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ORIGINAL ARTICLE

Evaluation of fungal culture filtrate containing chitinase as a biocontrol agent against *Helicoverpa armigera*

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biological control, biopesticide, chitinase, *Helicoverpa armigera,* Heliothis, submerged fermentation, *Trichoderma harzianum*.

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Abstract

Aims: To evaluate the biocontrol efficacy of culture filtrate containing chitinase from *Trichoderma harzianum* against Heliothis.

Methods and Results: *T. harzianum* was cultured by submerged fermentation using colloidal chitin as sole carbon source. The ability of the culture filtrate to hydrolyse colloidal chitin indicated the presence of chitinase as one of its components. Biocontrol assay on Heliothis showed that the culture filtrate is a potent antifeedant as it reduced the feeding rate and body weight of the larvae. It reduced the successful pupation and increased larval and pupal mortality in a dosage-dependent manner when applied topically. The highest mortalities (70%) were recorded for groups treated with 2000 U ml^{-1} chitinase activity. The percentage of adult emergence was zero for the highest chitinase concentration (2000 U ml^{-1}) tried.

Conclusions: The studies showed that the culture filtrate containing chitinase from *T. harzianum* is capable of negatively affecting the growth and metamorphosis of Heliothis larvae.

Significance and Impact of the Study: In view of the need for safer and environmentally friendly pest management tools, the present study could help in the development of enzyme-based biopesticides against Heliothis.

Introduction

Cotton bollworm (*Helicoverpa armigera* Hübner), commonly known as 'Heliothis' is one of the most serious pests on various crops, including cotton, tomatoes, sunflower, beans, maize and several cucurbitous and citrous crops. It is a multivoltine species with three to four generations requiring multiple control interventions per year (Xiulian *et al.* 2004). In central and northern India, it is the major pest affecting cotton. The larvae feed extensively on cotton plant parts including the newly emerging bolls causing severe loss of crop. Bollworm are relatively safe from natural enemies because of the cryptic feeding habits of the larvae within cotton bolls and because pupae are protected in the soil. Therefore, large numbers of *H. armigera* in cotton and other vegetables survive to adults that may disperse widely, producing progeny that damage high-value crops (Cabanillas and Raulston 1995; Michael and Donald 1996). Generally chemical pesticides are used to control the larvae, but the species rapidly attains resistance to many of the common pesticides used, resulting in the requirement of heavier dosages or newer pesticides. The use of chemical pesticides is also of significant environmental concern. Alternate strategies for control of the pest have also been tried extensively which include pheromone traps, pupa busting and use of plant-derived agents (e.g. extract of neem plant) with limited success. This being the status of efforts in control of Heliothis, it is interesting to study biological agents/products for the control of this pest. Biocontrol agents comprise an important element of many integrated pest management (IPM) programmes. Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans and other advantages (Liu *et al.* 2000). Biopesticides and predators have positive impacts on bollworm

population management (Ge and Ding 1996), although effective systems are not yet operative against *Helicoverpa* sp.

Chitin, an insoluble structural polysaccharide that occurs in the exoskeleton and gut linings of insects, is a metabolic target of selective pest control agents. One potential biocontrol agent is the insect moulting enzyme, chitinase, which degrades chitin to low molecular weight, soluble and insoluble oligosaccharides. Insect chitinase and its gene are now available for biopesticidal applications in the IPM programmes (Kramer and Muthukrishnan 1997). Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against chitin-containing proliferous and pathogenic organisms such as insects, nematodes and fungi (Carr and Klessig 1989; Linthorst 1991; Sahai and Manocha 1993). Resistance to undesired organisms can be imparted by degradation of their vital structures, such as the peritrophic membrane and cuticle of insects, the cell wall of fungal pathogens or by liberation of substances that subsequently elicit other type of defensive responses by the host (Boller 1987). The exoskeleton of insects primarily composed of chitin and protein, acts as a physicochemical barrier to environmental hazards and predators. However, some entomopathogenic fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Nomuraea rileyi* and so on can overcome these kinds of barriers by producing multiple extracellular degradative enzymes, including chitinolytic and proteolytic enzymes that help the pathogens to penetrate the barriers and expedite infection (El-Sayed *et al.* 1989; St. Leger *et al.* 1986, 1991). As these enzymes degrade chitin, it might be speculated that if applied on to the insect, or if it enters the gut of insect larvae, it can cause significant damage to the peritrophic membrane structure which will result in the larvae being not able to feed and consequently leads to death. Also if applied topically results in the disruption of cuticle which subsequently causes abnormal moulting. This projects the cocktail of enzymes as a potential agent to be included in the spraying type of biological control agents, provided the enzymes remain stable for sufficiently longer duration in the environment with retention of activity. Considering this, we initiated studies for a possible development of an enzyme-based biocontrol agent against *Helicoverpa* larvae.

The objective of the present study was to evaluate the efficacy of fungal culture filtrate as biocontrol agent against Heliothis. *Trichoderma harzianum* was cultured under submerged condition and the culture filtrate was analysed for both chitinase and protease activities. Process parameters were optimized for maximum chitinase production. Crude as well as lyophilized extracts were tested on the larvae. Effect of enzyme feeding on larval development was measured by changes in body weight, and the rate of pupation and death induced by the treatment. Effect of topical application of enzymes on larval development was measured by changes in body weight, rate of pupation, death and adult emergence.

Materials and methods

Micro-organism and maintenance

Trichoderma harzianum TUBF927, obtained from Technical University of Budapest, Hungary, was used for the study. The fungus was maintained on potato dextrose agar (PDA) slants, subcultured every 2 weeks and stored at 4°C.

Submerged fermentation

Spore inoculum was prepared by dispersing spores from fully sporulated culture on PDA slants in 0·1% Tween 80 under aseptic conditions. One millilitre of inoculum was transferred to 30 ml of sterilized production medium of pH 5·5 having the composition (g l^{-1}): $NaNO_3$ 1·0; KNO_3 5·0; $(NH_4)_2HPO_4$ 5·0; $MgSO_4.7H_2O$ 1·0, NaCl 1·0; colloidal chitin 1·0. To determine the optimum conditions for chitinase production, the strategy employed was to vary only one parameter at a time and monitor the enzyme yield and select the conditions that provide maximum yield during the process. The different process parameters optimized include incubation time, inoculum size and incubation temperature. For the optimization of incubation time, 1 ml of the spore inoculum was added to the medium and incubated for different intervals of time (0–120 h). The effect of inoculum size was studied by adjusting the spore count ranging from 4×10^5 to 8×10^5 spores per millilitre. The incubation temperature was studied by varying the temperature from 25° to 40°C. In each optimization step, the culture was agitated in a rotary shaker at 180 rev min^{-1} and was harvested by centrifugation. Each experiment was performed in triplicates and the mean value was taken. Standard deviation was calculated using functions in MS Excel (Microsoft Corporation) and is displayed as error bars. The clear supernatant was concentrated to powder (50 U mg^{-1} powder) by lyophilization. Upon requirement the powder was reconstituted with distilled water to obtain the required enzyme concentrations. The proteins present in the 96-h culture filtrate were analysed by 12% sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) and visualized by Coomassie Brilliant Blue R-250 staining. The chitinase activity was determined using 3,5,dinitrosalicylic acid (DNS) reagent (Miller 1959; Binod *et al.* 2005). Protease activity was measured with azocasein substrate and expressed in terms of velocity constant (K) (Tomarelli *et al.* 1949) calculated using the following equation:

$$K = 1/t \times 2{\cdot}3 \log C_1/C_2$$

where C_1 is the initial protein substrate concentration and C_2 is the final protein substrate concentration after t minutes of digestion.

Biocontrol studies

Maintenance of larvae

The Heliothis larvae were kept at approximately 14 : 10 (L : D) h of light, 27 ± 2°C and at 50–60% relative humidity, and were fed on cotton leaves unless otherwise indicated. Third instar larvae were used for antifeeding trials and fifth instar larvae for growth inhibition studies.

Antifeedant trials

The efficacy of culture filtrate as an antifeedant was tested by feeding larvae with cotton leaves coated with filtrate. Discs of known dimensions were made from cotton leaves and were dipped in enzyme preparation (1000 U ml^{-1} chitinase activity) for 1 min. The discs were spread on paper and air dried before being fed to the larvae. Leaf discs similarly treated in culture filtrate produced in the absence of colloidal chitin was used as control. Healthy third instar larvae of similar weight were taken for the experiment. Each experimental group consisted of a minimum of 10 larvae, starved for 24 h and then fed with filtrate-coated leaves. Feeding with treated leaves was continued for 4 days after which normal feeding was resumed. The weight of larvae was measured daily and observations regarding the general health of larvae, pupation and death were performed till the 14th day. Leaf area was monitored before and after feeding every day till normal feeding was resumed. Digital pictures of leaf discs were taken before and after feeding and the images were analysed using the software Scion Image 4·0·3·2 (Scion Corporation, Frederick, MD, USA). The rate of feeding was measured by noting the leaf area consumed by the larvae. Effect of feeding on larval development was measured by changes in body weight, and the rate of pupation and death induced by the treatment. The readings for increase in body weight, pupation and mortality were shown as mean values of the samples and standard deviation was calculated using functions in MS Excel and was displayed as error bars.

Growth inhibition studies

In the bioassay experiment for growth inhibition, lyophilized preparations and crude filtrate were used. The lyophilized filtrate was diluted in water to obtain final chitinase activities of 2000, 1000, 500, 250 and 100 U ml^{-1}. Five microlitres of each preparation was applied topically on the thorax back of the fifth instar larvae using a micropipette (10, 5, 2·5, 1·25 or 0·5 U per larvae). Five microlitres of culture filtrate produced in absence of colloidal chitin served as control. Topical application was continued for 3 days after which the larvae were left undisturbed. The weights of larvae were measured daily and observations regarding the general health of larvae, pupation, death and adult emergence were performed till the 17th day. Effect of filtrate applications on larval development was measured by changes in body weight, and the rate of pupation, death and adult emergence induced by the treatment. Standard deviation was calculated using functions in MS Excel and was displayed as error bars.

Results

Analysis of culture filtrate

The culture filtrate was analysed for chitinase and protease activities. The filtrate produced in the presence of colloidal chitin showed chitinase and protease activities whereas the control filtrate did not show any enzyme activity. The process parameter optimization resulted in maximum chitinase production at 96 h at the incubation temperature of 30°C with inoculum size of 5×10^5 spores ml^{-1} (Fig. 1). At 24 h, the chitinase production was 0·75 U ml^{-1} and it increased up to 96 h with activity of 10·2 U ml^{-1}. The protease activity was found maximum at 48 h (0·92 K) and at 96 h, it showed an activity of 0·4 K. SDS-PAGE analysis of culture filtrate showed protein bands only in the filtrate produced in presence of colloidal chitin (Fig. 2).

Antifeedant trials

Effect on larval feeding rate

The culture filtrate when used for coating the leaves resulted in a lesser consumption of feed by the larvae. At

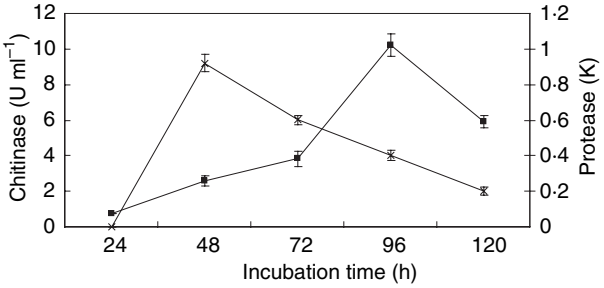

Figure 1 Extracellular protein production by *Trichoderma harzianum* TUBF 927 under submerged culture in presence of colloidal chitin as sole carbon source. Culture condition: incubation temperature: 30°C; inoculum size: 5×10^5 spores ml^{-1}. (■), chitinase (U ml^{-1}); (×), protease (K).

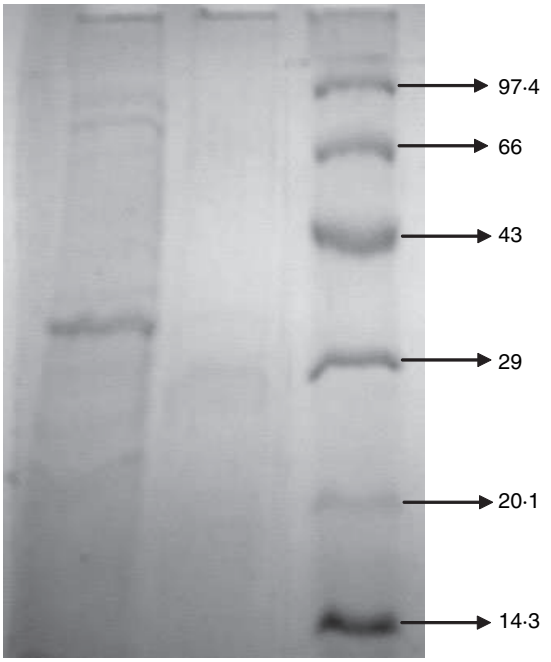

Figure 2 Sodiumdodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of proteins from culture filtrate. Lane I: 96-h filtrate of *Trichoderma harzianum* TUBF 927 cultured in presence of colloidal chitin as sole carbon source; lane II: 96-h filtrate of *T. harzianum* TUBF 927 cultured in absence of colloidal chitin (control); lane III: protein marker (Bangalore GeNei, PMWM).

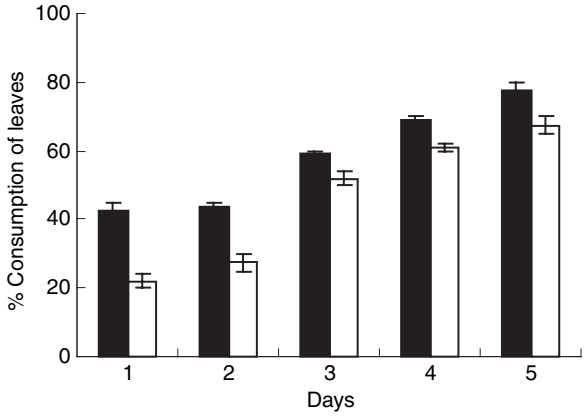

Figure 3 Effect of chitinase-antifeedant on larval feeding rate: (□) chitinase; (■) control.

1000 U ml^{-1} chitinase concentration, larval feeding was lower than in the case of the control (Fig. 3). When crude filtrate was used, the feeding rate was comparatively better but it was still lower than the control on all the days.

Effect on larval growth rate

Data presented in Fig. 4 show changes in mean body weights of experimental groups fed on leaves treated with

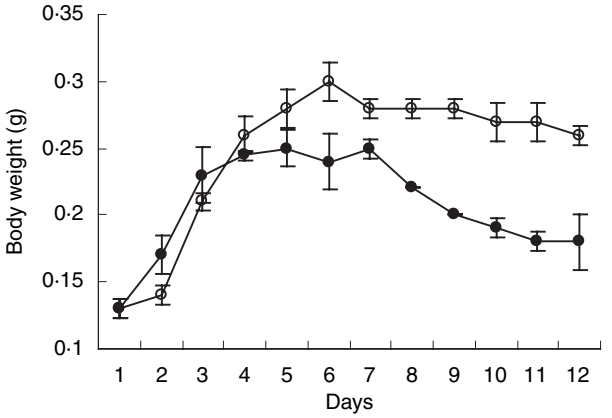

Figure 4 Effect of chitinase-antifeedant on larval growth rate: (●) chitinase; (○) control.

1000 U ml^{-1} of chitinase. Till day 7, the groups fed with chitinase showed retarded growth rate compared with the control. The control group performed better in terms of growth as indicated by increased body weight. It might be speculated that feeding on chitinase-treated leaves might have led to the destruction of peritrophic membrane which ultimately lead the larvae being not able to feed well and result in slow growth rate.

Growth inhibition studies

Effect on pupation

As a result of topical application of filtrate on thorax back of the larvae, the percentage of pupation in larvae decreased in proportion to the concentration of chitinase (Fig. 5). Pupation had started in experimental groups from day 3 onwards and reached the maximum by day 5. On day 17, the final day of observation, the percentage of successful pupation was measured as the

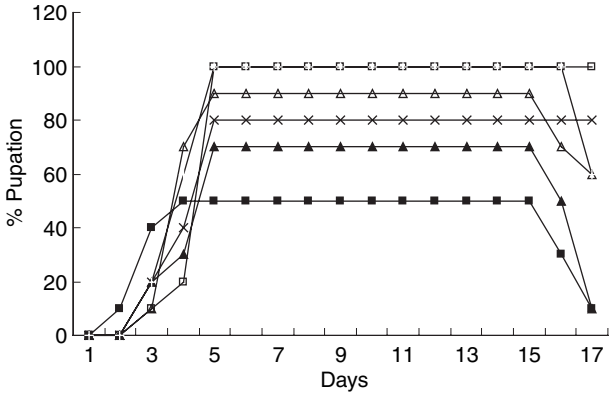

Figure 5 Effect of topical application of chitinase on larval pupation: (■), 2000 U ml^{-1}; (▲), 1000 U ml^{-1}; (×), 500 U ml^{-1}; (△), 250 U ml^{-1}; (⁘), 100 U ml^{-1}; (⁙), control.

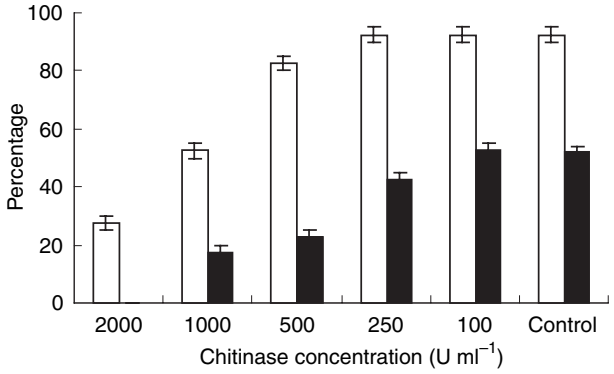

Figure 6 Effect of topical application of chitinase on larval growth rate: (□), per cent of successful pupation; (■), per cent of adult emergence.

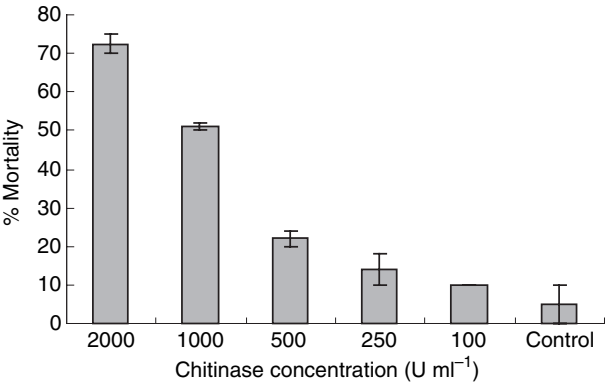

Figure 7 Effect of topical application of chitinase on larval mortality.

sum of live healthy pupae and adult emergence. The lowest pupation percentage was recorded for larvae treated with crude chitinase followed by 2000 U ml^{-1}. At the remaining concentrations, there was a dosage-dependent decrease in the number of pupae formed. The highest pupation rate was recorded in all groups from days 6 to 15. Data for this period showed that highest pupation efficiency of 100% was obtained in the control and 100 U ml^{-1} concentration of chitinase. The highest concentration may be speculated to accelerate pupation because of stress as the experimental group treated with this concentration showed 40% pupation even at day 3. So it might be concluded that at this concentration, though the larvae progress through pupation quickly because of stress, the pupae are not properly formed and finally die. Further studies on pupal morphology and biochemistry are needed to conclude the results. Overall, the results showed that the efficiency of successful pupation reduces with emerging doses of chitinase.

Effect on larval growth rate

The effect of treatment on larval development was represented as the percentage of successful pupation at the end of the test period. Successful pupation was defined as the formation of healthy pupae which can or have already developed into normal adults at the end of the test period (17 days). The percentage of successful pupation was calculated based on the sum of healthy pupae and adults on day 17 and is represented in Fig. 6. The figure also gives the percentage of adult emergence. It can be observed that the percentage of adult emergence was zero for the highest chitinase concentration tried. At this concentration only 25% of successful pupation was noted. It also indicated that chitinase affects successful pupation negatively in a dosage-dependent manner.

Effect on larval mortality

Mortality of larvae/pupae because of the treatment was measured on the 17th day. The larval development was affected which was indicated by lesser number of pupae which was proportionate to the concentration of chitinase used. Also at the end of the test period (day 17), the percentage of larval/pupal mortality was measured. The percentage mortality in chitinase-treated groups showed clear dosage dependence as presented in Fig. 7. The highest mortalities (70%) were recorded for groups treated with 2000 U ml^{-1}, whereas the control and the lowest chitinase concentration (100 U ml^{-1}) showed nearly 10% mortality. The groups treated with 1000 U ml^{-1} chitinase showed mortality rate of 50%.

Discussion

Chitinases are enzymes with a specific hydrolytic activity directed towards chitin. Enzymatic cleavage generally occurs randomly at internal locations over the entire length of the chitin polymer. The final products from chitinase catalysis are soluble, low molecular mass multimers of *N*-acetylglucosamine, such as chitotetraose, chitotriose and chitobiose. Chitinases have been detected in bacteria, fungi and plants and in the digestive systems of coelenterates, nematodes, polychaetes, molluscs and arthropods. Micro-organisms are generally preferred to plants and animals as sources of enzymes because the low production costs and enzyme contents of microbes are more predictable and controllable (Pandey *et al.* 1999). Another factor is easy availability of raw materials with constant composition for their cultivation. Many strains of the genera *Aspergillus*, *Penicillium* and *Trichoderma* have been isolated from soil and examined for their chitinase activity. Several reports are available on the production of chitinase under submerged condition using colloidal chitin as a sole source of carbon (Nampoothiri *et al.* 2004).

St. Leger *et al.* (1991) reported the presence of chitinase in the extracellular fluids from *Metarhizium anisopliae* grown on chitin as the sole carbon source. Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against chitin-containing pestiferous and pathogenic organisms such as insects, nematodes and fungi (Carr and Klessig 1989; Linthorst 1991; Sahai and Manocha 1993). Chitinolytic enzymes used by insects, nematodes, fungi, viruses and other organisms for moulting or penetration of structural barriers are potentially useful in pest management. Alternatively, the pathogen or pest can be exposed to chitinases at their developmental stages to increase their vulnerability to host defense.

As the occurrence of chitin as a structural component is limited to a few organs, such as the integuments of arthropods, nematodes and molluscs, the gut lining of insects and the cell walls of fungi and some algae, chitin metabolism is an excellent target for selective pest control strategies (Kramer and Koga 1986; Cohen 1987; Kramer *et al.* 1997). Because of the importance of chitin and its metabolic enzymes in insect growth and development, chitinolytic enzymes and their genes have received a great deal of attention in recent years. Their chemical, physical, kinetic and regulatory properties, as well as their potential for development as biopesticide and microbial biological control agents are being explored (Kramer *et al.* 1997).

Insect cuticle is a composite material consisting of a thin lipid–protein-rich epicuticle covering the bulky procuticle. The procuticle is composed of the exo- and endocuticle which are composed mainly of chitin and protein, wherein the exocuticle is generally melanized (Andersen 2002). Insects periodically shed their old exoskeletons and either continuously or periodically shed their peritrophic membranes and resynthesize new ones. This process is mediated by the elaboration of chitinases in the moulting fluid that accumulates in the space between the old cuticle and the epidermis and in the gut tissue. The *N*-acetylglucosamine-containing products of hydrolysis are ultimately recycled for the synthesis of a new cuticle. Often the larvae will ingest and digest the old cuticle or exuvium, the components of which are also recycled. This behaviour coincides with the period of chitinase expression in the gut. For penetration through the insect cuticle, deuteromycete fungi such as *Metarhizium* and others produce chitinase, protease and lipase, commonly referred to as cuticle-degrading enzymes (St. Leger *et al.* 1986; Krieger de Moraes *et al.* 2003). Early studies by Coudron *et al.* (1984) demonstrated that chitinolytic activity in several entomopathogens was important for growth and potentially needed for penetration. Chitinase activity compared with the rate of fungal development in isolates of *Nomuraea rileyi*, which is parasitic on larvae of *Trichoplusiani*, the cabbage looper, showed significantly higher levels of an endochitinase and β-1,4,*N*-acetylglucosaminidase in two virulent *N. rileyi* strains compared with an avirulent mutant strain grown over a period of 30 days (El-Sayed *et al.* 1989). In the virulent isolates, the chitinase activity/total protein ratio during germination (2 days) was as much as 35 times greater than that found in conidia at day 0. Chitinase activity was also present at the onset of the blastospore stage (3 days) which is a stage critical to penetration of the chitin-ladened host insect cuticle. Thus, it was speculated that chitinolytic enzymes play a role in dissolution of insect cuticles during penetration by entomopathogenic fungi (Sahai and Manocha 1993).

Chitinases have been purified from the moulting fluid and integument of the tobacco hornworm, *Manduca sexta*. The *M. sexta* chitinase gene is not active during the larval feeding period. The enzyme is expressed only during narrow time frames just before larval–larval, larval–pupal and pupal–adult moulting. The activity of this gene is regulated positively by ecdysteroid, and affected negatively by juvenile hormone (Kramer *et al.* 1993). The tight developmental and hormonal regulation of chitinase expression suggests that the presence or absence of this enzyme might be detrimental to insect growth if expressed at an inappropriate time or if not expressed at an appropriate time.

Reports are available for the degradation of insect gut peritrophic membrane by chitinase. Brandt *et al.* (1978) proposed that chitinases cause perforations in the membranes, thus facilitating entry of the pathogens into the tissues of susceptible insects. The addition of exogenous chitinase from *Streptomyces griseus* to the blood meal of the mosquito, *Anopheles freeborni*, prevented the peritrophic membrane from forming (Shahabuddin *et al.* 1993). Perforation of peritrophic membranes occurred *in vivo* after *Spodoptera* fifth instar larvae were fed on a diet containing recombinant *ChiAII*, a recombinant endochitinase encoded by *Serratia marcescens* (Regev *et al.* 1996).

Leaves excised from chitinase-positive and -negative transgenic tobacco plants expressing the *M. sexta* chitinase were fed to first instar larvae of the tobacco budworm, *Heliothis virescens* (Ding *et al.* 1998). After 3 weeks, the total mass of larvae recovered from chitinase-negative leaves was nearly sixfold higher than the mass of larvae surviving on chitinase-positive leaves. Larvae reared on plants lacking the *Manduca* chitinase gene consumed substantially more leaf tissue than did larvae feeding on plants expressing the gene. Overall, mortality was greater for larvae grown on chitinase-positive plants. In order to determine whether the *Manduca* chitinase from transgenic tobacco and several chitinases from other sources were

directly toxic to insects, a beetle-feeding study was conducted using the insect enzyme and chitinases from two bacterial and one plant species. Recombinant *Manduca* chitinase from transgenic tobacco and chitinases from *Serratia*, *Streptomyces* and *Hordeum* sp. were fed to neonate beetle larvae at a 1–2% level in the diet. The growth and survival of larvae consuming the microbial and plant chitinases were the same as those of larvae consuming the untreated diet, indicating that these chitinases have no adverse effects on beetle growth. On the other hand, all of the larvae consuming the recombinant insect chitinase supplemented diet were dead within a few days after egg hatch (Ding *et al.* 1998). Till now no investigations into the safety of food derived from transgenic plants with modified chitinase content have been published. In this context, more research towards the development of safer and environmentally friendly enzyme-based biopesticides are encouraged.

Microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic micro-organisms. Synergistic effects among chitinolytic enzymes and microbial insecticides have been known to occur since the early 1970s. Larvae of the spruce bud worm, *Choristoneura fumiferana*, died more rapidly when exposed to chitinase-Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff 1974). The mortality of gypsy moth (*Lymantria dispar*) larvae was enhanced when chitinase was mixed with Bt relative to a treatment with Bt alone, in laboratory experiments. The toxic effect was correlated positively with enzyme levels. Crude chitinase preparation from *Bacillus circulans* enhanced the toxicity of Bt *kurstaki* towards diamond back moth larvae (Kramer and Muthukrishnan 1997).

A major concern in developing commercial biopesticides is the speed of kill as compared with chemical insecticides. *Bacillus thuringiensis* takes 3–4 days and NPV (nucleopolyhedrosis viruses) 5–7 days for effective control of insect pests. However, both face limitations in their mode of action, i.e. the need to be ingested by the pest. Furthermore, the rapid build up of resistance in insect populations is a major concern. The studies conducted for testing the efficacy of chitinase as a biocontrol agent against the insect pest *H. armigera* have shown that the enzyme is capable of negatively affecting the growth and metamorphosis of the larvae. This is true when chitinase is used in feed or when topically applied. The conditions used for the experiment tried to simulate the field conditions of spraying the agent in a water base when the leaves are coated with enzyme or the larvae are directly exposed. It is evident that at the specific concentrations tested, chitinase can induce at least 50% mortality till pupa stage. It may still be speculated that the adults emerging from chitinase-treated larvae may be abnormal and incapable of normal life, although this needs further experimental evidence. In view of the need for new pest management tools, a possible alternative is insect pathogens that can secrete high levels of chitin-metabolizing enzymes. This might enable the use of chitinase sprays combined with other pesticide formulations to facilitate faster kill.

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