

Production of cuticle degrading enzymes by *Isaria fumosorosea* and their evaluation as a biocontrol agent against diamondback moth

Shaukat Ali · Zhen Huang · Shunxiang Ren

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Abstract Production and biocontrol efficacy of culture filtrates containing cuticle degrading enzymes from three isolates of *Isaria fumosorosea* against diamondback moth (*Plutella xylostella*) was observed during this study. This fungus when cultured in liquid medium having different carbon sources showed maximum biomass production when 1% chitin was added as carbon source. These isolates when grown in liquid culture conditions having 1% chitin as source produced cuticular degrading enzymes (proteases (Pr1 and Pr2), chitinases, chitosanase, and lipase) in a sequential manner and the production of these enzymes differed from control. Biocontrol assays with *P. xylostella* showed that the culture filtrates of *I. fumosorosea* were potent antifeedants because reduction in the feeding rate and body weight of the larvae was observed. Similarly, reduction in rates of successful pupation, adult emergence was observed when the culture filtrates were applied topically. At the end of the test period, the lowest ST₅₀ value (1.57 ± 0.20 days) was recorded for insect groups treated with culture filtrates from isolate IF28.2 when compared to

the control. In view of the need for safer and environmentally friendly pest management tools, the present study can help in the development of enzyme-based biopesticides against *P. xylostella*.

Keywords Cuticle degrading enzymes · Biocontrol program · Microbial control · Entomopathogenic fungi

Introduction

Diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) is a major pest of cabbage, broccoli, and canola. Each year, farmers worldwide spend more than \$1 billion to control this pest, primarily by using chemical insecticides (Henrik et al. 2000). As a result, natural antagonists are killed (Xu et al. 2004) and many populations of diamond back moth have become resistant to conventional insecticides (Shelton et al. 1993; Tabashnik 1994). In addition, *Bacillus thuringiensis* resistant field populations have been detected in several countries, such as, U.S.A., Central America, and Asia (Syed 1992; Ferré and van Rie 2002). Alternative control measures being investigated for diamondback moth include the use of entomopathogenic fungi (Cherry et al. 2004; Wright 2004; Muhammad et al. 2005). Isolates of different fungal species such as, *Zoophthora radicans*, *Paecilomyces fumosorosea*, *Metarhizium anisopliae*, *Fusarium* sp., and *Beauveria bassiana* can infect diamondback moth. *B. bassiana* is virulent against diamondback moth in screen house or field conditions (Pell et al. 1993; Ibrahim and Low 1993; Vandenberg and Ramos 1997; Vandenberg et al. 1998; Altre et al. 1999). *Isaria fumosorosea* (*Paecilomyces fumosorosea* designated as *Isaria* clade, Luangsa-Ard et al.

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Zhen Huang is joint first author.

S. Ali · Z. Huang · S. Ren (✉)
Engineering Research Center of Biological Control, Ministry of Education, College of Natural Resource and Environment, South China Agricultural University, Wushan Road, Guangzhou City 510642, People's Republic of China
e-mail: rensxcn@yahoo.com.cn

S. Ali
e-mail: alientomologist@gmail.com

Z. Huang
e-mail: hzscau@scau.edu.cn

2005) is one of the most promising fungal species for control of diamondback moth, whiteflies and other insect pests (Altre et al. 1999; Wraight et al. 2000; Ali et al. 2009).

The fungal infection is dependent upon numerous biological events which are initiated by the adhesion of fungal spores to the insect cuticle, spore germination, and hyphal growth. In order to breach the insect cuticle, fungal hyphae exert mechanical pressure and produce cuticle degrading enzymes (Altre et al. 1999). Several cuticle degrading enzymes such as proteases and chitinases have been detected in entomopathogenic fungi such as *B. bassiana*, *Numuraea rileyi*, and *M. anisopliae* (Brey et al. 1986; El-Sayed et al. 1989). In insects, chitin forms a structural barrier that must be overcome by extracellular chitinases so that the fungus can enter and be dispersed throughout the hemocoel. This suggests that chitinases might play an important role in both cuticle degradation and host penetration (St. Leger et al. 1996).

Proteolytic enzymes along with chitinases and lipases are important factors of entomopathogenic fungi (Samuels and Paterson 1995). Pr1, a serine protease, plays a major role in insect penetration and subsequent pathogenicity (St. Leger et al. 1988, 1996). Lipids are also integral to the insect cuticle and improvements in lipase production by an entomopathogen would be highly desirable for use of entomopathogens to be used as bioinsecticides (Clarkson and Charnley 1996). As these enzymes degrade chitin, it might be speculated that these enzymes can cause disruption of cuticle which subsequently causes abnormal molting. Similarly, if these enzymes enter the gut of insect larvae, they 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 (Binod et al. 2007). This projects that the cocktail of enzymes is a potential agent to be included in the spraying type of biological control agents, provided that the enzymes remain stable for sufficiently longer duration in the environment with retention of activity. Considering this, we initiated studies for the possible development of an enzyme-based biocontrol program against diamondback moth larvae.

The objective of the present study was to evaluate the efficacy of fungal culture filtrate against diamondback moth. *Isaria fumosorosea* was cultured under submerged condition and the culture filtrate was analyzed for Pr1, Pr2, chitinases, chitosanase, and lipase activities. Crude culture filtrates were also tested on the larvae for biological activity. Effect of enzyme feeding on larval development was measured by changes in amount of larval feeding and body weight. Effect of topical application of enzymes on larval development was measured by changes in rate of pupation, rate of adult emergence and larval mortality.

Materials and methods

Fungal strains

Isaria fumosorosea isolates (IF-28-2, IF-32, and IF-49) originally isolated from soil (Liu, 2006), deposited to the collection at Engineering Research Center of Biological Control, South China Agricultural University were used during these studies. To produce the inoculum for each assay *I. fumosorosea* was cultured on potato dextrose agar (Potato infusion 200 g/l; Dextrose 20 g/l and Agar 20 g/l) and incubated at $26 \pm 2^\circ\text{C}$ for 10 days. Conidia were harvested with distilled water containing 0.03% aqueous polysorbate monooleate (TweenTM 80), bought from Whiga chemicals Shanghai and sieved through filter paper into sterile vials. Conidia were counted in Fuchs-Rosenthal hemocytometer using a compound microscope in order to prepare a suspension of 1×10^7 conidia/ml.

Basal medium

Basal medium (pH 7.2) consisted of glucose 0.2% (w/v), peptone 0.5% (w/v), MgSO_4 0.01% (w/v), K_2HPO_4 0.1% (w/v) and SDS 0.25% (w/v).

Growth of entomopathogenic fungi on cuticle components

The effect of chitin (reagent grade from Whiga chemicals, Shanghai), chitosan (reagent grade purchased from Sinopharm chemical reagent, Shanghai, China), amino acids like alanine, isoleucine and glycine (Sinopharm chemical reagent, Shanghai, China) as a carbon source for biomass production by *I. fumosorosea* was studied by growing the fungal isolates in liquid medium described above containing carbon sources at 1% (w/v) in 50 ml of basal medium in 250 ml Erlenmeyer flasks, heat sterilized at 121°C for 15 min while the basal medium without any carbon source was used as a control. The flasks were inoculated with 1 ml of 1×10^7 spores/ml and incubated at 180 rpm and 30°C for 5 days. For biomass determinations the culture supernatants were separated from the mycelium by filtration through pre-weighed Whatman filter no. 1 and dried at 80°C until a constant weight.

Production cuticle degrading enzyme complex of entomopathogenic fungi on chitin

The cuticle degrading enzyme complex was produced by growing *I. fumosorosea* in liquid medium described above. Chitin (1% w/v) was added as a carbon source to previously sterilized basal medium (121°C , 15 min) while the basal medium without chitin served as control. The flasks

were inoculated with 1 ml of 1×10^7 spores/ml and incubated at 180 rpm and 30°C for 5 days. Samples were removed at 24 h intervals used for further enzymatic analysis. To prepare the culture filtrates for biological control studies the inoculum was harvested by centrifugation at 1,000 g for 10 min at 4°C in a Microfuge[®] 18 with a F241.5P rotor (Beckman Coulter, Inc, USA).

Enzyme assays

Protease assay

Isaria fumosorosea subtilisin and trypsin-like activities are referred to as Pr1 and Pr2, respectively. Subtilisin-like (Pr1) activity was assayed by using succinyl-(alanine)₂-proline-phenylalanine-*p*-nitroanilide (Sigma) as substrate, and trypsin (Pr2) activity using benzoylphenylalanine-valine-arginine-*p*-nitroanilide (Sigma). Each assay consisted of 0.05 ml substrate (1 mmol l^{-1}), 0.85 ml 15 mmol l^{-1} Tris-HCl buffer (pH 8.5) and 0.1 ml crude enzyme. The mixture was incubated for 1 h at 28°C and the reaction was terminated by adding 0.25 ml of 30% acetic acid and left to stand for 15 min on ice, after which the samples were centrifuged at 1,250 g for 5 min at 4°C in a Microfuge[®] 18 with a F241.5P rotor (Beckman Coulter, Inc, USA). The supernatants were transferred to the wells of microtitre plate and absorbance was measured using spectrophotometer (Beckman, Coulter) at 410 nm. One unit of activities was expressed as nanomoles nitroanalide (NA) released per mg per hour. Protein was measured using Coomassie Brilliant Blue G-250 (Dingguo chemicals, Beijing) according to Bradford (1976), with bovine serum albumin as standard.

Lipase assay

Lipase activity was determined as described by Pignede et al. (2000). The substrate emulsion was prepared with olive oil, 50 ml (obtained from Nan Hai oil Industry, Shenzhen, China) and gum Arabic (Jinhuadu chemical reagent company, Guangzhou, China.), 50 ml (10% w/v). The reaction mixture contained 1 ml enzyme (sample already obtained from culture flasks), 5 ml substrate emulsion and 2 ml of 50 mM phosphate buffer, pH 6.8, and were incubated for 1 h at 37°C with shaking. The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein (obtained from Jinhuadu chemical reagent company, Guangzhou, China) as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50 mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1 μmol of fatty acid per min.

Chitinase and chitosanase assay

Colloidal chitin was prepared by the method of Roberts and Seletrenikoff (1988) with some modification. One hundred grams of chitin flakes of reagent grade chitin (Whiga chemicals, Shanghai) were added slowly to 1.75 l concentrated HCl and agitated gently for 3 h on a magnetic stirrer. This solution was then filtered to 20 l of pre chilled distilled water with constant mixing and allowed to settle. A dense white precipitate formed was then centrifuged at 10,000 rpm for 10 min at 4°C. The precipitate was then washed in cold distilled water repeatedly until the pH of the wash reached near to 5.5. The supernatant was discarded and colloidal chitin was then kept in refrigerator for future use.

Chitinase assay was based on the estimation of reducing sugars released during the hydrolysis of colloidal chitin. The reaction mixture contained 0.5 ml of enzyme, 0.5 ml of 0.5% colloidal chitin, and 1.0 ml of citrate phosphate buffer pH 5.6. The mixture was kept in a water bath at 37°C for 1 h. The amount of reducing sugar liberated was estimated by Miller's (1959) method. One unit (U) of activity was defined as the amount of enzyme which catalyzed the release of 1 μg of reducing sugar per ml per minute under the reaction conditions. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

Chitosanase activity was estimated using acid swollen chitosan (purchased from Sinopharm chemical reagent, Shanghai, China) as the substrate. The acid swollen chitosan were prepared following the protocol used to make acid swollen chitin. The assay mixture will contain 1 ml 0.7% acid swollen chitosan, 1 ml 50 mM acetate buffer, pH 5.0, and 1 ml enzyme that were incubated at 37°C for 1 h. The reducing sugars produced were estimated using Somogyi method (Somogyi 1951) at 520 nm. One international unit of enzyme was defined as the activity that produced 1 μmol glucosamine equivalents per min.

Biocontrol studies

Insect cultures

Larvae of *P. xylostella*, were obtained from the stock cultures raised on *Brassica campestris* L. Plants were grown in plastic pots using regular soil. Slow release fertilizer (N: P: K = 13:7:15, Shenzhen Batian ecotypic engineering Co., LTD. Xili Shenzhen China) was added as required to maintain normal plant growth. The pots were incubated in an artificial climate room at 26°C and >95% R.H. Second instar larvae were used for antifeedant studies whereas fourth instar was used for growth inhibition studies.

Studies on antifeedant activity

The efficacy of fungal culture filtrate as an antifeedant was tested by feeding larvae with fresh *B. campestris* leaves coated with filtrate. Leaf discs (10 cm²) were dipped in enzyme preparation and air dried on paper before being fed to the larvae. Leaf discs similarly treated in culture filtrate produced in the absence of chitin were used as control. The leaf discs were replaced on daily basis. Feeding with treated leaves was continued for 4 days after which normal feeding were resumed. The insects were placed in an air-conditioned room at 26°C and >95% R.H. The rate of feeding was measured by noting the leaf area consumed by the larvae (using AM 300 portable leaf area meter, Dynamax Inc, USA) and was expressed as percent leaf consumption based on the following transformation.

$$\text{Percent leaf consumption} = \left(\frac{\text{Leaf area consumed}}{\text{Total leaf area}} \right) * 100$$

Effect of feeding on larval development was measured by changes in body weight during a 10-day observation period. Each treatment and control was repeated three times with a new batch of insects and new culture fluid, and for each repetition there were four leaves, each leaf having 20 diamondback moth larvae.

Studies on growth inhibition of diamondback moth

In the bioassay experiment for growth inhibition crude fungal filtrates were used. Five microliters of each preparation were applied topically on the thorax back of the 4th instar larvae using a micropipette. Five microliters of culture filtrate produced in absence of colloidal chitin served as control. After this larvae were left to air dry before being transferred to 20-cm diameter clean glass Petri dishes and a piece of filter paper (20 cm in diameter) was placed at the bottom of the dish with a few drops of water for maintenance of moisture. Topical application was continued for 3 days after which the larvae were left undisturbed. Leaf disks were replaced every 2 days except during the pupal stage. The insects were placed at 26°C and >95% R.H. and the effect of filtrate applications on larval development was measured by changes in rate of pupation, adult emergence, and mortality induced by the treatments.

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 proportion of larvae successfully pupating divided by the total number of larvae which can or have already developed into normal adults at the end of the test period (10 days).

Statistical analysis

Biomass production and enzymatic activities after 5 days, percent pupation and adult emergence were analyzed by Analysis of variance (ANOVA) and treatment means were compared by using Tukey's test for mean comparisons at 5% level of significance. Data regarding sequential production of different enzymes, percent larval feeding, and changes in larval body weight were analyzed by Repeated Measures Anova. For mortality data, Median survival time (ST50) estimates were obtained by using standard Kaplan–Meier survivorship analysis, and were subjected to ANOVA followed by the Dunnett's test. All statistical analysis was performed using SAS 8.01(SAS 2000).

Results

Growth of entomopathogenic fungi on cuticle components

The biomass produced by IF28.2 in the basal medium having 1% (w/v) solution or suspensions of different carbon sources after 5 days of inoculation was significantly different among different treatments and control ($F_{5,12} = 17.98$, $P < 0.0001$). The lowest biomass production (1.34 ± 0.34 mg/ml) was observed in control while the highest biomass (10.86 ± 1.02 mg/ml) was obtained from the basal medium having 1% chitin as a carbon source (Table 1).

Biomass production by *I. fumosorosea* isolate IF32 in the basal medium having different carbon sources differed significantly among different treatments and control ($F_{5,12} = 27.12$, $P < 0.0001$). The highest biomass production (9.22 ± 0.83 mg/ml) was observed for the basal medium having 1% chitin as a carbon source, whereas the

Table 1 Influence of different carbon sources on biomass production by *I. fumosorosea* after 5 days of fungal growth

Carbon source	Mean fungal biomass weight (mg/ml)		
	P28.2	P32	P49
Alanine	6.80 ± 0.28b	5.76 ± 0.62b	5.06 ± 0.58a
Chitosan	4.00 ± 0.74c	3.39 ± 0.47c	2.98 ± 0.37b
Chitin	10.86 ± 1.02a	9.22 ± 0.83a	8.11 ± 0.46c
Isoleucine	2.76 ± 0.24cd	2.33 ± 0.39d	2.04 ± 0.48cd
Glycine	2.23 ± 0.17d	1.88 ± 0.26d	1.64 ± 0.28d
Control	1.34 ± 0.34e	1.23 ± 0.13e	1.09 ± 0.18e

Means in the same column with different letters are significantly different from each other (Tukey's, $P < 0.05$)

±: Standard error (based on three replicates)

lowest biomass production (1.23 ± 0.13 mg/ml) was observed for control (Table 1).

The production of biomass by *I. fumosorosea* isolate IF49 in the basal medium having different carbon sources after 5 days of inoculation differed significantly among different treatments and control ($F_{5,12} = 32.12$, $P < 0.01$). The lowest biomass production (1.09 ± 0.18 mg/ml) was observed in control while the highest biomass (8.11 ± 0.46 mg/ml) was obtained from the basal medium having 1% chitin as a carbon source (Table 1).

Sequential production of cuticle degrading enzymes by entomopathogenic fungi

The pattern of extracellular enzymes production in culture containing 1% chitin was studied with three different *I. fumosorosea* isolates (Fig. 1a–e). In all cultures enzymes were produced sequentially and showed a distinct phase of rapid production. The analysis of repeated measures ANOVA resulted in significantly different interaction effect between different isolates and time intervals for Subtilisin-like (Pr1) activity ($F_{15,46} = 26.91$, $P < 0.0001$) and trypsin-like (Pr2) activity ($F_{15,46} = 36.30$, $P < 0.01$). These enzymes appeared first within 24 h and a rapid increase in production was observed after 48 h (Fig. 1a, b). Similarly a significantly different interaction effect between different isolates and time intervals was also observed for lipase ($F_{15,46} = 10.83$, $P < 0.024$) and chitinase activity ($F_{15,46} = 33.96$, $P < 0.0001$). These enzymes started to appear within first 24 h and then increased rapidly after 48 h (Fig. 1c, d). Interaction effect between different isolates and different time intervals proved to be significantly different for chitinase activity ($F_{15,46} = 20.59$, $P < 0.0001$). This enzyme started to appear between 24 and 48 h and there was a sudden increase in their production after 48 h which became almost stable between 96 and 120 h (Fig. 1e).

Range of cuticle degrading enzymes produced by entomopathogenic fungi

I. fumosorosea grew in liquid culture medium having 1% chitin as sole carbon source and produced a range of extracellular cuticular degrading enzymes corresponding to the major components of insect cuticle, i.e., chitin, protein, lipids. Enzyme activities in 5 day culture filtrates are shown in Table 2. Marked variations in enzyme levels between different isolates as well as different species were also found which may be accounted for difference in growth rates and sequential phasing of enzyme production.

As shown in Table 2, Subtilisin-like (Pr1) activity was exhibited by different *I. fumosorosea* isolates under liquid culture conditions; however, the amount of secreted

enzyme varied significantly from control ($F_{3,8} = 10.6$, $P < 0.021$). The highest level of Pr1 activity (29.14 ± 1.34 U/mg) was found in the supernatants from IF28.2 while the lowest Pr1 activity was detected for control (mean of Pr1 production by all three isolates in basal medium without chitin) having a mean value of 7.35 ± 0.49 U/mg.

The trypsin-like (Pr2) activity shown by different *I. fumosorosea* isolates in the basal medium after 5 days of inoculation was significantly different from control ($F_{3,8} = 24.21$, $P < 0.001$). The lowest Pr2 activity was observed for control (mean of Pr2 production by all three isolates in basal medium without chitin) having a mean value of 2.91 ± 0.61 U/mg whereas the highest Pr2 activity (14.67 ± 1.05 U/mg) was found in the supernatants inoculated with *I. fumosorosea* isolate IF28.2 (Table 2).

The lipase production by different *I. fumosorosea* isolates (except IF49) differed significantly from control ($F_{3,8} = 15.68$, $P < 0.001$). Maximum lipase activity (1.51 ± 0.36 U/mg) was shown by *I. fumosorosea* isolate IF28.2 whereas the lowest lipase activity was observed for control (mean of lipase production by all three isolates in basal medium without chitin) a mean value of 0.69 ± 0.13 U/mg.

Chitinases were produced in varying amounts by different *I. fumosorosea* isolates when compared to the control ($F_{3,8} = 28.01$, $P < 0.001$). *I. fumosorosea* isolate IF28.2 proved to be the most active producer of chitinases showing average activity of 16.86 ± 0.54 mU/mg while the lowest chitinases activity (4.94 ± 0.72 mU/mg) was observed for IF49 (Table 2).

Significant differences were also observed among different *I. fumosorosea* isolates for chitinase activity when compared to the control ($F_{3,8} = 14.88$, $P < 0.0001$). Maximum chitinase activity was shown by *I. fumosorosea* isolate IF28.2 having a mean value of 6.38 ± 0.70 mU/mg whereas the lowest chitinase activity (0.91 ± 0.37 mU/mg) was observed for control (mean of chitinase production by all three isolates in basal medium without chitin).

Studies on antifeedant activity

Effect on larval feeding rate

A significantly different interaction effect between the culture filtrates of different isolates used for coating the leaves and different time intervals was observed for cumulative percent leaf consumption ($F_{12, 38} = 13.03$, $P < 0.0001$). Lowest rates of % leaf consumption at different time periods were observed for *I. fumosorosea* isolate IF28.2. After 5 days feeding rates for IF49 were comparatively higher but these were still lower than the control (mean of all the three isolates in the basal medium without chitin) on that day (Fig. 2).

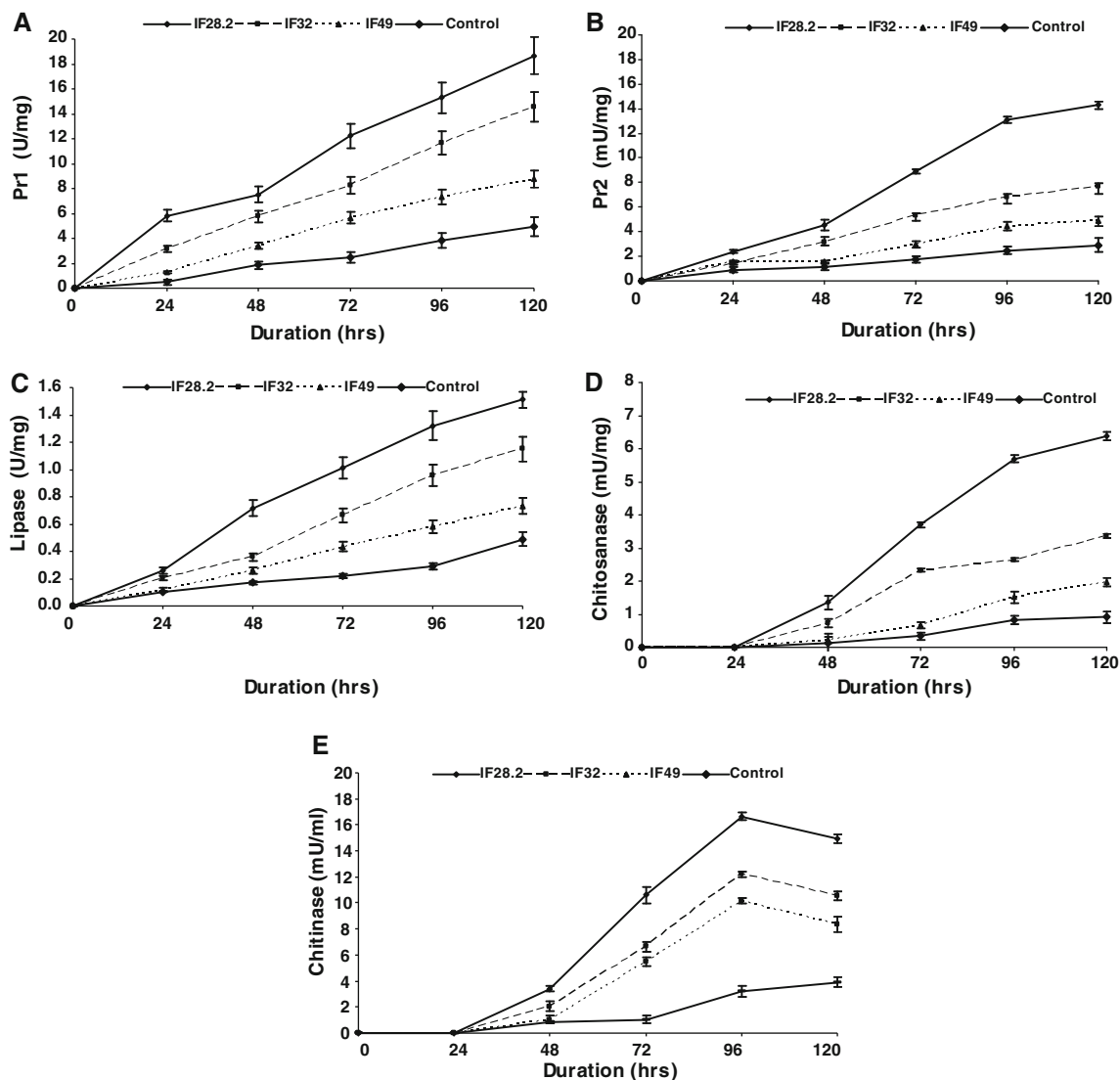


Fig. 1 Sequential production of Extracellular cuticle degrading enzymes in 5 day culture filtrates from isolates of *I. fumosorosea*. **a** Pr1; **b** Pr2; **c** lipases; **d** chitosanase; and **e** chitinase. * Control stands average activity of all the three isolates in the basal medium without chitin

Table 2 Extracellular cuticle degrading enzymes produced by different isolates of *I. fumosorosea* in basal medium having 1% chitin after 5 days of fungal growth

Isolate	Pr1 (U/mg)	Pr2 (U/mg)	Lipase (U/mg)	Chitinase (mU/mg)	Chitosanase (mU/mg)
IF28.2	29.14 ± 1.34a	14.67 ± 1.05a	1.51 ± 0.36a	16.86 ± 0.54a	6.38 ± 0.70a
IF32	18.65 ± 0.87b	7.67 ± 0.82b	1.15 ± 0.57ab	12.19 ± 0.35b	3.35 ± 0.43b
IF49	14.57 ± 0.69b	4.86 ± 0.78c	0.73 ± 0.09 bc	10.15 ± 0.63c	1.95 ± 0.39c
Control ^a	7.35 ± 0.49c	2.91 ± 0.61d	0.49 ± 0.13 c	3.93 ± 0.72d	0.91 ± 0.37c

Means in the same column with different letters are significantly different from each other (Tukey's, $P < 0.05$)

±: Standard error (based on three replicates)

^a Control stands average activity of all the three isolates in the basal medium without chitin

Effect on larval body weight

Interaction effect between the culture filtrates of different isolates used for coating the leaves and different time

intervals for increase in body weight by the larvae was significant ($F_{27,78} = 15.57$, $P < 0.001$). The control group performed better in terms of growth as indicated by increased body weight. Almost similar rates of increase in

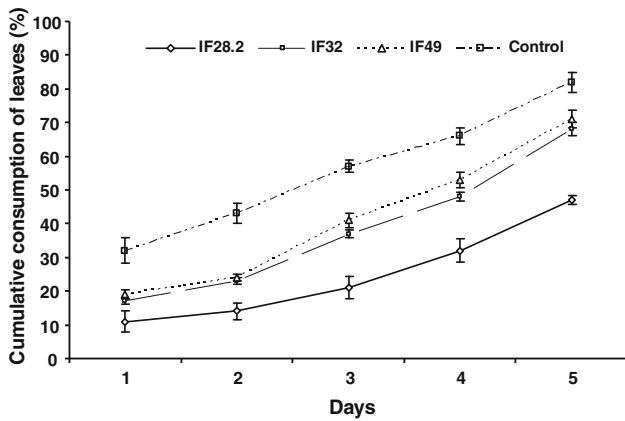


Fig. 2 Effect of hydrolytic enzyme culture filtrates of *I. fumosorosea* on larval feeding rate. Bars represent standard error of means (based on three independent replicates) * Control stands average activity of all the three isolates in the basal medium without chitin

body weight were observed for *I. fumosorosea* isolates IF32 and 49 while for IF28.2 the rates of larval growth were lowest when compared to the other treatments (Fig. 3).

Growth inhibition studies

Effect on larval development

The percentage pupation after 10 days culture filtrates application was significantly different among different *I. fumosorosea* isolates and control ($F_{3,8} = 40.42, P < 0.001$). The lowest rates of successful pupation were observed for IF28.2 having mean value of $11.50 \pm 0.64\%$ while the highest rates of % pupation ($74.30 \pm 1.08\%$) was observed for the control (mean of all the three isolates in basal medium without chitin) (Fig. 4).

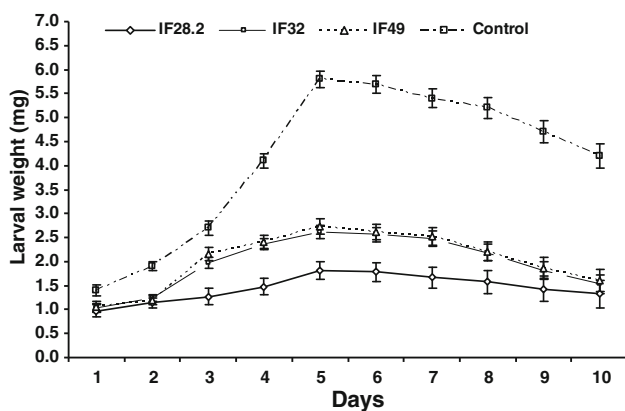


Fig. 3 Effect of hydrolytic enzyme culture filtrates of *I. fumosorosea* on larval body weight. Bars represent standard error of means (based on three independent replicates) * Control stands average activity of all the three isolates in the basal medium without chitin

Effect on adult emergence

Significantly different rates of % adult emergence were observed among culture filtrates of different *I. fumosorosea* isolates and the control ($F_{3,8} = 23.61, P < 0.001$). It can be observed that the percentage of adult emergence was lowest ($4.67 \pm 0.57\%$) for *I. fumosorosea* isolate IF28.2 where the highest rate of adult emergence was observed for control (Fig. 5).

Effect on larval mortality

Mortality of larvae/pupae due to the treatment was measured until the 10th day. At the end of the test period, the analysis of median survival time (ST_{50}) showed significant differences between different isolates and control ($F_{3,8} = 17.78, P < 0.001$). Culture filtrates from IF28.2 proved to be the most aggressive showing lowest ST_{50} value (1.57 ± 0.20 days), and those from control were the least virulent (Fig. 6).

Discussion

Effect of chitin, chitosan, and amino acids components (alanine, glycine, and isoleucine) of insect cuticle as a sole carbon source on biomass production by *I. fumosorosea* was determined. This approach was used to ensure that any possible delay or absence in enzyme production was not due merely to non utilization of possible inducing substrates. Higher biomass values were obtained for chitin and amino acid alanine which is similar to the findings of St. Leger et al. (1986b) who observed rapid and extensive growth of *M. anisopliae* isolate ME1 on NAG and alanine. They also observed good growth on olive oil but only in the presence of 0.2% $NaNO_3$ as nitrogen source.

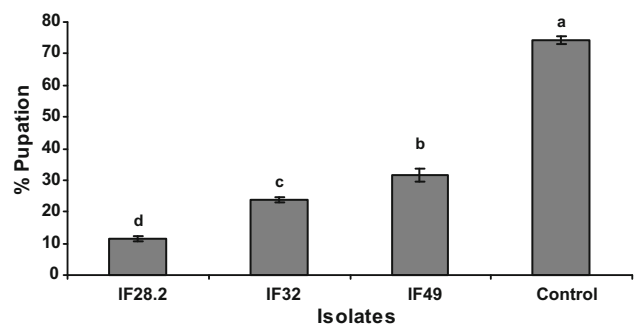


Fig. 4 Effect of topical application of culture filtrates of hydrolytic enzymes from *I. fumosorosea* on % pupation. Legends with different letters are significantly different from each other (Tukey's, $P < 0.05$); Bars represent standard error of means (Based on three independent replicates). * Control stands average activity of all the three isolates in the basal medium without chitin

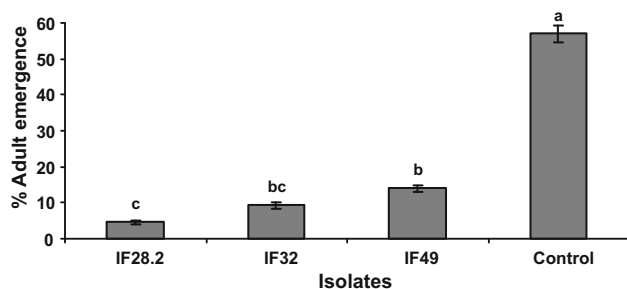


Fig. 5 Effect of topical application of culture filtrates of hydrolytic enzymes from *I. fumosorosea* on % adult emergence. Legends with different letters are significantly different from each other (Tukey's, $P < 0.05$); Bars represent standard error of means (Based on three independent replicates). * Control stands average activity of all the three isolates in the basal medium without chitin

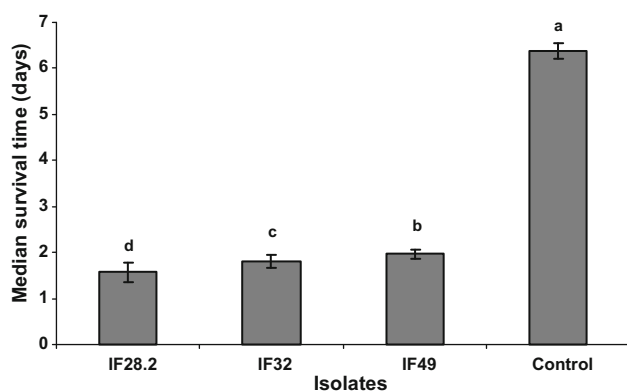


Fig. 6 Effect of topical application of culture filtrates of hydrolytic enzymes from *I. fumosorosea* on median survival time (ST50). Legends with different letters are significantly different from each other (Tukey's, $P < 0.05$); Bars represent standard error of means (based on three independent replicates). * Control stands average activity of all the three isolates in the basal medium without chitin

To gain an indication how host influences the growth of cuticle degrading enzymes; isolates of *I. fumosorosea* were grown on 1% chitin as a sole carbon source. During these studies higher levels of chitinase and protease were observed which are similar to the findings of St. Leger et al. (1986a) who also suggested that protease play an important role in cuticle as chitinolytic enzymes appear after proteolytic enzymes. Therefore, it was important to investigate the other chitin metabolizing enzymes such as chitosanase, NAGase, and lipase that can help in infection process. Detection of higher levels of these enzymes in the culture filtrates suggested that these enzymes are also involved in the infection process which is similar to the findings of St. Leger et al. (1986b) who also observed these enzymes in the fungal culture filtrates having insect cuticle or chitin as sole carbon source. Qazi and Khachatourians (2007) showed that *B. bassiana* can produce higher amount of

proteases and other cuticle degrading enzymes when aphid exuviae were added to the basal medium. Similarly, St. Leger et al. (1991) showed that conidia of *M. anisopliae* growing on insect cuticle have higher level of protease, chitinase, esterase in comparison to Sabouraud dextrose agar. A possible reason for higher levels of enzyme can be the composition of insect cuticle that comprises of about 70% protein and these enzymes may have an important role in host penetration (Gillespie et al. 1998).

Cuticle degrading enzymes were produced rapidly and sequentially in the culture medium. The first to appear were proteases and lipases and their rapidity increased after 24 h which can be an important factor in pathogenesis providing nutrients and enabling onset of infection process before host defenses become effective. Chitinase and other related enzymes, which are induced by chitin breakdown appears substantially later than proteases. These findings are similar to St. Leger et al. (1986a) who observed that appearance of extracellular enzymes was paralleled by the sequential solubilization of cuticle constituents. This sequence of enzyme production can be explained by the fact that naturally, fungal conidia are endowed for a robust mode of life, and process of pathogenesis only starts if the release of degradation products from cuticle exceeds the needs for fungal growth which can only occur in nutrient rich hemolymph and tissues but not during the pre-penetration phase (Qazi and Khachatourians 2008). Therefore, the appearance of Pr1 and Pr2 shows a clear advantage for different fungal isolates used during these studies in acquiring nutrients to enable the onset of cuticle degradation and fungal penetration.

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). Our results about the effects of culture filtrates having cuticle degrading enzymes showed that the control group performed better in terms of growth as indicated by increased body weight which are similar to the findings of Binod et al. (2007) who showed that hydrolytic enzyme culture filtrates from entomopathogenic fungi, *T. harzianum*, were capable of negatively effecting the growth and metamorphosis of *Heliothis* larvae.

Microbial cuticle degrading enzymes 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 toward diamond back moth larvae (Kramer and Muthukrishnan 1997). The studies conducted for testing the efficacy of fungal culture filtrates of cuticle degrading enzymes against the insect pest *P. xylostella* have shown that the cuticle degrading enzymes are capable of negatively affecting the growth and metamorphosis of the larvae. This is true when these enzymes are 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 enzymes or the larvae are directly exposed. It is evident that at the different isolates tested can induce very low ST₅₀ values when compared to the control. It may still be speculated that the adults emerging from 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 cuticle metabolizing enzymes. This might enable the use of cuticle metabolizing enzyme sprays combined with other pesticide formulations to facilitate with other pesticide formulations to facilitate faster kill.

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