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Full Length Article

Toxicity of Proteins Secreted by Entomopathogenic Fungi against *Plutella xylostella* (Lepidoptera: Plutellidae)

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ABSTRACT

The diamond back moth (DBM) (*Plutella xylostella* L.) is an important and cosmopolitan pest of cruciferous crops. The toxic crude proteins produced by *Isaria fumosorosea* were checked against DBM for its effectiveness as insecticidal and antifeedant characteristics. The crude proteins produced in the Czapek Dox liquid medium were used against 3rd instar larvae of DBM. The results indicated a considerable insecticidal and antifeedant activity by crude protein extracts. Notably the most active crude protein extract belong to the isolate CNZH that showed 83.3% mortality of 3rd instar larvae 6 days post treatment. CNZH was tested for its toxicity at different concentrations of crude protein and a maximum mortality percentage of 91.6 were recorded after 6 days of treatment. A significant level of increase in the antifeedant index was recorded with the increase of concentrations and time duration. At the concentration of 9 mg protein/mL the antifeedant index was observed to be significantly higher than that of 6 and 3 mg protein/mL ($P<0.05$). CNZH due its insecticidal characteristics can be recommended for the incorporation in the biological control program of DBM. Further studies regarding purification of the active microbial insecticidal compound and its mode of action need to be carried out. The complete purification of the protein and its insecticidal and antifeedant tests in the field can help to develop a natural product for plant protection. © 2012 Friends Science Publishers

Key Words: *Isaria fumosorosea*; Toxicity; DBM; Crude proteins; Antifeedant index

INTRODUCTION

Diamond back moth (DBM) is a significant insect pest of cauliflower all over the world (Shankar *et al*., 1996). The largest number of species of genus *Plutella* (Sch.) has been recorded in USA. Seven species of this pest were recorded in South America. *P. xylostella* is cosmopolitan in distribution (Bhalla & Dubey, 1986; Salinas, 1986). It is commonly thought to have originated in the Mediterranean region. The wide spread of synthetic insecticides on highly significant crucifer crops has resulted in high levels of resistance in *P. xylostella* in most parts of the world (Lim & Tan, 1986; Talekar & Shelton, 1993). The resistance ability against toxic agents is brought about either by genetic selection or by direct exposure to the selecting agent or by cross resistance resulting from selection by some other toxic agent. DBM has a long history of becoming resistant to every insecticide (Talekar & Shelton, 1993). On the other hand, continuous use of insecticide leads to environment hazards; therefore, an alternate method safe for environment and human beings for the control of DBM is direly needed.

As a result of different environmental and health hazards and insecticide resistance development, currently researchers have focused on searching natural compounds from flora and microbes as a substitute to usual chemicals for the management of insect on different crops (Quesada-Moraga *et al*., 2006). For the exploration of active natural compounds to control insect pests, the monitoring technique was employed by selecting the biotypes of different microorganisms (Schulz *et al*., 2002). Entomopathogenic fungi e.g., *Metahrizium anisopliae*, *I. fumosorosea* and *Beauveria bassiana* etc. are the part of nature that have a prospective to manage the pests without disturbing nature and are not harmful to life. Therefore, these can be used as an alternative source for the control of insect pests because these produce such kinds of chemicals that have insecticidal and antifeedant activities (Vey *et al*., 2001). Most of the fungi as *M. anisopliae*, *B. bassiana*, *I. fumosorosea* produce secondary metabolites that are low molecular weight compounds and the tests for their insecticidal and antifeedant activities have been done in previous researches (Amiri *et al*., 1999; Kim *et al*., 2002; Hafeez *et al.,* 2010). Recently Quesada-Moraga *et al*. (2006) used the crude protein extracts of *M. anisopliae* for the control of *Spodoptera litura*. Keeping in view the aforementioned discussion, the present study was designed to derive proteins/compounds that are toxic to *P. xylostella* from *I. fumosorosea* and other *Paecilomyces* spp. isolates.

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MATERIALS AND METHODS

Entomopathogenic fungi: Insect pathogenic fungi from genus *Isaria* selected for screening were originated from various locations generally from China and South Korea (Table I). The monoconidial slants were cultivated on potato dextrose agar (PDA) at 25±1°C as described earlier (Freed *et al.,* 2011a, b) for this, 100 µL of low density conidia were spread on PDA plates. After 24 h growth, a solitary grown conidium was identified. A part of the media around the required conidium was detached and shifted to PDA. The inoculated fungi were kept at 25±1°C, and then preserved at 4°C.

Insects: The lepidopteran species *P. xylostella* was reared at 21±1°C and 14:10 photoperiod in the insect rearing laboratory. The larvae were reared on cabbage leaves. The larvae were reared in cages (60 x 60 x 60 cm). The culture was maintained till the required age larvae. The adults were separated from the cages in other cages for mating and oviposition. Adults were fed on an artificial diet consisting of sucrose (50 g), Methyl p-hydroxy benzoate (1 g), vitamin (stock solution), 5 mL and water (500 mL). Cotton balls soaked in artificial diet solution were placed in the cages. The cabbage potted plants were placed in the cages for oviposition. The eggs were removed twice daily to minimize the age differences of larvae of each lot.

Production of toxic proteins of fungi in Czapek's media: The crude protein extracts were extracted from the fungi as described previously (Bridge *et al*., 1997; Quesada-Moraga *et al*., 2006). The fungi were grown from the single conidium on Petri plates of 9 cm diameter. After 15 days of development at 25±1°C, the conidia were scraped off into distilled water and the concentration of conidia was estimated by haemocytometer. For making initial fungal culture, conidia (≈ 1 mL) of *I. fumosorosea* isolates and other *Paecilomyces* spp. (1×10^7 spore/mL) was introduced in 25 mL of Czapek's media ($NaNO_3$ 3 g, $MgSO_4.7H_2O$ 0.5 g, K_2HPO_4 1 g, KCl 0.5 g, $FeSO_4.7H_2O$ 0.01 g, cane sugar 30 g, bacteriological peptone 5 g & water 1000 mL) in a 100 mL conical flasks and were incubated on a rotary shaker at 25°C at 110 rpm for 4 days period. For the implantation of resultant cultures for maximal development of different fungi, 2 mL of the initial fungal suspension were introduced to 1000 mL conical flasks having 250 mL of the Czapek's media and these were afterward incubated at same conditions for next 7 days. For getting the extract, toxic proteins from the filtration product of all fungus species used was mixed at 90% saturation of ammonium sulphate and centrifuged at 10,000 g for half an hour. For desalting the crude extracts, one part of fungal filtrate was separated with two parts of pure water for 24 h at 4°C. In order to perform this, sample was introduced to a permeable membrane with a cut-off 6-8 kDa. The resultant desalted extract was then concentrated by implanting the same membrane in polyethylene glycol 20000 at 4°C.

Insecticidal action of proteins secreted by the isolates of insect pathogenic fungi: The toxicity of protein extracts produced by divergent fungi was examined by cabbage leaf disc test. For this the newly molted third instar larvae for each replication were put in Petri plates (diameter = 9 cm) and was fed on a circular leaf (5 cm). All leaf discs were injected with 6 µL protein extract with at a concentration of 6 mg protein/mL. The control was injected with 6 µL of desalted CD media only. In both treatments, the cabbage leaves were changed every 24 h with freshly protein injected leaves for a six days. Four replications having twenty larvae each were employed in the whole trial. The test was performed at 21±1°C and 14:10 photoperiod. The mortality of the larvae was recorded daily and mortality percentage was calculated.

Toxicity crude protein of *I. fumosorosea* CNZH: CNZH was cultivated in CD liquid media in the same conditions as depicted above and the toxicity of protein was assessed by the cabbage leaf assay. Newly molted third instar *P. xylostella* were placed individually in the 9 cm diameter Petri dish with a 5 cm diameter leaf disc. Each leaf was injected 5 µL protein extract with concentrations of 3, 6, 9 mg protein/mL by with the help micro syringe. The control was also injected with same procedure but with 5 µL of the desalted CD media. In treated and non treated larvae, the cabbage leaves were changed after 24 h with a freshly treated leaf for six days. Four replications with twenty insects including control were employed. The trial was performed at the similar conditions as above. The mortality data were recorded after every 24 h to calculate the percent mortality. The antifeedant action of the protein extract of CNZH isolate was checked against third larval instar of *P. xylostella* by the above leaf no choice test. The leaf area consumed by the larvae for eating was measured by the nine quard paper. Areas of the treated and

Table I: Entomopathogenic fungal isolates used for crude protein extraction

Isolates	Fungal species	Origin	Source
CNIM	*Isaria fumosorosea*	Inner Mongolia, China	Riverside soil
CNZG	*Isaria fumosorosea*	Guangdong, Zhanjiang, China	Farm land soil
CNZH	*Isaria fumosorosea*	Zhejiang, Ningbo, China	Mountain soil
CNHG	*Isaria fumosorosea*	Gansu, Huixian, China	Farm land soil
CNBJ	*Isaria fumosorosea*	Beijing, China	Urban land soil
CNXN	*Isaria fumosorosea*	Xinjiang, China	Forest soil
SKCH-1	*Isaria fumosorosea*	Cheju Island, South Korea	Urban land soil
CNXM	*Paecilomyces lilacinus*	Xizang, Motuo, China	Forest soil
CNSH	*Paecilomyces marquandii*	Hubei, Shiyan, China	Mountain soil

non treated leaves were measured. The antifeedant index was evaluated by equation (Quesada-Moraga *et al*., 2006).

IA = C-T/C+T

Where C is the leaf area (mm^2) used in control, whereas T is the leaf area (mm^2) eaten in the treatments.

Protein concentration: The protein was quantified with the Bradford's method (Bradford, 1976) keeping bovine serum albumin as a standard. For the estimation of the quantity of protein a wave length of 280 nm was used.

Data analysis: Mortality data was analyzed by using one-way (ANOVA), and the Tukey's (HSD) test was performed to match means of the treatment. The collective mortality response throughout the evaluation phase was evaluated with Kaplan-Meier survival. The contact time required to kill 50% of the insects was done by Probit analysis. All the data were subject to statistical analyses by using SAS 8.0 (SAS, 2002).

RESULTS

Toxicity of protein produced by *I. fumosorosea*: The chronic effect (6 days mortality) of the protein extracts obtained from nine different isolates, belonging to three different fungal species (*I. fumosorosea*, *P. lilacinus* & *P. marquandii*) were evaluated against third instar larvae of *P. xylostella* via oral administration through cabbage leaf discs. There was a considerable effect of application of the crude extract on the leaf discs with the mortality of the insect ranging from 4.16% to 83.3%, compared with the control treatment that illustrated no mortality (Fig. 1). Amongst the isolates of *I. fumosorosea*, three isolates (CNIM, CNZH & CNHG) showed noteworthy oral toxicity to *P. xylostella*. Particularly the most active crude protein extract belong to *I. fumosorosea* CNZH isolate, that showed 83.3% mortality in cabbage leaf discs assay. The isolates CNZG, CNBJ and CNXN showed somewhat same percent mortality i.e., 47.91, 52.0 and 45.83% after 6 days of treatment. The two other isolates of other species i.e., *P. lilacinus* and *P. marquandii* were also evaluated for the toxicity test but up to 6 days of the treatment they showed only 22.9 and 14.5% mortality percentage.

Insecticidal action of crude protein of *I. fumosorosea* CNZH: The crude protein extract of CNZH isolate caused minimal percentage of mortality at one day post treatment. However, the consumption of the treated leaf discs by the larvae was comparatively lower than that of control discs. Mortality percentage increased with the increase of time and also the concentration (Fig. 2). There was a significant effect of the treatment with the crude soluble protein extract added to the leaf discs with mortality of larvae after treatment of 3, 6 and 9 mg protein/mL, varying from 64.5, 83.3 and 91.6% (DMRT; $P < 0.05$), compared with untreated larvae that showed no mortality after 6 days.

Antifeedant action of crude protein of *I. fumosorosea* CNZH: The evolution of the antifeedant index over 5 days

Fig. 1: Percentage mortality of third instar *P. xylostella* larvae after 6 days of feeding on cabbage leaf discs treated with the crude protein extracts from entomopathogenic fungi isolates

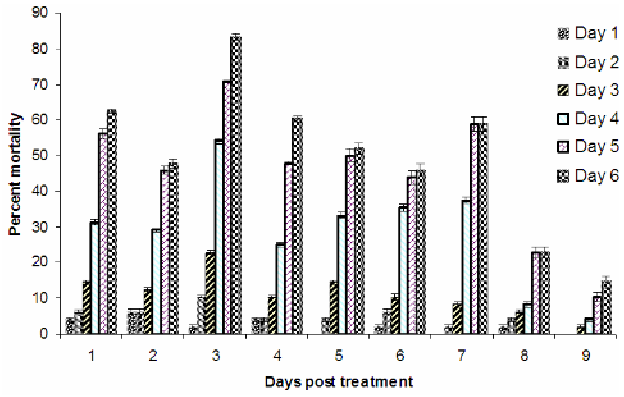
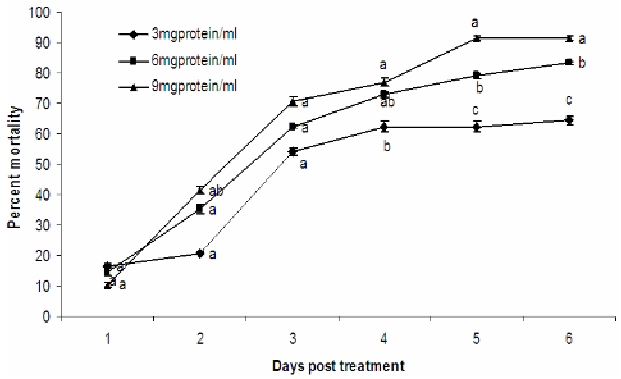
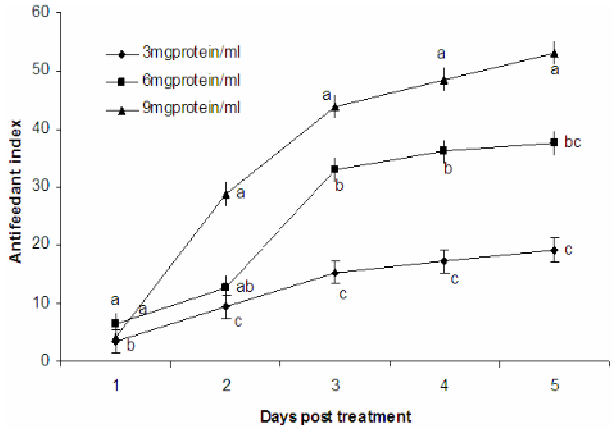


Fig. 2: Insecticidal activity of the crude soluble extract of *I. fumosorosea* isolate on third instar larvae after 6 days feeding of treated leaf discs. For each day the same letters are not significantly different (P<0.05) according to Duncan's Multiple Range Test (DMRT)



phase of consumption on treated cabbage leaves is shown in Fig. 3. The utilization of the leaves by the third instar larvae was more in the untreated as compared to that of the treated leaf discs. The data recorded showed that the antifeedant index of the protein extracts was directly related to the dose of the treatment, on the increase of dose the insect didn't feed on the leaf discs or feed minimal. It showed that the antifeedant index was dose dependent. At the concentration of 9 mg protein/mL, the antifeedant index was observed to be significantly higher than that of 6 mg protein/mL and 3 mg protein/mL. ($P < 0.05$) (Fig. 3). A significant level of increase in the antifeedant index was recorded as the concentrations increased. Statistically there was no significant difference among the concentrations of 3 and 6 mg protein/mL up to 2 days, but as the time increased these values became significantly different from each other (DMRT; $P < 0.05$). The antifeedant index data was taken up to six days but there was no difference among the values of day five and six.

Fig. 3: The antifeedant index of *P. xylostella* third instar larvae after 5 days of feeding on cabbage leaf discs treated with different concentrations of crude protein extract of *I. fumosorosea* isolate. For each day the same letters are not significantly different (P<0.05) according to Duncan's Multiple Range Test (DMRT)



DISCUSSION

The study shows that the crude proteins have insecticidal and antifeedant activities against the insect pests. Different *Paecilomyces* spp. isolates were tested for their crude protein extract toxicity against 3rd larval instar of *P. xylostella*. Out of nine total isolates, *I. fumosorosea* from different locations and hosts showed insecticidal activities against the insect, while the isolates of *P. lilacinus* and *P. marquandii* showed minute toxicity to *P. xylostella*. The extract produced by the isolate CNZH was the most active one and showed promisive tool for the control of *P. xylostella*. The protein extract showed dose related toxicities to the insect as previously reported by Bandani and Butt (1999) that destruxins have time related and dose related toxicities against the lepidopterons. Present study confirm the earlier studies done by Quesada-Moraga *et al*. (2006) that the crude protein extracts from the entomopathogenic fungi *M. anisopliae* have insecticidal activities against the lepidopteran insects pests especially to that of *Spodoptera litura*.

The studies on the secondary metabolites produced by the entomopathogenic fungi particularly *M. anisopliae,* which secretes a kind of metabolites that have contact toxicity to *S. litura* (Hu *et al*., 2007) and the lepidopteran insects are vulnerable to the destruxins, respectively (Brousseau *et al*., 1996; Thomsen & Eilenberg, 2000). The studies confirm our results that microorganisms like fungi, e.g., *Tolypocladium*, have proved to be toxic to *P. xylostella* (Bandani & Butt, 1999). The crude extract of the isolate CNZH showed antifeedant activities against the 3rd instar larvae of *P. xylostella*; the results showed that the antifeedant activities start to increase with the increase of concentrations and also with the time of contact. Maximum antifeedant activities were recorded at 9 mg protein/mL and also with the day of application as maximum antifeedant index were observed at 5th day of application. The swift refutation of toxic protein treated food was clear at the higher concentration (9 mg protein/mL) and it could be due to instantaneous suppression or rapid post-ingestive feedback as reported previously by Bernays *et al*. (2000) and Sadek (2003). The same kind of study was performed by Bandani and Butt (1999), who showed that the antifeedant activities of destruxin A and B occurred at low doses and also in dose related manner. The study also confirm the studies of Quesada-Moraga *et al*. (2006), which states that the toxic protein secreted by the *M. anisopliae* isolate 01/58-Su had antifeedant activities against the 2nd instar larvae of *S. litura* and it was also in dose dependent manner.

In conclusion, the rejection of the cabbage leaf discs at 9 mg protein/mL is the clear proof of the post ingestion feedback of *P. xylostella.* Further research for the purification and characteristics of the active protein and its evaluation in the field and green house condition is needed to be done.

REFERENCES

Amiri, B., L. Ibrahim and T.M. Butt, 1999. Antifeedant properties of destruxin and their potential use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocont. Sci. Technol.,* 9: 487–498

Bandani, A.R. and T.M. Butt, 1999. Insecticidal, antifeedant and growth inhibitory activities of efrapeptins, metabolites of the fungus Tolypocladium. *Bicont. Sci. Technol.,* 9: 499–506

Bernays, E.A., S. Oppenheimer, R.F. Chapman, H. Kwon and F. Gould, 2000. Taste sensitivity of insect herbivores to deterrents is greater in specialists than in generalists: a behavioural test of the hypothesis with two closely related caterpillars. *J. Chem. Ecol*., 26: 547–563

Bhalla, O.P. and J.K. Dubey, 1986. Bionomics of Diamondback moth in Northwestern Himalaya. *In*: Talekar, N.S. and T.D. Griggs (eds.), *Proc. 1st Int. Workshop, Taiwan*, pp: 55–61. Asian Vegetable Research and Development Center

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem*., 72: 248–254

Bridge, P.D., M.A.J. Williams, C. Prior and R.R.M. Paterson, 1993. Morphological, biochemical and molecular characterization of *Metarhizium anisopliae* and *M. flavovrodiae. J. Gen. Microbiol*., 139: 1163–1169

Brousseau, C., G. Charpentier and S. Belloncik, 1996. Susceptibility of spruce budworm, *Choristoneura fumiferana* Clemens, to destruxins, cyclodepsipeptidic mycotoxins of *Metarhizium anisopliae. J. Invert. Pathol*., 68: 180–182

Freed, S., F.L. Jin and S.X. Ren, 2011a. Determination of genetic variability among the isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins. *World J. Microbiol. Biotechnol.,* 27: 359–370

Freed, S., F.L. Jin and S.X. Ren, 2011b. Phylogenetics of entomopathogenic fungi isolated from the soils of different ecosystems. *Pakistan J. Zool.,* 43: 417–425

Hafeez, F., W. Akram, A. Suhail and M.A. Khan, 2010. Adulticidal action of ten citrus oils against *Aedes albopictus* (Diptera: Culicidae). *Pakistan J. Agric. Sci.,* 47: 241−244

Hu, Q.B., S.X. Ren, X.C. An and M.H. Qian, 2007. Insecticidal activity influence of destruxins on the pathogenicity of *Paecilomyces javanicus* against *Spodoptera litura*. *J. Appl. Entomol*., 131: 262–268

Kim, J.R., H.S. Yeon, H.S. Kim and Y.J. Ahn, 2002. Larvicidal activity against *Plutella xylostella* of cordycepin from the fruiting body of *Cordyceps militaris*. *Pest Manage. Sci*., 58: 713–717

Lim, G.S. and C.L. Tan, 1986. Occurrence of Diamondback moth on its parasitoid, within cabbage heads, MARDI. *Res. Bull. Mal*., 14: 158–163

Quesada-Moraga, E., J.A. Carrasco-Diaz and C. Santiago-Alvarez, 2006. Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep., Noctuidae). *J. Appl. Entomol.,* 130: 442–452

Sadek, M.M., 2003. Antifeedant and toxic activity of *Adhatoda vasica* leaf extract against *Spodoptera littoralis* (Lep., Noctuidae). *J. Appl. Entomol*., 127: 396–404

Salinas, P.J., 1986. Studies on the diamondback moth in Venezuela with reference to other Latin American countries. *In*: Talekar, N.S. and T.D. Griggs (eds.), "*Diamondback Moth Management: Proc. 1st Int. Workshop*", pp: 17–24. Asian Vegetable Research and Development Center

SAS Institute, 2002. *Version 9.0*. SAS Institute Inc., Cary, North Carolina, USA

Schulz, B., C. Boyle, S. Draeger, A.K. Rommert and K. Krohn, 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol. Res*., 106: 996–1004

Shankar, G.S., S. Mallikarjuappa, U.G. Bhatt, G.N. Kendappa and M.S. Miphyantha, 1996. *Bacillus thuringiensis Berliner Subsp; Kurstaki in the Management of DBM in India, Proc.,* 3rd edition, pp: 104–108. International Workshops, Kuala Lumpur, Malaysia

Talekar, N.S. and A.M. Shelton, 1993. Biology, ecology, and management of the diamond back moth. *Annu. Rev. Entomol.,* 38: 275–301

Thomsen, L. and J. Eilenberg, 2000. Time-concentration mortality of *Pieris brassicae* (Lepidoptera: Pieridae) and *Agrotis segetum* (Lepidoptera: Noctuidae) larvae from different destruxins. *Pest Manage. Samp*., 29: 1041–1048

Vey. A., R. Hoagland and T.M. Butt, 2001. Toxic metabolites of fungal biocontrol agents. *In*: Butt, T.M., C.W. Jackson and N. Magan (eds.), *Fungi as Biocontrol Agents*, pp: 311–345. CAB International, Wallingford, UK

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