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Combined application of chitinolytic bacterium *Paenibacillus* sp. D1 with low doses of chemical pesticides for better control of *Helicoverpa armigera*

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ABSTRACT

Helicoverpa armigera is a serious pest of *Cajanus cajan* in many parts of world. Rapid development of resistance against number of insecticides and cry toxin-based biocontrol agents has led to search for biocontrol agents with alternative mode of action. The ability of chitinolytic bacteria to degrade vital chitinous structure in insects suggests their potential in insect control. The present investigation was carried out to study insect control potential of a high chitinase producing bacterium, *Paenibacillus* sp. D1. Biocontrol studies with Helicoverpa larvae showed *Paenibacillus* sp. D1 and its chitinase to be potent antifeedant that reduced the feeding rate and body weight of the larvae. The decreased body weight was attributed to hydrolysis of the chitinous structures of the larvae. This was evident from decrease in the total chitin content and increased mortality of the larvae fed on the leaves treated with *Paenibacillus* sp. D1 and chitinase as compared to untreated controls. A combined dose of *Paenibacillus* sp. D1 or its chitinase with an organophosphate insecticide, acephate, was found to be more lethal than their individual treatments suggesting integrated insect control potential of the bacterium.

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Biocontrol; chitinase; *Helicoverpa armigera*; *Paenibacillus* sp. D1; synergism

Introduction

India is one of the major pigeon pea (*Cajanas cajan*) producers in the world accounting for almost 90% of the total world production. It is mainly consumed as dehulled splits (dhal) and is one of the important sources of protein in the diet, especially, of vegetarian population. Among 200 species of insects known to live and feed on this crop, *Helicoverpa armigera* (Hübner), commonly known as "legume pod borer" is the major cause of crop loss in India. Pigeon pea crop losses due to *Helicoverpa* can range from 20% to 100% on farmers' fields and are estimated to add up to more than US$310 million annually worldwide (http://www.icrisat.org/what-we-do/agro-ecosystems/aes-rb-pest-management.htm). *Helicoverpa armigera* has several key physiological and ecological characteristics which facilitate its survival in unstable environments. It is cosmopolitan, multivoltine, polyphagus, highly motile, exhibits fecundity and facultative diapauses (Fitt 1989). Moreover, *H. armigera* is relatively safe from natural enemies because of the cryptic feeding habits of the larvae within pods and protection of pupae in the soil (Binod et al. 2007). Conventionally, this pest is controlled by chemical insecticides. However, extensive and often indiscriminate use of insecticides has not only led to rapid development of resistance in these pests towards number of pesticides, but also has resulted in environmental degradation and adverse effects on human health (Kranthi et al. 2002). With the most reliable tool turning redundant, pest management experts have started exploring alternative strategies for its control. Although strategies based on physical methods like manual shaking of plants, pupa busting, pheromone traps etc. have been used with some success, use of biological control agents may prove to be a better alternative.

Biological control with cry toxin producing *Bacillus thuringiensis* strains initially proved to be effective against *H. armigera*, but some recent reports relating to development of cry protein resistance in some instances raises the need for search of biocontrol agents with some alternative mode of action (Narayanan 2004; Gunning et al. 2005; Shanmugam et al. 2007; Rajagopal et al. 2009).

During last few years chitinolytic microorganisms have stimulated considerable interest in biocontrol of insect pests (Gohel et al. 2006; Mubarik et al. 2010; Abdullah et al. 2014). Chitinase produced by these microorganisms hydrolyse chitin, an insoluble structural polysaccharide present in the exoskeleton and gut linings of insects leading to low feeding rate and finally resulting in death of the insect. Although a few entomopathogenic fungi have been used for chitinase-based insect biocontrol (St. Leger et al. 1986, 1991; El-Sayed et al. 1989), reports on use of chitinolytic bacteria for biocontrol of *H. armigera* are lacking.

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In our earlier research work, we have reported high chitinase production by isolate, *Paenibacillus* sp. D1 (Singh et al. 2009a). The purified chitinase from the bacterium exhibited good thermostability for potential applications at elevated temperature (Singh & Chhatpar 2011a). Chitinase form the isolate-exhibited strong tolerance towards number of commonly used insecticides for control of insect pests in fields suggesting its potential use in integrated pest control systems (Singh et al. 2009b). The present investigation was carried out to explore the potential of *Paenibacillus* sp. D1 and its chitinase for biocontrol of *H. armigera*, individually and in combination with an organophosphate insecticide, acephate.

Materials and methods

Organism and chitinase production

The bacterium used in the study was a potent chitinase-producing isolate of *Paenibacillus* sp. D1. The medium used for growth and chitinase production by bacterium was as mentioned earlier (Singh et al. 2009a). One litre culture, grown at 30 °C for 72 h, was centrifuged at 10,000 × *g* for 10 min (4 °C) and the culture supernatant was precipitated using ammonium sulphate (saturation up to 70%). The precipitate was dissolved in 100 ml 50 mmol l^{-1} sodium acetate buffer, pH 5.0 and dialysed overnight against the same buffer. Dialysed enzyme was further concentrated (10 folds) by ultrafiltration using 50 kDa cut-off Omega ultrafiltration membrane (Pall Corporation) and used for biocontrol studies.

Chitinase assay

Chitinolytic activity was estimated as described by Singh and Chhatpar (2011a). The assay system consisted of 10 mg of acid-swollen chitin, 50 μmol of acetate buffer (pH 5.0), and 100 μl of enzyme in a total volume of 2.0 ml. After incubation at 50 °C for 10 min, the products were estimated by the Nelson method (1944). One unit of chitinolytic activity was defined as the amount of enzyme required to liberate 1 μmol of *N*-acetyl-D-glucosamine equivalent per h at 50 °C.

Zymogram analysis of the culture filtrate

For zymogram analysis, the samples were mixed with 5 × Laemmli sample buffer without reducing agent. The samples were loaded on 10% polyacrylamide gel containing 0.1% sodium dodecyl sulphate (SDS PAGE), 0.01% ethylene glycol chitin and 0.001% calcofluor white M2R and electrophoresed at 4 °C. After electrophoresis, the gel was immersed in 50 mM acetate buffer (pH 5.0) containing 2.5% triton X-100 for 15 min. Triton X-100 was removed by three washes with acetate buffer. The gel was then again immersed in the same buffer for 1−3 h and then visualised under ultraviolet light (302−312 nm). Clear zone on a fluorescent background indicated chitinase activity.

Insect culture

Helicoverpa armigera larvae were collected from pigeon pea fields at Model farm, Anand Agricultural University, Vadodara, India. They were maintained at approximately, 28 ± 2 °C, 60% relative humidity and a photoperiod of 14 h light and 10 h dark and fed on pigeon pea leaves and pods. Adults were sexed and paired to get eggs and neonate larvae. The pairs were kept separately in transparent glass jars (30 cm × 15 cm) to provide natural light. The wall of the jar had a window covered with fine mesh net to allow ventilation.

Antifeedant trials

For antifeedant trials, healthy third instar larvae of approximately equal weight were used. The efficacy of bacterial isolate, its chitinase and acephate as antifeedant was tested by feeding larvae with pigeon pea leaves of equal known dimensions (2 cm × 2 cm), coated with culture (10^6 cells), chitinase (1000 U) and 50 μg (recommended concentration for field application, RFC) (Singh & Chhatpar 2011a) of acephate, respectively. Leaves treated with sterile medium, acetate buffer and distilled water were used as control for culture, chitinase and insecticide treatments, respectively. All the experimental treatments were done using 10 larvae in triplicates in three independent trials (n = 90, where n is total number of larvae). The larvae were starved for 24 h to clear their gastrointestinal tract of any residual food and then fed on treated leaves for 4 days, after which normal feeding was resumed. The weight of larvae was measured daily and observations regarding death were performed till the 14th day as described by Binod et al. (2007). The area of the leaf consumed was also measured graphically for the first five days. The original leaf area was measured by tracing it on a graph paper. After being fed by the insect, the leaf outline was again traced on to the same graph paper and the amount of leaf consumed by larvae was measured by calculating the missing area in mm^2.

Determination of combined toxicity of Paenibacillus *sp. D1/chitinase and acephate*

The efficacy of combined treatment of the *Paenibacillus* sp. D1 or its chitinase with acephate was determined using half the dose used for individual treatments of each of the agents.

The interaction of *Paenibacillus* sp. D1 or chitinase with insecticide, with respect to larval mortality, was

determined using Limpel's formula (1) (Richer 1987),

$$E_e = (X + Y) - (XY)/100, \quad (1)$$

where E_e is the expected effect from additive responses of two inhibitory agents, say *Paenibacillus* sp. D1 (or chitinase) and insecticide; X and Y represent the percentage inhibition caused by *Paenibacillus* sp. D1 (or chitinase) and insecticide, respectively.

The synergy factor (SF) is calculated by Abbott's formula (2) (Abbott 1925),

$$\text{SF} = \text{Observed inhibition}/\text{Expected inhibition}, \quad (2)$$

where SF > 1 for synergistic reaction, SF < 1 for antagonistic reaction and SF = 1 for additive reaction.

Chitin estimation in insects

Chitin content in the insects was estimated as described by Cauchie (2002). Freeze-dried animals were first decalcified by means of HCl 0.5 N for 4 h. Proteins were then extracted in the residue using a NaOH 0.5 N solution at 100 °C for 6 h. The insoluble residue remaining after the successive HCl and NaOH treatments was repeatedly incubated in a solution of purified chitinase (Sigma 6137; 1 mg ml^{-1} distilled water) at 37 °C for 8 h. The supernatant was then incubated in a solution of *N*-acetylglucosaminidase (Sigma A6805) for 4 h at 37 °C. The *N*-acetylglucosamine (GlcNAc) monomers liberated by the enzymatic hydrolysis of chitin were measured by the colorimetric method as described by Nelson (1944).

Statistical analysis

All the experimental data were subjected to analysis of variance (ANOVA) followed by Fisher LSD (least significant difference) test ($p < 0.05$) using SIGMASTAT version 3.5 (Systat Software Inc., Pint Richmond, CA).

Results

Analysis of the culture filtrate

The concentrated culture filtrate of *Paenibacillus* sp. D1 exhibited very high chitinase activity of 900 units/ml. Zymogram analysis of the concentrate revealed presence of three different chitinases (Figure 1).

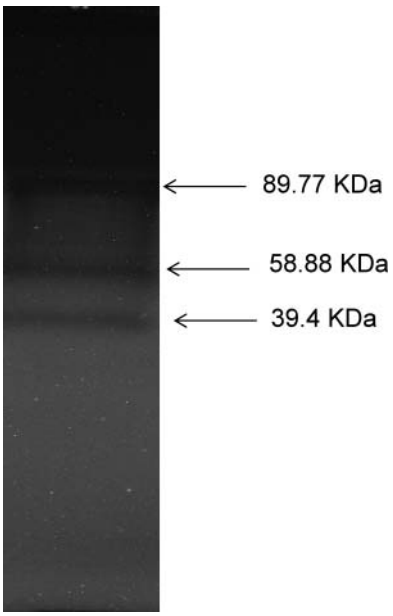

Figure 1. Zymogram analysis of crude chitinase from *Paenibacillus* sp. D1. Arrows indicate zone of clearance due to chitin degradation by the three isoforms of chitinase.

Antifeedant trails

Effect on larval feeding rate

Feeding the larvae on leaves coated with chitinase and acephate resulted in lower feeding rates compared to untreated control leaves. However, coating the leaves with *Paenibacillus* sp. D1 had very little or no effect on feeding rate of larvae for the first five days (Figure 2). Statistical analysis using ANOVA and Fisher LSD test revealed the antifeedant effect of chitinase and acephate to be significantly higher compared to control. The p value, degree of freedom (DF) between treatments and F value were <0.02, 3 and 4.3, respectively.

Effect on larval body weight

The larvae fed on leaves coated with chitinase or acephate exhibited reduced body weight compared to control from the first day whereas body weights of larvae feed on *Paenibacillus* sp. D1 started decreasing only after the sixth day (Figure 3).

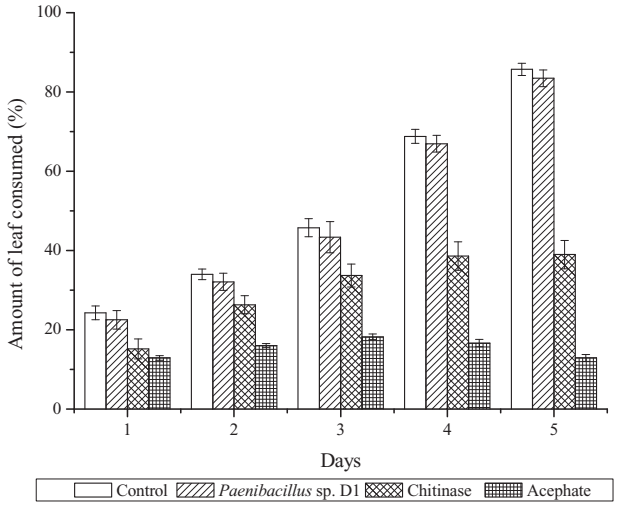

Figure 2. Effect of various antifeedants on larval feeding rate in terms of leaf consumption.

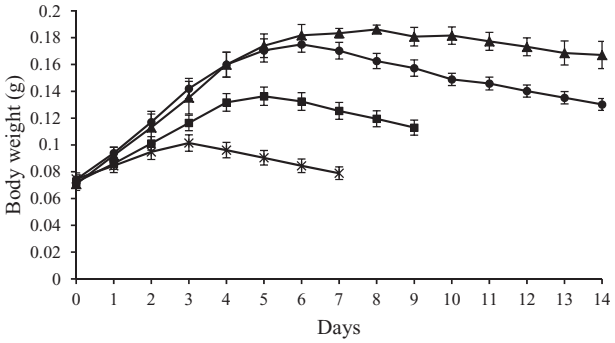

Figure 3. Effect of different antifeedants on body weight of larvae. Triangle: control, circle: *Paenibacillus* sp. D1, square: chitinase, cross: acephate. Error bars indicate standard deviation.

Chitin content of larvae

The chitin content of the larvae was estimated at the end of the experiment. Chitin content (9.3 ± 2.11 g/kg of dry weight) of larvae fed on chitinase treated leaves was reduced by more than 80% compared to control (46.67 ± 5.06 g/kg of dry weight). The chitin content of larvae fed on *Paenibacillus* sp. D1 and acephate-treated leaves were 16.18 ± 0.52 and 34.14 ± 5.15 g/kg of dry weight which contributed to 65.35% and 26.90%, respectively (Figure 4).

Effect on larval mortality

Among all the treatments maximum mortality was observed with acephate (90.0 ± 10.0%) followed by treatment with chitinase (63.33 ± 5.78%). *Paenibacillus* sp. D1 treated larvae exhibited mortality rate of 40.33 ± 5.78%. The killing effect of acephate, chitinase and *Paenibacillus* sp. D1 treatments were highly significant as revealed by statistical analysis (DF, 8; *F* value, 63.7 and *p* value, <0.001). A combined treatment of *Paenibacillus* sp. D1 or chitinase with acephate (with half the doses of each agent used for individual

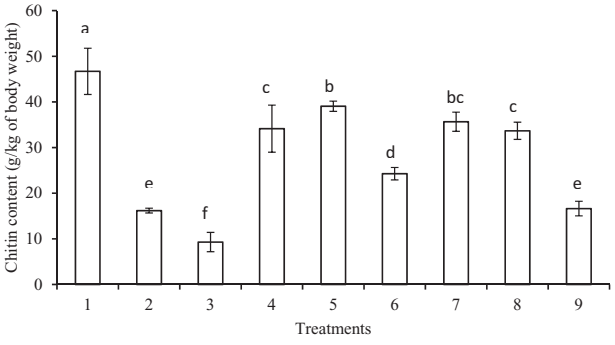

Figure 4. Effect of various antifeedants on total chitin content of larvae. (1) Control, (2) *Paenibacillus* sp. D1, (3) chitinase, (4) acephate, (5) 50% dose of *Paenibacillus* sp. D1, (6) 50% dose of chitinase, (7) 50% dose of acephate, (8) combined 50% doses of *Paenibacillus* sp. D1 and acephate, and (9) combined 50% doses of chitinase and acephate. Bars with similar alphabets do not differ significantly (Fisher LSD, $p < 0.05$).

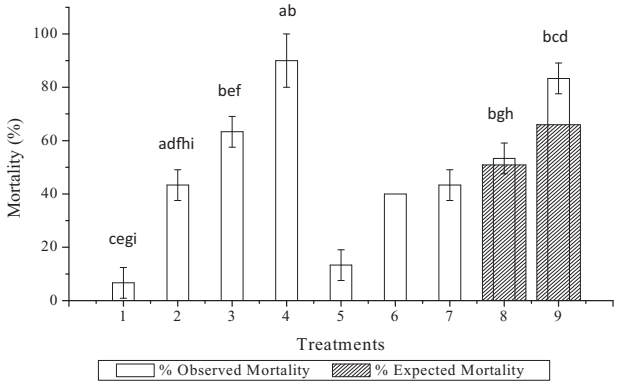

Figure 5. Effect of different antifeedants on larval mortality. (1) Control, (2) *Paenibacillus* sp. D1, (3) chitinase, (4) acephate, (5) 50% dose of *Paenibacillus* sp. D1, (6) 50% dose of chitinase, (7) 50% dose of acephate, (8) combined 50% doses of *Paenibacillus* sp D1a and acephate, and (9) combined 50% doses of chitinase and acephate. Bars with similar alphabets do not differ significantly (Fisher LSD, $p < 0.05$).

treatments) was found to be more effective in killing the larvae compared to their individual treatments, suggesting synergistic interaction between the bacterium/chitinase and the insecticide for larval mortality. Combined treatment of chitinase with acephate resulted in higher mortality of larvae compared to combined treatment of *Paenibacillus* sp. D1 and acephate evident from values of expected and observed mortality rates and SF of their interaction. The value of expected and observed mortality of larvae on combined treatment of chitinase and *Paenibacillus* sp. D1 with acephate were 66.0%: 83.33 ± 1.26% and 50.88%: 53.33 ± 5.78%, respectively, while the values of SF were 1.26 and 1.04, respectively (Figure 5).

Discussions

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyse the hydrolytic degradation of chitin, an insoluble linear β-1,4-linked polymer of GlcNAc. Chitinase have broad distribution in nature and have been reported in bacteria, fungi, nematodes, plants, insects, fish and humans. The physiological functions of chitinases depend on their source (Bhattacharya et al. 2007). Since chitin is a major constituent of exoskeleton and gut lining of insects and cell wall of some fungal phytopathogens, chitinolytic bacteria have received increased attention as biocontrol agents against fungal and insect pests (Gohel et al. 2006). Although number of entomopathogenic fungi such as *Metarhizium anisopliae*, *Beauveria bassiana* and *Nomuraea rileyi* have been studied for chitinase-based insect biocontrol, reports on chitinolytic bacteria are rare (St. Leger et al. 1986, 1991; El-Sayed et al. 1989). Thus, the present investigation was carried to evaluate the biocontrol potential of a high chitinase producing isolate, *Paenibacillus* sp. D1 and its chitinase. Since our earlier work

has shown extreme tolerance and stability of *Paenibacillus* sp. D1 and its chitinase towards acephate, an organophosphate insecticide, integrated pest management potential of *Paenibacillus* sp. D1 and its chitinase with acephate was also studied (Singh et al. 2009b).

Chitinolytic bacteria are known to produce multiple chitinases for efficient chitin digestion (Itoh et al. 2002; Suzuki et al. 2002; Kudan & Pichyangkura 2009). The chitinolytic system of bacteria generally comprises of endochitinases and exochitinases. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyse the progressive release of diacetylchitobiose starting at the non-reducing end of chitin microfibril and β-(1,4) *N*-acetylglucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Dahiya et al. 2006). Synergistic interactions between these enzymes are required for efficient degradation of chitin (Suzuki et al. 2002). *Paenibacillus* sp. D1 was found to produce multiple chitinase as revealed by zymogram analysis of concentrated culture supernatant.

Antifeedant studies with *Paenibacillus* sp. D1, chitinase and acephate showed considerable decrease in the feeding rate and body weight of the *H. armigera* larvae. Decrease in feeding rate and body weight of the larvae treated with *Paenibacillus* sp. D1 and chitinase can be attributed to disruption of chitinous peritrophic membrane lining the gut epithilium of the larvae. Disruption of peritrophic membrane has been reported to result in reduced feeding rates, decrease of body weight ultimately leading to death of the larvae (Carozzi & Koziel 2005). Decrease in the body weight of the larvae fed on leaves treated with *Paenibacillus* sp. D1 was observed after the sixth day, while larvae fed on chitinase and insecticide exhibited reduced body weight from the first day of feeding. The delay in action of *Paenibacillus* sp. D1 may be due to the time required for the bacterium to overcome insect defences and colonise the gut. Inside the gut, presence of chitinous peritrophic membrane can induce the chitinolytic system of the bacterium. Decrease in total chitin content of larvae fed on *Paenibacillus* sp. D1 and chitinase-treated leaves compared to control further highlighted the role of chitinase in larval mortality.

Moreover, combined treatment of *Paenibacillus* sp. D1 or its chitinase with acephate resulted in higher mortality of the larvae compared to their individual treatments. Disruption of the peritrophic membrane by chitinase can facilitate better uptake of insecticides by the insect tissues accounting for the synergism in larval mortality observed with combined treatment of the bacterium and chitinase with acephate. Regev et al. (1996) have reported synergistic interaction δ endotoxin of *Bacillus thuringiensis* and endochitinase of *Serratia marcescens* for control of *Spodoptera littoralis* larvae. There are number of reports on synergistic control of fugnal phytopathogens with combined doses of fungicides and chitinases (Someya et al. 2007; Chien-Jui & Chen 2008; Singh & Chhatpar, 2011b). However, reports on synergistic insect control with combined application of chitinase and insecticides are lacking. The present investigation revealed the biocontrol potential of *Paenibacillus* sp. D1 and its chitinase against *H. armigera* larvae. Furthermore, interaction of *Paenibacillus* sp. D1 and its crude chitinase with test acephate was found to be highly synergistic for control of *H. armigera* larvae. Such synergistic interactions will be advantageous for developing new fungicide formulations and application strategies which can reduce the dose of toxic agrichemical in fields.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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