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Evaluation of Chitinase from *Metarhizium anisopliae* as Biopesticide Against *Plutella xylostella*

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Abstract.- Chitinases are virulence determination factors of entomopathogenic fungi which perform critical functions during degradation of insect cuticle. The present study was conducted to evaluate the biocontrol efficacy of fungal culture filtrate containing chitinase from *M. anisopliae* against *P. xylostella*. *M. anisopliae* was cultured by submerged fermentation using colloidal chitin as sole carbon source. Maximum chitinase yield (105.32±1.19 mU/ml) was recorded at 96 h of incubation showing the ability of the culture filtrate to hydrolyse the colloidal chitin. Biocontrol assay against *P. xylostella* showed that the culture filtrates were potent antifeedants by reducing 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 larval mortalities (67.89±3.11 %) were recorded for groups treated with 600 mU ml^{-1} chitinase activity. The maximum reduction in cuticular weight (75±1.34%) and reduction in chitin content/larva (69±2.11) was also observed for the highest chitinase concentration (600 mU ml^{-1}) tried. Our results showed that the culture filtrate containing chitinase from *M. anisopliae* are capable of negatively affecting the growth and metamorphosis of *P. xylostella* larvae. 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.*

Key Words: Biocontrol, cuticle degrading enzymes, biopesticides, Cordycipitaceae, Plutellidae.

INTRODUCTION

Insect cuticle is a composite structural material with mechanical properties that are optimal for their biological functions. The cuticle consists of a thin outer epicuticle containing lipids and proteins and a thick procuticle consisting mainly of chitin and proteins (Andersen *et al*., 1995; Samson *et al*., 1988). Entomopathogenic fungi enter their hosts through direct penetration of the cuticle, which is a barrier against most microbes. Consequently, fungal pathogens have a potential as a biological means of controlling sap-sucking insects that have not been easily controlled with chemical pesticides. During the fungal penetration through the host cuticle, hydrolytic enzymes such as proteases, chitinases, and lipases are produced and secreted and are important for the initiation of the infection process (Schagger and von Jagow, 1987; Yang and Yeh, 2005). Chitinases catalyze the hydrolysis of chitin, which is a b-(1,4)-linked polymer of N-acetyl-D-glucosamine and one of the important structural components of insect cuticle (Tsigos and Bouritos, 1995). Chitinases are produced by a large number of organisms including plants, fungi, and bacteria, and play an important role in the defense mechanism of plants against pathogens and in the mycoparasitic process of fungi. They also play an important role in nutrition, development, and morphogenesis of fungi. However, the role of the chitinases in the host infection process is not yet fully understood.

Metarhizium anisopliae Sorokin (Clavicipitaceae; Hypocreales) is one of the most promising fungal species currently being investigated as a biological control agent against diamondback moth, whiteflies and other insect pests (Altre *et al.,* 1999). A range of extracellular enzymes that can degrade the components of the insect cuticle are produced when *M. anisopliae* is grown *in vitro* with cuticle as the sole carbon source (Clarkson and Charnley, 1996). The regulation of cuticle-degrading enzyme is probably complex and may involve a combination of carbon/nitrogen induction and/or repression (Ali *et al*., 2009). Although much work has been carried out on the chitinolytic activity as well as characterization of chitinases produced by different entomopathogenic

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fungi yet the role of purified enzymes in fungal pathogenicity has not been well studied.

The objective of the present study was to evaluate the efficacy of fungal culture filtrate as biocontrol agent against diamondback moth. *M. anisopliae* isolate M408 was cultured under submerged condition and the culture filtrate was analyzed for both chitinase and protease activities. Lyophilized extracts were tested on the larvae. Effect of enzyme feeding on larval development was measured by observing the rates of feeding and changes in body weight induced by the treatment. Effect of topical application of enzymes on larval development was measured by changes rate of pupation, adult emergence, mortality and rates of cuticle degradation induced by the treatments.

MATERIALS AND METHODS

Fungal strains

M. anisopliae isolates (M408) 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 *M. anisopliae* was cultured on potato dextrose agar (Potatoes, infusion 200 g/L; Dextrose 20 g/L and Agar 20 g/L) and incubated at 26±2°C for 10 days (Ali *et al.,* 2009). Conidia were harvested with distilled water containing 0.03% Tween 80 and sieved through filter paper into sterile vials. Conidia were counted using a compound microscope in hemocytometer (0.0625m m^2; Fuchs-Rosenthal Merck Euro Lab, Darmstadt, Germany) to calibrate a suspension of 1×10^7 conidia/ml.

Insect cultures

Larvae of *P. xylostella* were obtained from the stock cultures kept in greenhouse of the Engineering Research Center of Biological Control, South China Agricultural University on *Brassica campestris* L., respectively. Plants were grown in plastic pots having a diameter of 15 cm. Sufficient 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. Second instar larvae were used for antifeedant studies whereas fourth instar was used for growth inhibition studies.

Submerged fermentation and preparation of enzyme concentrations

The chitinase was produced by growing *M. anisopliae* in liquid. Basal medium (pH 7.2) which 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). As a carbon source 1% chitin was added to previously sterilized basal medium (121°C, 15 min) The flasks were inoculated with one ml of 1 x 10^7 spores/ml and incubated at 180 rpm and 30°C for 5 days. Samples were removed at 24 hrs intervals used for further enzymatic analysis. To prepare the culture filtrates for biological control studies the inoculum was harvested by centrifugation at 1000 x g for 10 min at 4°C in a Microfuge®18 with a F241.5P rotor (Beckman Coulter, Inc, USA). The clear supernatant was concentrated to powder (600 mU ml^{-1}) by lyophilization. The powder was reconstituted with distilled water to obtain the other enzyme concentrations (400, 200, 100, 50 mU ml^{-1} powder) and distilled water was used as a control.

Determination of antifeedant activity

The efficacy of fungal culture filtrate as an antifeedant was tested by feeding larvae with fresh *B. campestris* leaves coated with different concentration of chitinase. Leaf discs (10 cm^2) 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 feedings 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 percent transformed by the following relationship

Percentage leaf consumption = (Leaf area consumed/total leaf area) x 100%

Effect of feeding on larval development was measured by changes in body weight. Each

treatment and control was repeated three times with a new batch of insects and new conidial suspensions, and for each repetition with four leaves, each leaf with 20 diamondback moth larvae.

Studies on growth inhibition of diamondback moth

In the bioassay experiment for growth inhibition five microlitres of each preparation was applied topically on the thorax back of the 4th instar larvae (20 larvae/treatment) using a micropipette. Five micro litres of distilled water served as control. After the application of different treatments 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 to maintain the 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 in an air-conditioned room 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 different treatments 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 (10 days).

Determination of reduction in cuticle weight and chitin contents

Five microlitres of each concentration was applied topically on the thorax back of the 4th instar larvae using a micropipette whereas the application of distilled water 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. Leaf disks were replaced every 2 days except during the pupal stage. The insects were placed in an air-conditioned room at 26°C and >95% R.H. and the rate of % mortality induced by the treatments were observed at every 24hrs interval. Larvae were observed for mortality up to 5 days and dead larvae were kept at 4°C. After 5 days, the cuticle of all the larvae was removed in Ringer's solution (NaCl, 0.9%; KCl, 0.04%; $CaCl_2$, 0.02%). The amount of chitin in larval cuticle was estimated according to Nahar (2004). The percent reduction in cuticular weight as well as % reduction in chitin contents was calculated relative to control.

Analytical determinations

Colloidal chitin was prepared by the method of Roberts and Selitrenikoff (1988) with some modification. One hundred grams of chitin flakes were added slowly to 1.75 liter concentrated HCl and agitated gently for 3 hours on a magnetic stirrer. This solution was then filtered to 20 liter 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 at 560 nm. One unit (U) of activity was defined as the amount of enzyme which catalyzed the release of one 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.

Chemicals and reagents

All the chemicals were obtained from Guangzhou Jinhuadu chemical reagent company, Guangzhou, China. Chitin obtained from crab shell was purchased from Sinopharm Chemical Reagent Company, Shanghai, China.

Statistical analysis

Protein production and enzymatic activities after 5 days, % 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, % larval feeding and changes in larval body weight were analyzed by Repeated Measures ANOVA. Mortality data was percent transformed and subjected to ANOVA followed by the Tukey's test. All statistical analyses were performed using SAS 8.01 (SAS, 2000).

RESULTS

Chitinase production by M. anisopliae *in the basal medium having 1% chitin as carbon source*

The chitinase activity shown by *M. anisopliae* in the basal medium differed significantly at different incubation periods ($F_{5,12}$ = 29.47, P<0.001). Maximum chitinase yield (105.32±1.19 mU/ml) was recorded at 96 h of incubation. Beyond this period, the enzyme yield was found to be decreasing as shown in Table I. At 120 h, the yield was reduced when compared to the yield obtained at 96 h however, the amount of secreted protein varied significantly at different time intervals ($F_{5,12}$ = 10.6, P<0.021) and gradually increased with the passage of time having highest protein concentration (124.14±1.23 U/ml) after 120h (Fig.1).

Table I.- Effect of different concentrations of chitinase from *I. fumosoroseus* on cuticle degradation of *P. xylostella*

Concentrations (mU/ml)	Reduction cuticle weight (%)	Reduction in chitin content (%)
600	75±1.34 a	69±2.11 a
400	69±1.82 b	60±1.76 b
200	59±1.65 bc	51±1.36 c
100	42±1.23 c	34±1.51 d
50	26±1.15 d	22±0.87 e
F; df; P	12.97; 4; <0.001	14.69; 4; <0.001

Mean (± SE; n = 3) in the same column with different letters are significantly different from each other (Tukey's, P<0.05)

Effect of chitinase on larval feeding rate

The different concentrations of chitinase when used for coating the leaves resulted in a lesser consumption of feed by the larvae (Fig.3). A significantly different interaction effect between the culture filtrates of different concentrations used for coating the leaves and different time intervals was observed for % leaf consumption ($F_{20, 60}$ = 17.92, P<0.0001). For chitinase concentration of 600 mU/mL, larval feeding was lowest than in the case of other treatments as well as control, while statistically similar rates of feeding were observed for 100 and 200 mU/mL. After 5 days feeding rate for 50 mU/mL was comparatively higher but these were still lower than the control on that day (Fig. 2)

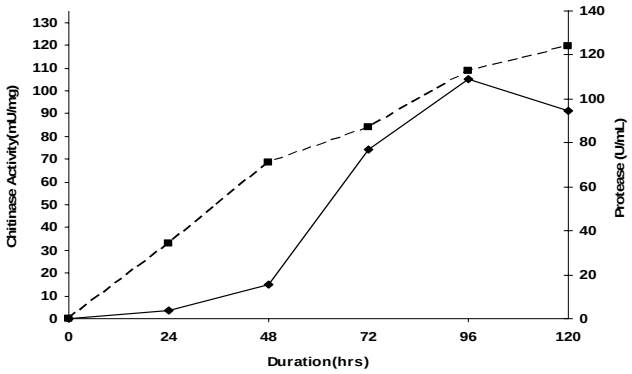

Fig 1. Chitinase activity of *M.anisopliae* at different temperatures after 5 days of growth.

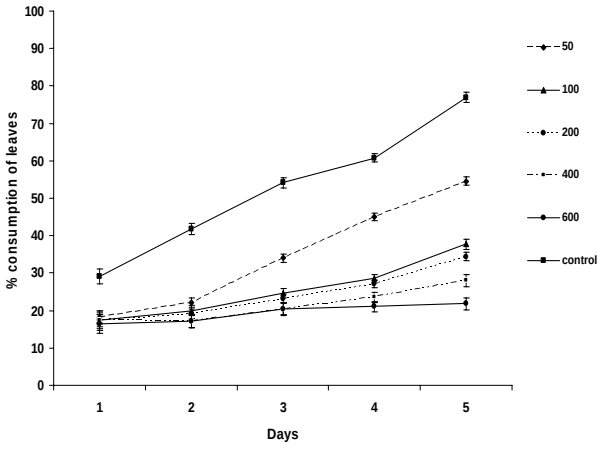

Fig 2. Effect of different concentrations of chitinase on larval feeding rate. Bars represent standard error of means (Based on three independent replicates).

Effect on larval body weight

Data presented in Figure 3 shows significantly different changes in mean body

weights of experimental groups fed on leaves treated with different chitinase concentrations. 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 ($F_{45,129} = 21.97$, $P<0.001$). The control group performed better in terms of growth as indicated by increased body weight. It might be speculated that feeding on 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. Almost similar rates of increase in body weight were observed for enzyme concentrations of 200 and 100 mU/mL while for 600 mU/mL the rates of larval growth were lowest when compared to the other treatments (Fig. 3).

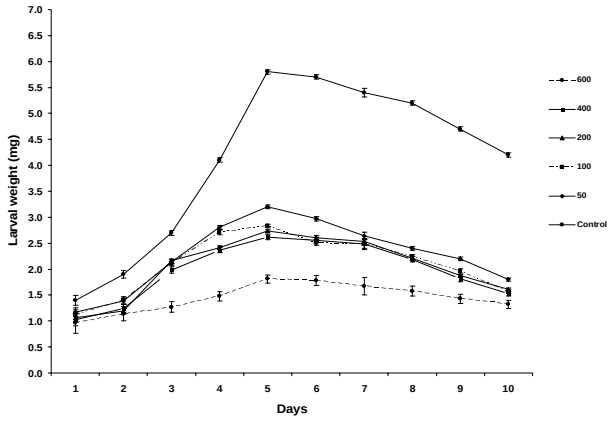

Fig. 3. Effect of different concentrations of chitinase on larval weight. Bars represent standard error of means (based on three independent replicates).

Effect on larval development

The percentage of successful pupation was calculated based on the sum of healthy pupae and is represented in Figure 4. The percentage pupation after 10 days culture filtrates application was significantly different among different treatments and control ($F_{5,12} = 31.12$, $P<0.001$). The lowest rates of successful pupation were observed for 600 mU/mL having mean value of 11.50±0.64%, while the highest rates of % pupation (74.30±1.08%) was observed for the control.

Effect on adult emergence

Significantly different rates of % adult emergence were observed among enzyme concentrations and control ($F_{5,12} = 27.51$, $P<0.001$). The percentage of adult emergence was lowest (4.67±0.57%) for enzyme concentration of 600 mU/mL while the highest rate of adult emergence was observed for control (Fig. 4).

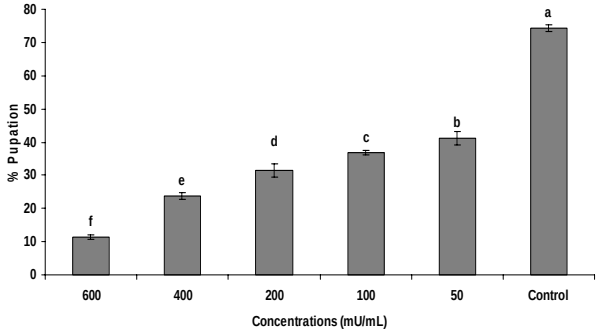

Fig. 4. Effect of different concentrations of chitinase on % pupation. Legends different letters are significantly different from each other (Tukey's, $P<0.05$); Bars represent standard error of means (based on three independent replicates)

Effect on larval mortality

Mortality of larvae / pupae because of chitinase treatments was measured on the 10th day. At the end of the test period, significantly different rates of percentage mortality were observed between different treatments and control ($F_{5,12} = 19.76$, $P<0.001$). The lowest mortality was (3.80±1.096 %) was recorded for control and the highest mortality was observed for chitinase concentration 600 mU/mL having a mean value of 67.89±3.11% (Fig.5).

Effect of chitinase on cuticle degradation of P. xylostella

The weight of the cuticle /larvae as well as chitin content/larvae was significantly affected by the application of different concentrations of chitinase from *M. anisopliae*. With the increase in concentration of chitinase, the loss in weight of cuticle/larva and chitin content/larval cuticle

became more pronounced suggesting the degradation of chitin in the cuticle due to chitinase (Table I). It can be observed that the percentage of reduction in the cuticular weight was lowest (26±1.15 %) for 50mU/mL while the maximum reduction (75±1.34%) was observed at chitinase concentration of 600mU/mL. Similarly the highest reduction in chitin contents/larva (69±2.11 %) was observed for 600 mU/mL, whereas the lowest reduction was observed for control (Table I).

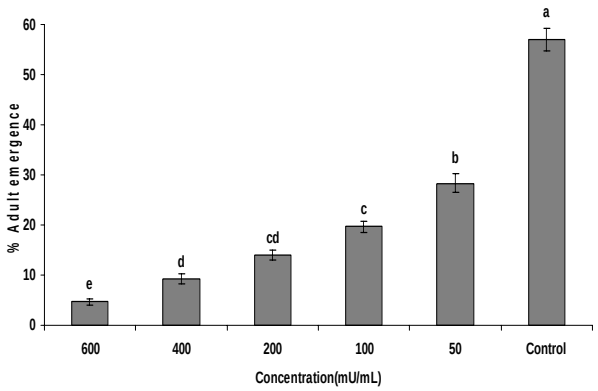

Fig 5. Effect of different concentrations of chitinase on % adult emergence. Legends different letters are significantly different from each other (Tukey's, P<0.05); Bars represent standard error of means (based on three independent replicates).

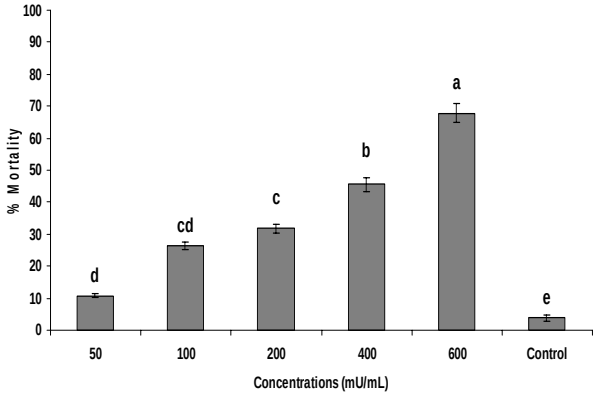

Fig 6. Effect of topical application of different concentrations of chitinase on percentage mortality of *P. xylostella.* Legends different letters are significantly different from each other (Tukey's, P<0.05); Bars represent standard error of means (based on three independent replicates).

DISCUSSION

Chitin, a naturally abundant mucopolysaccharide and the supporting material of crustaceans, insects etc consist of 2-acetamido 2-deoxy-*β*-D-glucose (N-acetyl glucosamine) through a β(1–4) linkage (Majeti, 2000). The complete enzymatic hydrolysis of chitin to free N-acetyl glucosamine is performed by a chitinolytic system consisting of two fractions, endochitinase and chitobiase. The physiological functions of chitinase (EC 3.2.1.14) depend on their source. In bacteria, chitinases play roles in nutrition and parasitism whereas in fungi, protozoa and invertebrates they are involved in morphogenesis. Chitinases are involved in the defense mechanism of plants and invertebrates (Gooday, 1995). During the last decade, chitinases have received increased attention because of their wide range of applications. The major applications include use of chitinases for the biocontrol of plant pathogens (Lorito *et al.,* 1993; Mathivanan *et al*., 1998) and for developing transgenic plants (Lorito and Scala, 1999; Bolar *et al*., 2000).

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. In order to penetrate penetration through the insect cuticle, deuteromycete fungi such as *Metarhizium* 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).

Perforations of the peritrophic membrane was proposed by Brandt *et al*. (1978), thus facilitating entry of pathogen into the susceptible insects. Perforation of peritrophic membrane was also observed by Regev *et al*. (1996) when 5th instar *Spodoptera* larvae were fed on a diet containing a recombinant endochitinase encoded by *Serratia marcescens*. Binod *et al.* (2005) showed that the culture filtrates containing chitinase from *T. harzianum* negatively effected the growth and metamorphosis of *Heliothis* larvae. Our studies are also in line with the above mentioned findings showing that the different enzyme levels are capable of causing an inhibitory effect on the growth and metamorphosis of *P. xylostella*. It is also evident from our findings that a considerable reduction in the cuticular weight, % chitin contents and percentage mortality until the pupal stage. It can also be speculated that the emerging adults from treated larvae may be abnormal or incapable of normal life. Keeping in mind the very slow speed of kill of entomopathogenic fungi, there is a need to find alternate ways of using these pathogens in biological control of insects and a possible alternate can be the use of the use of chitinase sprays combined with other pesticide formulations to facilitate faster kill of insect pests.

The results described above confirm the degradative action of chitinase preparation on the insect cuticle, which ultimately led to insect death. But so far, the effect of cuticle degrading enzyme complex of *M. anisopliae* in the control of *P. xylostella* was not demonstrated. Therefore, it can be concluded that the study of chitinase in *M. anisopliae* is important because it perform critical functions by their involvement in growth and degradation of the fungal cell wall and insect cuticle, as chitin is a major component of both and are virulence determination factors (Kachatourians, 1991, 1996; Charnely, 1997). Implication of chitinases in degradation of insect cuticle could thus be an important tool in the knockdown of insects, especially *P. xylostella* in shorter time. However, stability of the enzyme preparation under field conditions would be a major concern and needs further studies in the future.

REFERENCES

ALI, S., HUANG, Z. AND REN, S.X., 2009. Production and regulation of extracellular proteases from the entomopathogenic fungus *Isaria fumosoroseus* (Cordycipitaceae: Hypocreales) in the presence of diamondback moth cuticle. *Biocontr. Sci. Technol.*, **19**: 523-535.

ALTRE, J.A., VANDENBERG, J.D. AND CANTONE, F.A., 1999. Pathogenicity of *Paecilomyces fumosoroseus* isolates to diamondback moth, *Plutella xylostella*: correlation with spore size, germination speed, and attachment to cuticle. *J. Inverteb. Path*., **73**: 332-338.

ANDERSEN, S.O., 2002. Characteristic properties of proteins from pre-ecdysial cuticle of larvae and pupae of the mealworm *Tenebrio molitor*. *Insect Biochem. mol. Biol*., **32**: 1077-1087.

ANDERSEN, S.O., HOJRUP, P. AND ROEPSTORFF, P., 1995. Insect cuticular proteins. *Insect Biochem. mol. Biol*., **25**: 153-176.

BINOD, P., PUSZTAHELYI, T., NAGY, V., SANDHYA, C., SZAKACS, G., POCSI, I. AND PANDEY, A., 2005. Production and purification of extracellular chitinases from *Penicillium aculeatum* NRRL 2129 under solid-state fermentation. *Enz. Microb. Technol*., **36**: 880-887.

BOLAR, J.P., NORELLI, J.L., WONG, K.W., HAYES, C.K., HARMAN, G.E. AND ALDWINCKLE, H.S., 2000. Expression of endochitinase from *T. harzianum* in transgenic apple increases resistance to apple scab and reduced vigor. *Phytopathology*, **90**: 72.

BRADFORD, M.M., 1976. A rapid and sensitive method for

the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248-254.

BRANDT, C.R., ADANG, M.J. AND SPENCE, K.D., 1978. The peritrophic membrane: ultrastructural analysis and function as a mechanical barrier to microbial infection in Orgyia pseudotsugata. *J. Inverteb. Path.*, **32**: 12-24.

CHARNELY, A. K., 1997. Entomopathogenic fungi and their role in pest control. In: *Environmental and microbial relationships, the Mycota IV* (eds. D.T. Wicklow and B.E. Soderstorm)., Springer, Heildelberg, pp.185-201.

CLARKSON, J.M. AND CHARNLEY, A.K., 1996. New insights into mechanisms of fungal pathogenesis in insects. *Trends Microbiol.*, **4**:197-204.

COUDRON, T.A., KROHA, M.J. AND IGNOFFO, C.M., 1984. Levels of chitinolytic activity during development of three entomopathogenic fungi. *Comp. Biochem. Physiol.*, **79**: 339-348.

EL-SAYED, G.N., COUDRON, T.A., IGNOFFO, C.M. AND RIBA, G., 1989. Chitinolytic activity and virulence associated with native and mutant isolates of the entomopathogenic fungus, *Nomuraea rileyi*. *J. Inverteb. Path.*, **54**: 394-403.

GOODAY, G.W., 1995. Diversity of roles for chitinases in nature. In: *Chitin and Chitosan* (eds. M.B. Zakaria, W.M. Wan, Muda and M.P. Abdullah). Malaysia Penerbig Universitiy, Kebangsaan, pp. 191-202.

KHACHATOURIANS, G.G., 1991. Physiology and genetics of entomopathogenic fungi. In: *Handbook of applied mycology*, *vol 2* (eds. D.K. Arora, L, Ajello and K.G. Mukerji). Marcel Dekker Inc., New York, pp. 613-661.

KHACHATOURIANS, G.G., 1996. Biochemistry and molecular biology of entomopathogenic fungi. In: *Human and animal relationships* (eds. D.H. Howard, J.D. Miller), Mycota VI, Springer, Heidelberg, pp. 331-363.

KRIEGER DE MORAES, C., SCHRANK, A. AND VAINSTEIN, M.H., 2003. Regulation of extracellular chitinase and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. *Curr. Microbiol.*, **46**: 205-210.

LIU, S.Y., 2006. *Investigating and screening the intensive pathogenicity of the fungi in genera* Paecilomyces *and* Metarhizium. Masters thesis, Department of Entomology, South China Agricultural University, Guangzhou, China.

LORITO, M., HARMAN, G.E., HAYES, C.K., BROADWAY, R.M., TRONSMO, A., WOO, S.L. AND DIPIETRO, A., 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology*, **83**: 302-307.

LORITO, M. AND SCALA, F., 1999, Microbial genes expressed in transgenic plants to improve disease resistance. *J. Plant Path.*, **81**: 73-88.

MAJETI, N.V.R., 2000. A review of chitin and chitosan applications. *React. Funct. Polym.*, **46**: 1-27.

MATHIVANAN, N., KABILAN, V. AND MURUGESAN, K., 1998. Purification, characterization and antifungal activity of chitinase from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*. *Canadian J. Microbiol.*, **44**: 646-651.

MILLER, G.L., 1959. Use of dinitrosalicylic acid for estimation of reducing sugar. *Anal. Chem.*, **31**: 426-428.

NAHAR, P.B., 2004. *Development of biocontrol agents for the control of pests in agriculture using chitin metabolism as target*. Ph.D. thesis, Division of Biochemical sciences, National chemical laboratory, Pune, India.

REGEV, A., KELLER, M., STRIZHOU, N., SNEH, B., PRUDOVSKY, E., CHET, I., GINZBERG, G. AND KONCZ- KALMAN, Z., 1996. Synergistic activity of *Bacillus thuringiensis* delta endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Appl environ. Microbiol.*, **62**: 3581-3586.

ROBERTS, W. A. AND SELETRENIKOFF, C. P., 1988. Plant and bacterial chitinase differ in antifungal activity. *J. Gen. Microbiol.*, **134**: 169-176.

SAHAI, A.S. AND MANOCHA, M.S., 1993. Chitinases of fungi and plants: their involvement in morphogenesis in host-parasite interactions. *FEMS Microbiol. Rev.*, **11**: 317-338.

SAMSON, R.A., EVANS, H.C. AND LATGE, J.P., 1988. *Atlas of entomopathogenic fungi*. Springer-Verlag, New York.

SAS INSTITUTE, 2000. *SAS user's guide*: *Statistics*. Cary, North Carolina, U.S.A.

SCHAGGER, H. AND VON JAGOW, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, **166**: 368-379.

ST. LEGER, R.J., COOPER, R.M. AND CHARNLEY, A.K., 1986. Cuticle-degrading enzymes of entomopathogenic fungi: Regulation of production of chitinolytic enzymes. *J. Gen. Microbiol.*, **132**: 1509-1517.

TSIGOS, I. AND BOURIOTIS, V., 1995. Purification and characterization of chitin deacetylase from *Colletotrichum lindemuthianum*. *J. biol. Chem.*, **270**: 26286-26291.

YANG, A.H. AND YEH, K.W., 2005. Molecular cloning, recombinant gene expression, and antifungal activity of cystatin from taro (Colocasia esculenta cv. Kaosiung no.1). *Planta,* **221**: 493-501.

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