml
** In a column means followed by similar letters are not significantly different ($p<0.05$) by DMRT
s Mean of three replications, n=30
ST: Seed treatment FA : Foliar application DI: Days interval
DE. Day of experiment DAT: Days after treatmen *Pf* - *P. fluorescens*

Biochemical assays

The phenol and tannin content and the peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activities in leaves, squares and bolls showed significant differences between *P. fluorescens* included and excluded conditions. The content of metabolites and defense related enzyme oxidase and lyase activities were significantly higher in treatment involving ST followed by FA of *P. fluorescens* consistently than other treatments (Figures 1 and 2). Ability of the *P. fluorescens* to induce defense related enzymes like peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and chemicals like phenols and tannins in tomato and hot pepper was reported by Ramamoorthy, Raguchander and Samiyappan (2002). The correlation analysis revealed significant negative relationship of mortality with biochemical constituents. Tannin content (R^2 = 0.944), POD (R^2 = 0.956) and PAL (R^2 = 0.928) activity in leaves were found negatively correlated with larval mortality due to HaNPV, while phenol content (R^2 = 0.945) in squares, and PPO activity in squares (R^2 = 0.9384) and bolls (R^2 = 0.946) were negatively correlated with larval mortality (Table 4)

Decreased pathogenicity of *Lymantria dispar* NPV (LdNPV) was reported on oak foliage due to increased hydrolysable tannin content of the leaf material (Keating, Yendol and Schultz, 1988) and aggregation of LdNPV viral occlusion bodies by the leaf phenolic contents (Keating, Hunter and Schultz, 1990) was also reported. Felton and Duffey (1990) found that the quinones formed by the action of PPO in insect damaged plant tissues of tomato covalently bonded to the occlusion bodies of HzSNPV and reduced their digestibility and solubility under alkaline conditions. Similarly, a negative correlation between percentage mortality and biochemical factors like foliar pH, phenols, tannins and protein

binding capacity of chickpea, tomato, redgram, cotton and bhendi were established against *H. armigera* by Ramarethinam *et al.* (1998). In our studies, the antagonism observed between *P. fluorescens* and HaNPV is not direct as simultaneous treatment of HaNPV+*Pf*-FA (DE) caused similar mortalities as that of HaNPV alone in leaves, squares and bolls assay. (Tables 1, 2 and 3). *P. fluorescens* induced systemic resistance in cotton plants, prior to experimentation has led to altered biochemical status and rendered the plant parts less suitable for *H. armigera* feeding. As a consequence, there was reduced intake of the viral occlusion bodies and corresponding decrease in larval mortality in ST + FA of *P. fluorescens* and HaNPV treatment.



S - Seed treatment; F - Foliar Application; S+F - Seed+Foliar treatment:
C+*Pf* - Foliar application of *Pf* on the day of experiment; C - Control
Pf - *P. fluorescens*

Fig. 2. Changes in biochemical constituents of *P. fluorescens* treated cotton: Enzyme activity

Table 4. Analysis of the relationship between larval mortality due to HaNPV and biochemical constituents in cotton (LRA 5166) treated with *P. fluorescens*

Particulars	R^2	Intercept	Regression co-efficient	Standard error	t-value
Phenol					
Leaf	0.86 NS	120.02	-3.801	1.315	-3.151 NS
Square	0.945*	131.15	-3.500	0.656	-5.521*
Boll	0.857 NS	218.63	-5.805	1.6725	-3.471 NS
Tannin					
Leaf	0.944*	168.67	-7.373	1.270	-5.815*
Square	0.886 NS	153.28	-5.713	1.444	-3.952 NS
Boll	0.737 NS	182.64	-7.389	3.192	-2.668 NS
POD					
Leaf	0.956*	93.012	-1.4782	0.293	-5.130*
Square	0.880 NS	101.56	-1.246	0.369	-3.379 NS
Boll	0.889 NS	125.83	-2.119	0.527	-4.019 NS
PPO					
Leaf	0.881 NS	75.44	-7.096	2.151	-3.705 NS
Square	0.9384*	93.37	-12.424	2.269	-5.888*
Boll	0.946*	121.05	-25.529	4.281	-5.962*
PAL					
Leaf	0.928*	101.35	-85.366	13.858	-6.401*
Square	0.833 NS	106.30	-67.5	21.360	-3.160 NS
Boll	0.843 NS	149.46	-145.903	44.456	-3.281 NS

NS – Non significant; * significant at p = 0.05; n=20

CONCLUSIONS

Important constraints in the use of nucleopolyhedroviruses for pest control include loss of efficacy due to plant mediated chemicals and reduced intake of virus when larvae experience feeding difficulties. This is a set back in integrating plant resistance and this biopesticide. *P. fluorescens* in Indian situation is gaining importance in view of its control potential and ISR against plant diseases and phytoparasitic nematodes. Rabindra and Jayaraj (1988) advocated the use of phagostimulants for NPVs in suppressive environments. This study enlarges the scope of using compounds with organoleptic and buffering principles that enhance acquisition of the viral occlusion bodies and retention of viral activity in altered biochemical environment in insect gut. Formulation of nucleopolyhedroviruses with additives like PPO inhibitors, quinone trapping agents and antioxidants could also offer solutions for incompatibility.

ACKNOWLEDGEMENTS

The authors are grateful to Tamil Nadu Agricultural University, Coimbatore, India for providing the research facilities. The authors are thankful to Dr.R.Samiyappan, Professor of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India for the supply of *Pseudomonas fluorescens*.

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