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# PLANT GROWTH PROMOTING RHIZOBACTERIA OF COTTON AFFECTING THE DEVELOPMENTAL STAGES OF *HELICOVERPA ARMIGERA*

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**Abstract:** Rhizobacteria isolated from cotton roots exhibiting antagonism towards seedling blight and leaf blight of cotton were tested for their efficiency against the insect pest American bollworm (*Helicoverpa armigera*). The bioformulation developed using *Bacillus subtilis* (isolate DGL9) + chitin was found to be detrimental to the developmental stages of *H. armigera* (larva, pupa and adult) by causing larval mortality, pupal and adult malformation with reduced adult emergence. Generally, the larvae exhibited antifeeding behaviour when fed on bolls collected from rhizobacterial treatments. Hence, the developmental stages were altered leading to early pupation. Further, the efficacy of the isolate DGL9 was confirmed by culturing the bacteria in a suitable medium and incorporating the cell suspension and supernatant obtained form the broth culture in larval diet. The larvae fed to the diet exhibited defective developmental stages which was more significant in case of diet incorporated with supernatant. The percentage of pupal malformation, adult emergence and adult malformation was high at 96 h of incubation with the supernatant.

**Key words:** cotton, *Helicoverpa armigera*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, bioformulation

## INTRODUCTION

Members of the genus *Helicoverpa* (Mathews 1991) are pests of world wide significance. There are 60 cultivated species and 67 other species which serve as host plants for *H. armigera* (Reed and Pawar 1982). *H. armigera* is the predominant species in peninsular India which is widely distributed throughout Africa, Middle East, Southern Europe, Central and South East Asia, New Zealand and many Eastern Pacific Islands (Filt 1989). The wide host range allows the pest to breed throughout the year in India leading to extensive and continued damage (Regupathy *et al.* 1997) and it had become India's major agricultural pest (Regupathy *et al.* 2003).

More than 75 per cent of the insecticides used in cotton are targeted towards *H. armigera* of which, synthetic pyrethroids constitute 50–70 per cent (Jayaswal 1989) and this high selection pressure has led to the development of resistance. Resistance in *H. armigera* to commonly used insecticides is one of the major constraints in cotton production in Tamil Nadu (Regupathy *et al.* 1999).

The damage of *H. armigera* is identified by the presence of irregular holes in ovaries, squares and young flower buds and excreta near the bore holes. The newly hatched larvae feed on the leaves, flower buds or flowers which are eventually hollowed out. The growing larva prefers buds and young bolls. The boll worms thrust their head inside the bore holes, with granular faecal pellets outside the bore holes (Regupathy *et al.* 1998).

Rhizobacteria have been studied as plant growth promoters for agricultural production as well as biocontrol agents against both diseases and pests (Kloepper and Beauchamp 1992). Zehnder *et al*. (1997b) reported that cucumber beetles (*Diabrotica undecimpunctata* Howardi) transmitting the bacterial wilt disease *Erwinia tracheiphila* when fed to cucumber plants treated with PGPR strains in greenhouse experiments exhibited reduced consumption of stems and cotyledons leading to reduced wilt severity compared to non treated plants. Delivery of *Pseudomonas fluorescens* through seed, soil, root or foliage reduced the incidence of both sheath blight and leaf folder in rice. Moreover, the leaffolder infested plants treated with *P. fluorescens* induced proteins with molecular weight of 41 kDa that were inhibitory to the insect (Radjacommare *et al.* 2002). With this prelude an investigation was made to control bollworm infestation on cotton utilising rhizobacteria from cotton which were earlier found to be antagonistic to root rot and leaf blight pathogens.

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## MATERIALS AND METHODS

**Preparation of talc – based formulation of plant growth promoting rhizobacterial (PGPR) strains of cotton**

Based on earlier studies *in vitro*, among the 60 rhizobacterial isolates collected from cotton growing regions of Tamil Nadu , India, the isolates DGL9 (*B. subtilis*) and K4 (*Pseudomonas aeruginosa*) were found to be highly inhibitory to the growth of *Rhizoctonia solani* that causes seedling blight and *Alternaria macrospora* which incites leaf blight. These PGPR isolates were used in to study their effect on *H. armigera*. *P. fluorescens* (Pf1) used for comparison was obtained from the Department of Plant Pathology, TNAU, Coimbatore. A loopful of bacterium from culture was inoculated either into nutrient or KB broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28±2°C). After incubation the broth containing $9x10^8$ cfu/ml was used for the preparation of talc – based formulation. To 400 ml of bacterial suspension, 1 kg of talc (sterilized at 105°C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxy methyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, following the method of Vidhyasekaran and Muthamilan (1995). After drying in shade overnight the formulation was packed in polypropylene bag and sealed. At the time of application the population of bacteria in talc formulation was 2.5 to $3.0x10^8$ cfu/g.

**Chitin amendment with talc – based formulations**

Five g of crab shell chitin (Sigma, USA) was slowly added to 100 ml of cold 0.25N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glass wool into 200 ml of ice cold ethanol at 4°C with rapid stirring. The resultant chitin suspension was centrifuged at 10,000 rpm for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral. One ml of colloidal chitin was added to 100 ml of KB or nutrient broth containing $9x10^8$ cfu/ml of the PGPR. After 48 h of incubation it was used for the preparation of talc based formulation as described above.

**Mass rearing of *H. armigera***

Second instar larvae of *H. armigera* were obtained from the Department of Biocontrol, Tamil Nadu Agricultural University and reared on chickpea based semisynthetic diet (Sathiah 2001). The sterilized semi synthetic diet was dispensed aseptically into sterile glass vials (5 ml). The diet was also poured into sterile plastic trays (30x15x5 cm). The hot diet in the glass vials and plastic trays were allowed to solidify and cool to room temperature under aseptic conditions. The second instar larvae were released into the plastic trays and trays were kept in an inverted position. The larvae were allowed to grow till third instar and used for further studies.

**Effect of rhizobacterial isolates against *H. armigera in vitro***

The talc based formulations containing the bacterial isolates with or without chitin were assessed for their efficacy against bollworm on cotton variety LRA 5166 in glasshouse. The crop was raised in 20 cm-diameter pots containing the potting mixture (red soil: sand: farm yard manure, 1:1:1 w/w/w). Foliar spray was given at 90 days (boll formation stage) after sowing with talc based formulation at 0.5% dissolved in water and endosulfan was used for comparison The treatment details were: T1 – *P. aeruginosa*. (K4), T2 – *B. subtilis*. (DGL 9), T3 – *P. fluorescens* (Pf1), T4 – *P. aeruginosa* spp. (K4) + chitin, T5 – *B. subtilis*. (DGL 9) + chitin, T7 – chitin, T8 – endosulfan (0.05%), T9 – Control (without spray treatment).

Cotton bolls were collected one day after treatment spray and bolls of uniform size from each treatment were placed inside ten plastic cups individually. The treatments were replicated three times. The third instar larvae were starved for 2 h and released inside each plastic cup and allowed to feed on the bolls. Larval mortality was recorded after 24 h and observations were continued till adult emergence.

**Influence of incubation period of rhizobacteria on developmental stages of *H. armigera***

The *B. subtilis* isolate DGL 9 was inoculated in nutrient broth. After 0, 24, 48, 72, 96, 120 and 150 h of incubation the bacterial culture was centrifuged at 6,000 rpm for 15 min. The bacterial cells were suspended in sterile distilled water and the cell concentration was estimated at 595 nm. Ten microliters of the bacterial cell suspension was added to the glass vials and smeared on the diet surface. After 15 min third instar larvae were released individually inside the vial and plugged tightly with cotton. Similarly, the supernatant obtained after centrifugation of the bacterial pellet was also added to the diet, 10 µl/vial, and tested for its efficacy. The larvae were held in the vial till pupation. The larval mortality was observed after 24 h and observations were continued till adult emergence. Each treatment was replicated three times and ten larvae were used per replication.

## RESULTS

**Effect of rhizobacterial isolates against *H. armigera in vitro***

When bolls collected from the treatments imposed on cotton plants were fed to larvae of *H. armigera* the feeding rate was affected considerably in endosulfan (0.05%) and rhizobacterial treatments. The feeding behaviour indicated a general antifeeding effect on the larvae. The larval mortality recorded was higher for chemical treatment (89.93 %) followed by DGL9 + chitin (16.67 %) and DGL9 (15.00 %) (Table 1). There was a considerable reduction in pupal weight and the chemical treatment recorded 0.10 g as against 0.28 g in control. The pupal weight in DGL9 + chitin was 0.16 g. An average of 10.17% and 8.24% pupal malformation was recorded for endosulfan and DGL9 + chitin treatments, respectively. Adult emergence was not noticed for chemical treatment whereas DGL9 + chitin recorded an emergence of 80.83%. There was 100% emergence from K4 and Pf1 (with or without chitin amendment) and control but there were few cases of pupal and adult malformations in K4 + chitin and Pfl + chitin.

Table 1. Effect of bioformulations against *H. armigera in vitro*

| S. No. | Treatment | Larval mortality* [%] | Pupal weight* [g] | Pupal malformation* [%] | Adult emergence* [%] | Adult malformation* [%] |
|---|---|---|---|---|---|---|
| 1 | K4 | 0.00 d (0.47) | 0.26 f | 0.00 f (0.46) | 100.00 a (89.12) | 0.00 e (0.47) |
| 2 | DGL9 | 15.00 c (22.78) | 0.17 c | 6.00 c (14.17) | 82.50 b (65.27) | 19.19 b (25.98) |
| 3 | PF1 | 0.00 d (0.47) | 0.27 g | 0.00 f (0.46) | 100.00 a (89.12) | 0.00 e (0.47) |
| 4 | K4 + chitin | 0.00 e (0.47) | 0.24 d | 1.67 e (7.42) | 100.00 a (89.12) | 4.16 c (11.76) |
| 5 | DGL 9 + chitin | 16.67 b (24.09) | 0.16 b | 8.24 b (16.68) | 80.83 c (64.03) | 25.25 a (30.16) |
| 6 | Pf1 + chitin | 0.00 d (0.47) | 0.25 e | 2.50 d (9.20) | 100.00 a (89.12) | 3.33 d (10.51) |
| 7 | Chitin | 0.00 d (0.47) | 0.25 e | 0.00 f (0.46) | 100.00 a (89.12) | 0.00 e (0.47) |
| 8 | Endosulfan (0.05 %) | 89.93 a (72.47) | 0.10 a | 10.17 a (18.59) | 0.00 d (0.47) | 0.00 e (0.47) |
| 9. | Control | 0.00 d (0.47) | 0.28 h | 0.00 f (0.46) | 100.00 a (89.12) | 0.00 e (0.47) |

*mean of the three replications. In each replication ten larvae were used
In a column, means followed by same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test
Figures in parentheses are arcsine transformed values

**Influence of cell suspension and supernatant of DGL9 (*B. subtilis*) on developmental stages of *H. armigera***

Cell suspension and supernatant of bacteria were added to the larval diet and the developmental stages were observed. The larval mortality was 17.50% in diet treated with supernatant from 72 h of incubation followed by 96 h (16.67%) (Table 2). There was reduction in pupal weight up to 0.11 g and 0.16 g in supernatant from 96 h and 72 h, respectively. There was a considerable percentage of pupal malformation (13.00) and adult malformation (21.05) at 96 h of incubation with the supernatant compared to other incubation periods. In case of diet treated with cell suspension, on comparing all the incubation periods, the larval mortality, pupal and adult malformation were considerable at 96 h wherein the adult emergence was 92.50%. Between cell suspension and supernatant, the latter was more detrimental to the bollworm development.

Table 2. Influence of cell suspension and supernatant of *B. subtilis* (DGL9) on developmental stages of *H. armigera*

| Incubation period [h] | *Bacillus subtilis* (DGL9) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | cell suspension* | | | | | supernatant* | | | | |
| | larval mortality [%] | pupal weight [g] | pupal malformation [%] | adult emergence [%] | adult malformation [%] | larval mortality [%] | pupal weight [g] | pupal malformation [%] | adult emergence [%] | adult malformation [%] |
| 0 | 0.00 e | 0.30 f | 0.00 d | 100.00 a | 0.00 e | 0.00 f | 0.30 f | 0.00 f | 100.00 a | 0.00 g |
| 24 | 0.00 e | 0.29 e | 0.00 d | 100.00 a | 0.00 e | 0.00 f | 0.21 d | 0.00 f | 88.33 d | 3.33 f |
| 48 | 1.67 d | 0.25 c | 1.67 c | 96.67 c | 4.24 c | 10.00 c | 0.17 c | 2.77 d | 90.00 c | 5.66 d |
| 72 | 7.50 a | 0.23 b | 2.50 b | 91.67 e | 8.18 b | 17.50 a | 0.16 b | 10.10 b | 79.17 e | 14.58 b |
| 96 | 5.83 b | 0.22 a | 4.17 a | 92.50 d | 12.61 a | 16.67 b | 0.11 a | 13.00 a | 78.33 f | 21.05 a |
| 120 | 2.50 c | 0.28 d | 2.50 b | 97.50 b | 3.42 d | 9.17 d | 0.22 e | 6.42 c | 92.50 b | 12.96 c |
| 150 | 0.00 e | 0.29 e | 0.00 d | 100.00 a | 0.00 e | 7.50 e | 0.22 e | 1.80 e | 100.00 a | 4.50 e |
| Control | 0.00 e | 0.31 g | 0.00 d | 100.00 a | 0.00 e | 0.00 f | 0.30 f | 0.00 f | 100.00 a | 0.00 g |

*mean of the three replications. In each replication ten larvae were used
In a column, means followed by same letter do not differ significantly ($p < 0.05$) according to Duncan's Multiple Range Test
For statistical calculations arcsine transformed values were used

## DISCUSSION

The results of the study indicated that when DGL9 + chitin and DGL9 treated bolls were fed to larvae of *H. armigera* it led to larval mortality. The larvae fed on bolls collected from rhizobacterial treatments showed antifeeding behaviour. Hence, the developmental stages were altered leading to early pupation. The treatment DGL9 + chitin caused low pupal weight with increased pupal and adult malformations. However, the larval mortality was high in endosulfan treatment where the emerging adults were absent. Similar results were obtained by Zehnder *et al.* (1997a, 1997b) wherein the PGPR treated cucumber plants showed reduced consumption by cucumber beetles with reduction in the percentage of wilted vines both in the glasshouse and field experiments. The altered feeding behaviour of the insects was due to reduction in the palatability. The antifeeding behaviour in the PGPR treatments was due to the reduction in the feeding stimulant

i.e., synthesis of cucurbitacin caused by alteration in the plant metabolic pathway which elicited the induction of plant defense compounds (Zehnder *et al*. 2001).

*H. armigera* fed on *Pseudomonas gladioli* treated cotton plants resulted in reduction of relative growth rate, consumption rate and digestibility of feed (Qingwen *et al.* 1998). Radjacommare (2000) recorded lower incidence of sheath blight and leaffolder in rice treated with PGPR strains cultured in chitin amended medium. Radjacommare *et al.* (2002) also demonstrated that rice leaves treated with *P. fluorescens* altered the feeding behaviour of leaffolder with reduction in larval and pupal weight. Increased larval mortality and incidence of malformed adults were also recorded *in vitro*. In order to analyse the possible cause behind the antifeeding behavior, particularly to correlate the involvement of the bacterial cells or the secondary metabolites produced by the bacterium in culture media leading to mortality and malformation, the study was carried out using the cell suspension and the supernatant of 24 h old culture of *B. subtilis* (DGL9). Cell suspension and supernatant of DGL9 isolate were analysed for their effect on the developmental stages of *H. armigera* by incorporating them in semisynthetic diets. The supernatants obtained from different incubation periods were more effective than cell suspensions in causing larval mortality. The larvae deterred from feeding for 4–5 days and were forced to feed upon starvation leading to alterations in developmental stages. The pupal weight and adult emergence was low at 96 h with more pupal and adult malformations. The treatment with cell suspension obtained, at 72 h and 96 h caused more larval mortality which might be attributed to increased bacterial cell concentration. Bong and Sikorowski (1991) had observed that the diet contaminated with the bacterium *P. maltophila* not only resulted in 60% reduction in adult emergence but also recorded higher pupal and adult malformations. Chestnut sprayed with *P. fluorescens* resulted in 20% weevil mortality (Yaman *et al*. 1999). Biological control of pests using bacteria has been demonstrated by several other researchers (Fahey *et al.* 1991; Racke and Sikora 1992; Zehnder *et al.* 1997a, 1997b; Rajendran 2003). Reduction in consumption of boll tissues of cotton treated with bioformulation of *Bacillus* by *H. armigera* has been observed (Bhuvaneswari 2005). The reason attributed to the enhanced activities of PGPR as observed in the present study was due to the increased multiplication of PGPR and enhanced activity of chitinase in chitin amended formulations.

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## POLISH SUMMARY

### WPŁYW BAKTERII KORZENIOWYCH STYMULUJĄCYCH WZROST ROŚLIN (PGPR) Z BAWEŁNY NA ROZWÓJ *HELICOVERPA ARMIGERA*

Podczas badań testowano ryzobakterie wyizolowane z korzeni bawełny, które wykazują antagonizm w stosunku do zarazy rozsad i liści bawełny. Bakterie badano pod względem ich skuteczności przeciwko owadziemu szkodnikowi bawełny, amerykańskiej słonecznicy orężówce (*Helicoverpa armigera*). Biopreparaty sporządzone z użyciem *Bacillus subtilis* (izolat DGL9) + chityna okazały się szkodliwe dla stadiów rozwojowych *Helicoverpa armigera* (larwy, poczwarki oraz osobniki dorosłe) powodując śmiertelność larw, deformacje u poczwarek i dorosłych osobników oraz ograniczenie pojawiania się tych ostatnich. Ogólnie larwy wykazywały zachowanie antyfidantne w czasie żerowania na bawełnie traktowanej preparatem. Stadia rozwojowe ulegały zatem zmianom powodując przyspieszenie przepoczwarzania. Skuteczność izolatu DGL9 potwierdzono poprzez hodowlę bakterii w odpowiednim medium i wprowadzeniu do pożywienia larw zawiesiny komórek i supernatantu otrzymanego z bulionu z hodowli. Larwy żerowały na takim pożywieniu wykazując deformacje w stadiach rozwojowych, które były znaczniejsze w przypadku wprowadzenia supernatantu. Procent zdeformowanych poczwarek była wysoka po 96 godzinach inkubacji z supernatantem.