Pakistan J. Zool., vol. 43(6), pp. 1203-1213, 2011.

Production and Regulation of Extracellular Proteases from the Entomopathogenic Fungus *Metarhizium anisopliae* (Cordycipitaceae; Hypocreales) in the Presence of Diamondback Moth Cuticle

Shaukat Ali, Zhen Huang, Wang QeZang and Shun Xiang Ren*

Engineering Research Center of Biological Control, Ministry of Education, College of Natural Resource and Environment, South China Agricultural University. Wushan Road, Guangzhou City, P.R. China, 510642

Abstract.- Extracellular proteases of entomopathogenic fungi have been implicated as components of the insect infection process. To elucidate their role in the infection process, it is essential to characterize these enzymes. During the current studies synthesis and regulation of extracellular proteases (Pr1 and Pr2) by *Metarhizium anisopliae* (Cordycipitaceae; Hypocreales) isolate IF28.2 were investigated as a function of carbon source (with special reference to diamondback moth cuticle), temperature and pH. The highest level of Pr1 and Pr2 activity were found in the supernatants from 1% glucose plus 1% diamondback moth cuticle (18.83±1.25 and 12.44±1.36 U/mg/h for Pr1 and Pr2, respectively). Maximum Pr1 production by the depressed mycelia was observed from the supernatants having diamondback moth cuticle as a nutrient source whereas depressed mycelia showed maximum Pr2 activity from the cultures having chitin as the basic nutrient. The optimum pH for Pr1 and Pr2 activity was 8 while 35°C was the best temperature for protease production.

Key Words: *Metarhizium anisopliae*, protease, carbon sources, diamondback moth, *Plutella xylostella.*

INTRODUCTION

Diamondback moth (DBM), *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 insecticides (Henrik *et al*., 2000). As a result, natural enemies are being sacrificed (Xu *et al*., 2004) and many populations of diamondback 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 regions, such as Hawaii (Tabashnik *et al*., 1990), Central America (Perez and Shelton, 1997), 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 *Zoophthora radicans* (Brefeld) Batko (Pell *et al*., 1993), *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Altre *et al*., 1999), *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Fusarium* species, and *Beauveria bassiana* (Balsamo) Vuillemin can infect diamondback moth under screenhouse or field conditions (Ibrahim and Low, 1993; Vandenberg and Ramos, 1997; Vandenberg *et al*., 1998; Shelton, *et al*., 1998).

Entomopathogenic fungi invade their hosts through the external skeleton (cuticle) which is a complex, composite structure with high-protein content. Through the combined action of hydrolytic enzymes such as chitinase, protease and lipase, the fungal mycelia are able to penetrate through these barriers (Bidochka and Khachatourians, 1987; St. Leger *et al*., 1986a). The importance of any one of these enzymes is dependant upon the cuticular characteristics of insects and invasion mechanism of the fungus. Several cuticle-degrading enzymes such as proteases and chitinases have been detected in entomopathogenic fungi *i.e.*, *B. bassiana, Numuraea rileyi* and *M. anisopliae* (Brey *et al*., 1986; El-Sayed *et al*., 1989). Proteolytic enzymes along with chitinases and lipases are important factors in virulence of entomopathogenic fungi (Samuels and Paterson, 1995). Thus, particular attention has been focused on the role of proteases in the penetration process (Charnley, 2003). Entomopathogenic fungi produce distinct extracellular serine proteases, such

* Corresponding author: rensxcn@yahoo.com.cn ; alientomologist@gmail.com
Wang Qezhang is joint first author.
0030-9923/2011/0006-1203 $ 8.00/0

as subtilisin-like proteases, trypsin-like proteases, metalloproteases, as well as several families of exo-acting peptidases that are believed to be important for host cuticle degradation (St. Leger *et al.,* 1995, 1996b). The subtilisin protease family Pr1 is the main enzyme produced by entomopathogenic fungi during the infection process, although the trypsin-like protease Pr2 is the first to appear during in vitro growth on the cuticle (Gillespie *et al.,* 1998). Antiserum against Pr1 protease interferes with penetration of the host cuticle and reduces infection, indicating that the level of active Pr1 may determine the capacity of the fungus to cause disease (St. Leger *et al.,* 1988).

Insect cuticle contains many different proteins, which vary between types of insect (Andersen *et al*., 1995; Dombrovsky *et al*., 2003). Thus, while Pr1 enzymes may not be prerequisites for pathogenicity, isolate virulence or specificity could depend in part on having proteases with high activity for host-specific cuticular proteins. Just a few strategic amino acid substitutions can affect cuticle binding and activity of Pr1 from *M. anisopliae* (St. Leger *et al*., 1986a, 1992). The regulation of genes encoding cuticle-degrading enzyme isoforms is probably complex and may involve a combination of carbon/nitrogen induction and/or repression (Screen *et al*., 1998).

Isolate virulence or specificity could depend in part on the ability to produce a battery of proteolytic enzymes with specificity for the range of proteins in the cuticle of a particular insect, in response to particular host cues at the correct stage in penetration. The present work is an attempt to explore the potential for this by comparing the regulation and activity of proteases produced by *Metarhizium anisopliae* Sorkin. *M. anisopliae* was chosen for this study because this pathogen is one of the most promising fungal species for control of diamondback moth under screen house or field conditions (Wu *et al*., 2010). Therefore, the aim of this study was to investigate the effects of carbon sources and/or diamondback moth cuticle, growth temperature, pH of the growth medium, on production and regulations of Pr1 and Pr2 by *M. anisopliae* isolate M408, which may increase our knowledge about protease production and secretion by this fungus.

MATERIALS AND METHODS

Insect cultures and cuticle preparation

Fourth instar larvae of *P. xylostella*, were obtained from the stock cultures kept in the greenhouse of the Engineering Research Center of Biological Control, South China Agricultural University on *Brassica campestris* L. 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.

Clean samples of the cuticle from fourth instar *P. xylostella* larvae were obtained according to the method of Missios *et al*. (2000). Approximately 50g of fourth instar *P. xylostella* larvae were homogenized in a Waring blender in 250 ml of a high salt buffer (0.5 M NaCl, 50 mM Tris, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; a serine protease inhibitor) and saturated with phenylthiourea (a tyrosinase inhibitor). Cuticles were collected and washed on a fine mesh sieve using ice-cold deionized water. The cuticle preparations were extracted immediately or dried at room temperature for subsequent analysis.

Fungal strains

For all assays, *M. anisopliae* isolate-IF28.2 originally isolated from soil (Liu, 2006), maintained in tubes containing Sabouraud's dextrose agar (SDA) and deposited at the Engineering Research Center of Biological Control, South China Agricultural University, were cultured on potato dextrose agar (PDA) and incubated at 26±2°C for 10 days. Conidia were harvested with deionized water containing 0.03% Tween 80 and sieved through filter paper into sterile vials. Conidia were counted using a hemocytometer (0.0625m m^2; Fuchs-Rosenthal Merck Euro Lab, Darmstadt, Germany) to calibrate a suspension of 1×10^6 conidia/ml.

Culture medium

The basal medium (pH 5.4) consisted of peptone 0.2% (w/v), $MgSO_4$ 0.01% (w/v), yeast extract 0.1% (w/v), casein hydrolysate 0.1% (w/v),trace element solution 5% (v/v) and vitamins

2% (v/v). The trace element and vitamin solutions used were those described by Cove (1966).

Effect of different carbon sources on protease activity and biomass production

Effect of carbon source on protease activity and biomass produced by *I. fumosoroseus* was studied by growing the fungal isolates in liquid basal medium (50 ml in 250 ml Erlenmeyer flasks) supplemented with yeast extract. As carbon sources, glucose (1%) or *N*-acetylglucosamine, GlcNac (1%) was added. Complex substrates such as diamondback moth cuticle (1%), chitin (1%), combinations of chitin plus glucose (1%), chitin 1% plus GlcNac (1%), diamondback moth cuticle (1%) plus glucose (0.8% or 1.5%) and diamondback moth cuticle (1%) plus GlcNac (1%) were also used while basal medium without any additional carbon source served as a control. The flasks were inoculated with one ml of $1x10^6$ spores/ml and incubated at 180 rpm and 28°C for 5 days.

Protease production by depressed mycelia of M. anisopliae

One hundred millilitres of basal medium having 1% diamondback moth cuticle as a carbon source in 250 ml conical flasks was inoculated with 1 ml of 10^6 spores/ml and incubated at 28°C in an orbital shaker (180 rpm) for 4 days to allow extensive fungal growth. The mycelial biomass was harvested by sieving through two layers of sterile muslin, washed with sterile basal salts medium, and transferred to 100 ml basal salts medium without nutrients for 24 h to achieve metabolite depression (referred to as depressed mycelia). All manipulations were conducted under aseptic conditions. The fungus was then supplied with a nutrient source [viz. diamondback moth cuticle, chitin, KOH chitin (chitin hydrolyzed in 30% potassium hydroxide at 80°C for 2 h to remove residual proteins) or bovine serum albumen (BSA)] at 1% (w/v) and the culture supernatant was assayed for Pr1 and Pr2 -like activities at 18 and 36 h.

Evaluation of best pH and temperature for protease activity and biomass production

The effect of pH on enzyme activity and biomass production was determined by varying the pH of the basal media having 1% diamondback moth cuticle as a carbon source, using with 0.1M sodium phosphate in the following pHs: 6, 7, 8 and 9 whereas non buffered basal medium was used as control (pH 5.4). Fifty milliliters of sterile growth medium inoculated with 1 ml of 1×10^6 spores/ml was incubated for 5 days in an orbital shaker operating at 180 rpm and 28°C.

The range of optimum temperature for protease activity and biomass production was determined by incubating 50 ml of basal medium (having 1% diamondback moth cuticle as a carbon source) inoculated with one ml of 10^6 spores/ml and incubating for 5 days at three different temperature (25, 35, 45 and 55°C) in an orbital shaker operating at 180 rpm.

Analytical determinations

M. anisopliae subtilisin and trypsin-like activities are referred to as Pr1 and Pr2, respectively. Subtilisin (Pr1) activity was assayed using succinyl-(alanine) $_2$-proline-phenylalanine-*p*-nitroanilide as substrate (St. Leger *et al*., 1987), and trypsin (Pr2) activity using benzoylphenylalanine-valine-arginine-*p*-nitroanilide, as described by Gupta *et al*. (1992). 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 one hour 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 1250 g for 5 min at 4°C in a Microfuge®18 with a F241.5P rotor (Beckman Coulter, Inc, USA). The supernatants were read 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 according to Bradford (1976), with bovine serum albumin as standard.

For biomass determinations, the culture supernatants were separated from the mycelium by filtration through Whatman filter paper #1 and dried at 80°C until constant weight.

Chemicals and reagents

Succinyl-(alanine)$_2$-proline-phenylalanine-*p*-nitroanilide and enzoylphenylalanine-valine-

arginine-*p*-nitroanilide were purchased from Sigma (St Louis, MO, USA). All other 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.

Experimental design and statistical analysis

Each study was conducted three times with freshly prepared fungal suspensions. Biomass produced as well as Pr1 and Pr2 activities under different conditions were analyzed by analysis of variance (ANOVA) and treatment means were compared using Tukey's studentized range test for mean comparisons at 5% level of significance. Treatment means of Pr1 and Pr2 activities for restricted mycelia experiments were compared by using Fishers pair wise comparison. All statistical analysis was performed using SAS 8.01(SAS, 2000).

RESULTS

Effect of different carbon sources on protease (Pr1 and Pr2) activity

The effects of different carbon sources on subtilisin (Pr1) like protease activity by *M. anisopliae* were tested in medium supplemented with simple or complex carbon sources individually or in combination. As shown in Table I, Pr1 activity was exhibited by *M. anisopliae* in all media tested; however, the amount of secreted enzyme varied significantly among different treatments and control. The highest level of Pr1 activity (261.04±2.44; 206.74±2.12 and 181.86±2.16 U/mL/h for M408, M440, and M460, respectively) was found in the supernatants from 1% *N*-acetylglucosamine (GlcNAc) plus 1% diamondback moth cuticle. When 1% diamondback moth was added to the media, without GlcNAc, Pr1 activity was also detected at comparatively higher levels, suggesting an induction of Pr1 enzymes. Similarly, when chitin was added to the media, lower level of Pr1 activity were observed as compared to the enzyme activity observed from the supernatant having 1% *N*-acetylglucosamine (GlcNAc) plus 1% chitin as sole carbon source (Table I). When glucose was used as a sole carbon sources, very low levels of chitinase activity was also detected even when the fungus was grown in high concentration of this compound but higher rates of chitinase activity were observed when glucose was used in combination with 1% diamondback moth (Table I).

Table I.- Effect of carbon sources on subtilisin-like (Pr1) protease activity of *M. anisopliae.*

Carbon sources	Pr1(U/mL/h)		
	M408	M440	M460
Glucose 1%	28.44± 1.60 c	32.92± 1.10 c	28.96± 1.58 c
GlcNac1%	83± 2.28 bc	78.00± 2.61 bc	63.00± 2.41 bc
Chitin 1%	123.96± 2.75 b	98.17± 1.70 b	86.36± 1.69 b
DBM cuticle 1%	234.06± 1.24 a	185.38± 1.83 b	163.06± 2.18 a
Glucose 1% + DBM cuticle1%	197± 1.34 ab	171.00± 1.78 b	141.00± 1.81 ab
GlcNac 1% + DBM cuticle 1%	261.04± 2.44 a	206.74± 2.12 a	181.86± 2.16 a
Glucose 1% + chitin 1 %	129.79± 1.76 b	102.79± 1.54 b	90.42± 1.24 b
GlcNac 0.8%+ chitin 0.8%	153.13± 2.91 b	121.27± 1.84 b	106.68 ± 1.09 b
Control	21.15± 1.67 c	16.75± 1.05 c	19.81 ± 1.07 c
F, df, P	53.67; 8 <0.0001	48.78; 8 <0.0001	69.90; 8 <0.001

Means in the same column with different letters are significantly different from each other (Tukey's, P<0.05)
±: Standard error (Based on three independent replicates).

The effects of different carbon sources on trypsin like (Pr2) protease activity by *M. anisopliae* were tested in medium supplemented with simple or complex carbon sources individually or combined As shown in Table II, *M. anisopliae* produced biomass in all media tested; while; its amount of varied significantly among different treatments and control. The highest levels of trypsin like (Pr2) protease activity for *M. anisopliae* isolates M408, M440, and M460 were found in the supernatants from 1.0% chitin having mean values of 103.84±2.92, 85.17±2.46 and 73.07±2.04 U/mL/h, respectively. When 1% diamondback moth cuticle was added to the media, without GlcNAc, Pr2 production was detected at comparatively lower levels. When different concentrations of glucose

were used as a sole carbon sources, very low levels of Pr2 were detected even when the fungus was grown in high concentration of this compound but higher rates of biomass production were observed when glucose was used in combination with 1.0% chitin (Table II).

Table II.- Effect of carbon sources on trypsin-like (Pr2) protease activity of *M. anisopliae.*

Carbon sources	Pr2(U/mL/h)		
	M408	M440	M460
Glucose 1%	47.41± 1.65 d	38.93± 1.82 d	33.34± 1.27 d
GlcNac1%	31.87± 1.86 e	31.64± 1.62de	27.18± 1.51de
Chitin 1%	103.84± 2.92 a	85.17± 2.46 a	73.07± 2.04 a
DBM cuticle 1%	64.59± 1.73 bc	52.91± 1.82bc	45.32± 1.01bc
Glucose 1% + DBM cuticle 1%	57.84± 2.13bcd	47.48± 1.79bcd	40.60± 1.49 c
GlcNac 1% + DBM cuticle 1%	70.41 ± 1.33 bc	57.76 ± 1.49 bc	49.57± 1.52bc
Glucose 1% + chitin 1 %	75.68± 1.59 b	62.32± 2.03 b	53.16± 1.42 b
GlcNac 1%+ chitin 1%	67.03± 1.88bc	52.76± 2.11 bc	45.74± 1.19bc
Control	28.16 ± 1.41 e	23.15 ± 0.96 e	19.81± 1.07 e
F, df, P	25.10;8; 0.0108	29.28;8; 0.0027	26.27;8; 0.0063

Means in the same column with different letters are significantly different from each other (Tukey's, P<0.05)
±: Standard error (Based on three independent replicates)

Protease production by depressed mycelia

The response of *M. anisopliae* isolates M408, M440 subtilisins-like (Pr1) activity to different substrates at 18 and 36 h was in the order of KOH chitin = Chitin> DBM cuticle> BSA> control *viz.*. Thus, M408 and M440 appeared to be more responsive to chitin as compared to the specific substrate like diamondback moth cuticle showing that these isolates can also be used as generalist fungal pathogens in future biocontrol programs. This isolate exhibited a marked increase in enzyme activity from 18 h to 36h in all the cultures except the control where the increase in Pr1 production was almost negligible (Figs. 1A, B). *M. anisopliae* isolate M460 produced significantly higher subtilisin-like protease activity in cultures containing DBM cuticle than in control at 18 and 36h (Fig. 1C). The order of enzyme production for M460 was DBM cuticle > KOH chitin > Chitin = BSA > control. The data about this isolate showed some interesting results as almost same rates of Pr1 production were observed for chitin as well as BSA and this pattern of Pr1 production was totally different from the other isolates used in this study (Fig. 1).

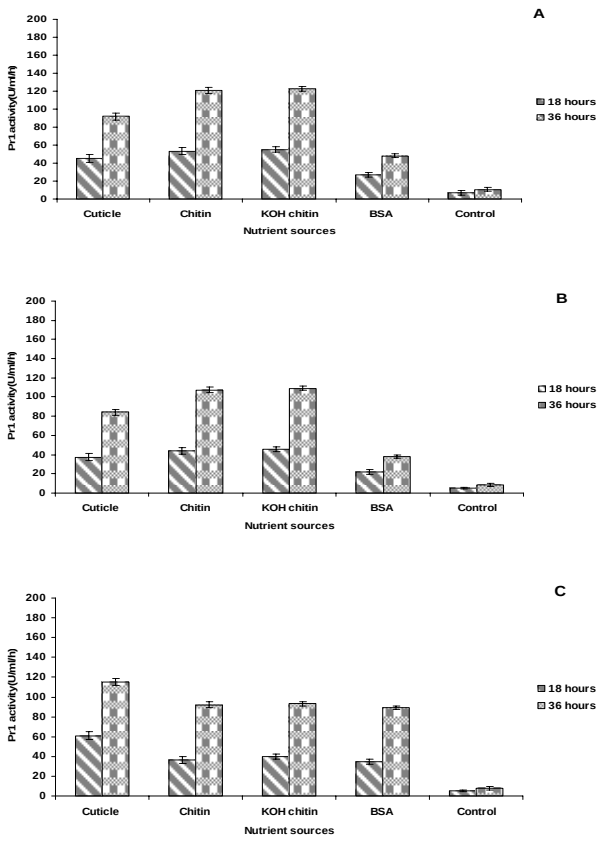

Fig. 1. Subtilisin like (Pr1) protease activity by depressed mycelia of *M. anisopliae* isolates on different polymeric substrates. (A) M408, (B) M440 and (C) M460. Bars represent standard error of means (based on three independent replicates).

The response of *M. anisopliae* isolates M408, M440 activity to different substrates at 18 and 36 h for trypsin-like (Pr2) activity was in the order of chitin ≥ KOH Chitin>DBM cuticle>BSA>control. These isolates exhibited a marked increase in

enzyme activity from 18 h to 36h in all the cultures except the control where the increase in Pr1 production was almost negligible. Thus, M408 and M440 appeared to be more responsive to chitin as compared to the specific substrate like DBM cuticle showing that these isolates can also be used as generalist fungal pathogens in future biocontrol programs (Fig. 2A, B). For *M. anisopliae* isolate M460, the order of Pr2 production on different substrates after 18 and 36 hours was in the order KOH chitin > cuticle>BSA>chitin>control.M460 produced the lowest Pr2 activity overall and showed an effect of mycelial depression on Pr2 activity (Fig.2).

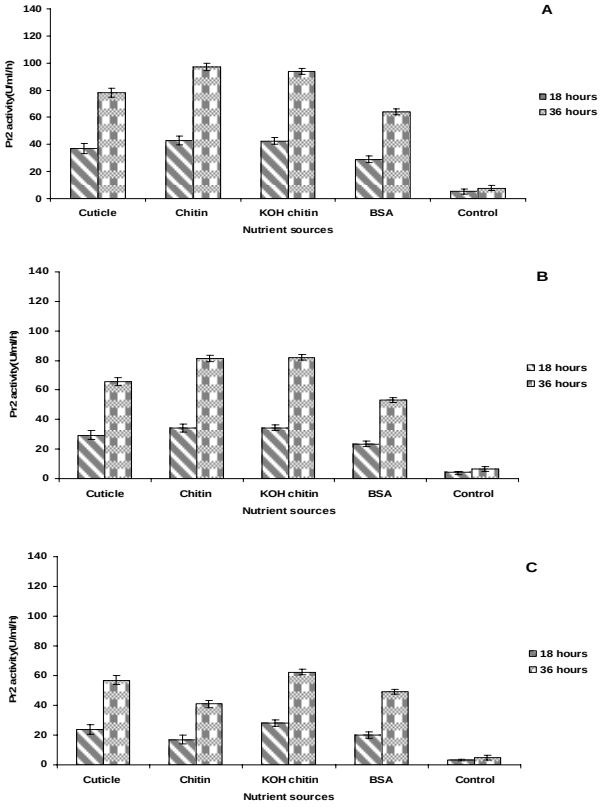

Fig. 2. Trypsin like (Pr2) protease activity by depressed mycelia of *M. anisopliae* isolates on different polymeric substrates. (A) M408, (B) M440 and (C) M460. Bars represent standard error of means (based on three independent replicates).

Evaluation of best pH for protease activity

There were significant differences in Pr1 activity of *M. anisopliae* associated with different growth pH regimes (Fig. 3). The best Pr1 production for all the isolates was obtained when media was buffered to the pH 8.0, having mean Pr1 activity of 177.89±6.18, 139.36±4.74 and 118±3.29 U/mL/h for M408, M440 and M460, respectively. Statistically similar rates of Pr1 production in the basal medium were observed at pH 6 and 9, whereas the lowest Pr1 activity for all the isolates was observed in control (Fig. 3A).

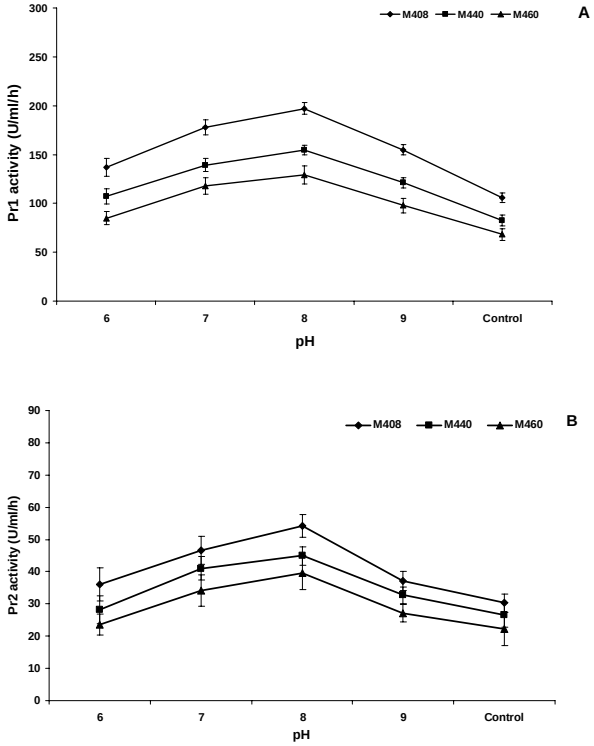

Fig. 3: Effect of pH on protease activity of *M. anisopliae* (A) Pr1 activity, (B) Pr2 activity. Bars represent standard error of means (based on three independent replicates).

There were significant differences among growth temperature when compared for Pr2 activity of *M. anisopliae*. For all the isolates used in these studies, the highest Pr2 activity (54.21±4.43, 44.88±3.68 and 39.69±4.17 U/mL/h for M408, M440, and M460, respectively) was observed when the growth medium was incubated at pH 8.0, The best pH range for Pr2 production in the basal medium was from 7-8 and after 8.0 there was a sudden drop in the Pr2 production (Fig.3B).

Evaluation of optimum temperature for protease activity

There were significant differences in Pr1 activity of *M.anisopliae* associated with different growth temperatures (Table III). The best Pr1 production for all the isolates was obtained when media was grown at a temperature of 35°C having mean Pr1 activity of 212.91±1.51, 174.96±1.49 and 139.28±1.12 U/mL/h for M408, M440 and M460, respectively. Statistically similar rates of Pr1 production in the basal medium were observed at 25 and 45°C, whereas the lowest chitinase activity for all the isolates was observed at 55°C

Table III.- Effect of temperatures on subtilisin-like (Pr1) protease activity of different *M. anisopliae* isolates.

Temp. (°C)	Pr1(U/mL/h)		
	M408	M440	M460
25	147.23±1.02ab	120.99±1.22ab	96.32±1.59 ab
35	212.91±1.51 a	174.96±1.49 a	139.28±1.12 a
45	169.61±1.97ab	139.37± .12ab	110.96±1.03ab
55	122 ± 1.81b	100.82± 1.59b	80.27 ± 1.43 b
F, df, P	6.03; 3; 0.018	4.89; 3; 0.03	4.34;3; 0.02

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

There were significant differences among growth temperature when compared for Pr2 activity of *M. anisopliae*. For all the isolates used in these studies, the highest Pr2 activity (61.61±1.29, 49.71±1.71 and 41.51±1.05 U/mL/h for M408, M440, and M460, respectively) was observed when the growth medium was incubated at 35°C, which was significantly similar to the Pr2 production at 45°C. The lowest lipase activity for all the isolates was observed at a 55°C (Table IV).

DISCUSSION

Fungal pathogenesis is a complex and multi-factorial phenomenon, with particular virulence factors coming into play at various stages of infection and death. Like most fungal pathogens, *M. anisopliae* might use a combination of enzymes to penetrate the cuticle and access the nutrient-rich host haemocoel. The extracellular protease of *M. anisopliae* has been implicated as a component of the insect infection process (Bidochka and Khachatourians, 1990), and in this study we report on the regulation of Pr1 and Pr2 protease production by three different isolates of *M. anisopliae* in liquid culture, as a function of carbon source (with special reference to diamondback moth cuticle), temperature and pH.

Table IV.- Effect of temperatures on trypsin-like (Pr2) protease activity of different *M. anisopliae* isolates.

Temp. (°C)	Pr2(U/mL/h)		
	M408	M440	M460
25	45.47±1.90 b	36.69±1.17 bc	30.64±1.69ab
35	61.61±1.29 a	49.71±1.71a	41.51±1.55 a
45	57.94±1.79 a	46.75±1.23 a	39.04±1.52 a
55	39.61±1.81 b	31.95±0.96 c	26.69±1.19 b
F, df, P	16.79; 3; .008	13.78; 3; 0.001	12.24;3;0.01

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

M. anisopliae extracellular hydrolytic enzymes are important for the degradation of host cuticle during infection, assisting penetration and providing nutrients for further growth (Bogo *et al.,* 1998; St. Leger *et al.,* 1995). The effects of different carbon sources on protease (Pr1 and Pr2) secretion and biomass production by *M. anisopliae* were tested in medium supplemented with simple or complex carbon sources individually or in combination. As shown in Table I, significantly different levels of protease activity were shown by *M. anisopliae* in all media tested; however, the amount of secreted enzymes varied. High levels of subtilisin (Pr1) activity were observed in cultures supplemented with diamondback moth cuticle or chitin plus 1% glucose, and lower levels were observed in cultures containing 1% glucose, 1% GlcNAc or when diamondback moth cuticle and chitin were used in combination with 1 % GlcNAc whereas a high subtilisin (Pr1) activity was observed from the supernatant having 1% diamondback moth cuticle as a sole carbon source. The highest level of trypsin like (Pr2) activity was found in medium having 1% diamondback moth cuticle plus 1% glucose (Table I). Since arthropod cuticles comprise about 70% protein, this enzyme activity may have an important role in host

penetration (Gillespie *et al.,* 1998).

Several studies used insect cuticles as substrates to analyze how host cuticles influence production of cuticle-degrading enzymes. Dias *et al.* (2008) suggested that subtilisin-like (Pr1) and trypsin–like (Pr2) proteases produced by *B. bassiana* were induced by the components of coffee berry borer cuticle. De Moraes *et al*. (2003) analyzed the secretion of proteases in single and combined carbon sources as compared to the complex substrates like chitin and *Boophilus microplus* cuticle. They observed highest level of Pr1 and Pr2 activities from the supernatant having *Boophilus microplus* cuticle and chitin as sole carbon source respectively. Possible reasons of lower protease activities from the supernatants having GlcNAc as a carbon source in combination with chitin or diamondback moth cuticle can be the composition of these chemicals in insect cuticle which is composed of more heterogeneous components than the polysaccharides hydrolyzed by the above cases. Yet an analogous situation of sequential hydrolysis of insect cuticle by entomopathogenic fungi exists, and concerted regulation by functionally dissimilar extracellular enzymes may be the case here. Our data show that extracellular protease synthesis is regulated by products of chitin degradation. One possibility is for *B. bassiania* extracellular protease to be under the control of the multiple regulatory system described earlier (Bidochka and Khachatourians, 1988).

Our findings on the production of extracellular protease (Pr1 and Pr2) by depressed mycelia clearly indicate that higher Pr1 and Pr2 are observed from the supernatants containing diamondback moth cuticle and chitin respectively. A similar pattern of extracellular protease production was observed by Bye and Charnley (2008) when different isolates of *Lecanicillium* spp. were grown in the presence of locust and peach aphid cuticle. At first sight, it seems counter intuitive that proteases should be induced by chitin. However, given that the cuticle consists primarily of chitin fibrils embedded in a protein matrix (Neville, 1984) and that fungal chitinases are also usually induced by chitin (Smith and Grula, 1983; St. Leger *et al*., 1986b), co-coordinated regulation of enzymes that hydrolyze the two main constituents of cuticle may prove most efficient under some circumstances. The subtilisin like Pr1 from the mushroom pathogen *Trichoderma harzianum* is induced also by chitin in fungal cell wall preparations and is repressed in the presence of casein or BSA (Geremia *et al*., 1993). Since chitin is an insoluble polymer, the most likely inducer is the monomer NAG which plays a similar role for extracellular protease production by *B. bassiana* (Geremia *et al*., 1993) and *Metarhizium* endochitinase (St. Leger *et al*., 1986b).

Protease (Pr1 and Pr2) activity of *M. anisopliae* was also affected by pH changes in the basal medium with the diamondback moth cuticle as a carbon source. The production of these proteases seemed to be induced when the external pH is alkaline (Table II). These data suggest that both proteases are induced by specific components of the cuticle, and that their detection occurs at pH levels close to 8. Similar findings were also shown by Bidochka and Khachatourians (1987) who observed maximum Pr1 protease activity in *B. bassiana* at a pH 8.5. St. Leger *et al.* (1998) also described that *M. anisopliae* produces extracellular proteases only at the pH at which they are active. According to these authors, there is evidence for a concerted action of pH and presence of cuticle on enzyme induction in *M. anisopliae*. Recently Dias *et al*. (2008) also described higher Pr1 and Pr2 activities when *B. bassiana* was grown at a pH 8 in the presence of coffee berry borer cuticle.

The extracellular Pr1 and Pr2 activities were more active between 35 and 45°C which is similar to the findings of Bidochka and Khachatourians (1987) who observed that optimum temperature for protease activity ranged between 37 and 42°C. The protease inactivated at higher temperatures, approximately 40% activity remained after 30 min 50°C, while protease was rapidly inactivated at 60°C. Ito *et al.* (2007) also studied the effect of different temperatures on extracellular protease production by *B. bassiana* and observed that extracellular proteases were more active between 40 and 60°C.

The study of the regulation of virulence factors in entomopathogenic fungus is of particular importance because pathogenic specialization may operate by way of regulatory controls that allow their expression. Furthermore, studies on the timing

of the production of proteases and other factors in the presence of cuticular substrates could provide information about the role of the accumulated hydrolytic enzymes during pathogenesis. Similarly, controlling the induction-repression of extracellular protease by means of a single regulatory system would be the most effective way of adjusting the rate of insect cuticle degradation. During cuticle penetration, extracellular proteases would hydrolyze proteins. The results presented in this study increase the knowledge on protease production in *M. anisopliae* (isolate-IF28.2), opening new avenues for the study of the role of extracellular proteases (Pr1 and Pr2) in virulence against the diamondback moth during the infection process.

ACKNOWLEDGEMENTS

This research was funded by grants from National Natural Science Foundation of China (No. 31071685) and China Postdoctoral Science Foundation (No. 20100480760).

REFERENCES

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

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

BIDOCHKA, M.J. AND KHACHATOURIANS, G.G., 1987. Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana. Appl. environ. Microbiol.,* **53**: 1679-1684.

BIDOCHKA, M.J. AND KHACHATOURIANS, G.G., 1988. Regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassian. Exp. Mycol.,* **13**:161-168.

BIDOCHKA, M.J. AND KHACHATOURIANS, G.G., 1990. Identification of *Beauveria bassiana* extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, *Melanoplus sanguinipes. J. Inverteb. Pathol.,* **56**:362-370.

BOGO, M.R., ROTA, C.A., PINTO, J. H., OCAMPOS, M., CORREA, C.T., VAINSTEIN, M.H. AND SCHRANK, A., 1998. A chitinase encoding gene (*chit1 Gene*) from the entomophatogen *Metarhizium anisopliae*: isolation and characterization of genomic and full-length cDNA. *Curr. Microbiol.,* **37**:221–225.

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.

BREY, P.T., LATGE, J.P. AND PREVOST, M.C., 1986. Intergumental penetration of the pea aphid, *Acyrthosiphon pisium*, by *Conidiobolus obscurus* (Entomophthoraceae). *J. Invert. Pathol*., **48**: 34–41

BYE, N.J. AND CHARNLEY, A.K., 2008. Regulation of cuticle degrading subtilisin proteases fron the entomopathogenic fungi, *Lecanicillium* spp: implications for host specificity. *Arch. Microbiol.,* **189**: 81-92.

CHARNLEY, A.K., 2003. Fungal pathogens of insects: cuticle degrading enzymes and toxins. *Adv. bot. Res.,* **40**: 241–321.

CHERRY, A.J., MERCADIER, G., MEIKLE, W., CASTELO-BRANCO, M. AND SCHROER, S., 2004. The role of entomopathogens in DBM biological control. In: *Improving biocontrol of* Plutella xylostella (eds. A.A. Kirk and D. Bordat), Proceedings of International Symposium. Montpellier, France, pp. 51-70.

COVE, D.J., 1966. The induction and repression of nitrate reductase in fungus *Aspergillus nidulans*. *Biochem. biophys. Acta,* **113**: 51-56.

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

DIAS, B.A., NEVES, P.M.O.J., MAIA, F. AND FURLANETO, M.C., 2008. Cuticle degrading proteases produced by the entomopathogenic fungus *Beauveria bassiana* in the presence of coffee berry borer cuticle. *Braz. J. Microbiol.,* **39**: 301-306.

DOMBROVSKY, A., HUET, H., ZHANG, H., CHEJANOVSKY, N. AND RACCAH, B., 2003, Comparison of newly isolated cuticular protein genes from six aphid species, *Insect Biochem. mol. Biol.,* **33**: 709–715.

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 an entomopathogenic fungus *Nomuraea rileyi*. *J. Invert. Pathol.,* **54**: 394–403.

FERRÉ, J. AND VAN RIE, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuaringiensis. Annu. Rev. Ent.,* **47**: 501-533.

GEREMIA, R.A., GOLDMAN, G.H., JACOBS, D., ARDILES, W., VILA, S.B., VANMONTAGU, M. AND HERRERAESTRELLA, A., 1993. Molecular characterization of the proteinase-encoding gene, Prb1, related to mycoparasitism by *Trichoderma harzianum. Mol. Microbiol.,* **8**: 603–613.

GILLESPIE, J.P., BATEMAN, R. AND CHARNLEY, K., 1998. Role of cuticle degrading proteases in the

virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria. J. Inverteb. Pathol.,* **71**: 128–137.

GUPTA, S.C., LEATHERS, T.D., El-SAYED, G.N. AND IGNOFFO, C.M., 1992. Insect cuticle-degrading enzymes from the entomogenous fungus *Beauveria bassiana, Exp. Mycol.,* **16**: 132–137.

HENRIK, U.S., PITTENDRIGH, B.R., KROYMANN, J., WENIGER, K., FRITSCHE, J., ANTJE BAUKE, A. AND MITCHELL-OLDS, T., 2000. Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. *Plant Physiol.,* **124**: 1007-1018.

IBRAHIM, Y.B. AND LOW, W., 1993. Potentional of mass-production and field efficacy of isolates of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyes fumosoroseus* against *Plutella xylostella. Int. J. Pest Managem.*, **39**:288-292.

ITO, E.T., VARéA-PEREIRA, G., MIYAGUI, D.T., PINOTTI, M.H.P. AND NEVES, P.M.O.J., 2007. Production of Extracellular Protease by a Brazilian Strain of *Beauveria bassiana* Reactivated on Coffee Berry Borer, *Hypothenemus hamper. Braz. Arch. Biol. Technol.,* **50**: 217-223.

LIU, S.Y. 2006. *Investigating and screening the intensive pathogenicity of the fungi in genera* Paecilomyces *and* Metarhizium. M.Sc. thesis, South China Agricultural University, Guangzhou, People's Republic of China.

MISSIOS, S.H. DAVIDSON, C., LINDER, D., MORTIMER, L., ANTHONY, O. O. AND JOHN, S. D., 2000. Characterization of cuticular proteins in the red flour beetle, *Tribolium castaneum, Insect Biochem. mol. Biol.,* **30**: 47-52.

MUHAMMAD, S., ANDREW, B.K. AND LLOYD, M.D., 2005. Biological control of the diamondback moth, *Plutella xylostella*: A review. *Biocont. Sci. Technol.*, **15**: 763-789.

NEVILLE, A.C., 1984, Cuticle: organization. In: *Biology of the integument* (eds. B.J. Hahn, A.G. Maroksy and K.S. Richards), Springer, Berlin, pp. 611–625.

PELL, J.K., WILDING, N., PLAYER, A.L. AND CLARK, S.J., 1993. Selection of an isolate of *Zoophthora radicans* (Zygomycetes: Entomophthorales) for biocontrol of the diamondback moth. *J. Inverteb. Pathol.,* **61**: 75-80.

PEREZ, C.J. AND SHELTON, A.M., 1997. Resistance of (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* Berliner in Central America. *J. econ. Ent.*, **90**: 87-93.

SAMUELS, R.I., AND PATERSON, I.C., 1995. Cuticle degrading proteases from insect moulting fluid and culture filtrates of entomopathogenic fungi. *Comp. Biochem. Physiol.,* **110**: 661-669.

SAS INSTITUTE 2000, *SAS user's guide, statistics*, Cary, North carolina, SAS Institute.

SCREEN, S.E., BAILEY, A., CHARNLEY, K., COOPER, R. AND CLARKSON, J., 1998. Isolation of a nitrogen response regulator gene (nrr1) from *Metarhizium anisopliae*. *Gene,* **221**: 17-24.

SHELTON, A.M., VANDENBERG, J.D., RAMOS, M. AND WILSEY, W.T., 1998. Efficacy and persistence of *Beauveria bassiana* and other fungi for control of diamondback moth on cabbage seedlings. *J. econ. Ent.*, **33**: 142-151.

SHELTON, A.M., WYMAN, J.A., CUSHING, N.L., APFELBECK, K., DENNEHY, T.J., MAHR, S. AND EIGENBRODE, S.D., 1993. Insecticide resistance of diamondback moth in North America. *J. econ. Ent.,* **86**: 11-19.

SMITH, R.J. AND GRULA, E.A., 1983. Chitinase is an inducible enzyme in *Beauveria bassiana, J. Inverteb. Pathol.,* **42**: 319–326.

ST LEGER, R.J., JOSHI, L., BIDOCHKA, M.J. AND ROBERTS, D.W., 1998. Ambient pH is a major determinant in the expression of cuticle degrading enzymes and hydrophobin by *Metarhizium anisopliae. Appl. environ. Microbiol.*, **64**: 709-713.

ST. LEGER, R.J., CHARNLEY, A.K. AND COOPER, R.M., 1986a. Cuticle-degrading enzymes of entomopathogenic fungi mechanisms of interaction between pathogen enzymes and insect cuticle. *J. Inverteb. Pathol.,* **47**: 295–302.

ST. LEGER, R.J., CHARNLEY, A.K. AND COOPER, R.M., 1987. Characterization of cuticle degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. *Arch. Biochem. Biophys.*, **253**: 221–232.

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

ST. LEGER, R.J., DURRANDS, P.K., CHARNLEY, A.K. AND COOPER, R.M., 1988. Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta*. *J. Inverteb. Pathol.,* **52**: 285–293.

ST. LEGER, R.J., FRANK, D.C., ROBERTS, D.W. AND STAPLES, R.C., 1992. Molecular cloning and regulatory analysis of the cuticle- degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *Eur. J. Biochem.*, **204**: 991–1001.

ST. LEGER, R.J., JOSHI, L., BIDOCHKA, M. AND ROBERTS, D.W., 1995. Protein synthesis in *Metarhizium anisopliae* growing on host cuticle. *Mycol. Res.,* **99**: 1034–1040.

ST. LEGER, R.J., JOSHI, L., BIDOCHKA, M., RIZZO, M.J. AND ROBERTS, D.W., 1996b. Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles, *Appl. environ. Microbiol.,* **62**: 1257–1264.

SYED, A.R., 1992. Insecticide resistance on diamondback moth in Malaysia. In: *Diamondback moth and other crucifer pests* (ed. N.S. Talekar), Proceedings of 2nd International Workshop, AVRDC, Taiwan, pp. 437-442.

TABASHNIK, B.E., 1994. Evolution of resistance to *Bacillus thuringiensis. Annu. Rev. Ent.*, **39**: 7-79.

TABASHNIK, B.E., CUSHING, N.W., FINSON, N. AND JOHNSON, M.W., 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth. *J. econ. Ent.*, **83**: 1671-1676.

VANDENBERG, J.D. AND RAMOS, M.R., 1997. Screening of fungal isolates against diamondback moth larvae. *Arthrop. Managem. Tests*, **22**: 420-421.

VANDENBERG, J.D., RAMOS, M.R. AND ALTRE, J., 1998. Dose-response and age and temperature-related susceptibility of the diamondback month (Lepidoptera: Plutellidae) to two isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae). *Environ. Ent.*, **64**: 1402-1412.

WRIGHT, D.J., 2004. Biological control of *Plutella xylostella*, In: *A global perspective. Improving biocontrol of Plutella xylostella (*eds. A.A. Kirk and D. Bordat), Proceedings of International Symposium, Montpellier, France, pp. 9-16.

WU, J.H., ALI, S., HUANG, Z. AND REN, S.X. 2010. Media composition influences on growth, enzyme activity and virulence of the entomopathogen *Metarhizium anisopliae* (Hypocreales; Clavicipitaceae). *Pakistan J. Zool.*, **42**: 451-459.

XU, Y.Y., LIU, T.X., LEIBEE, G.L. AND JONES, W.A., 2004. Effects of selected insecticides on *Diadegma insulare* (Hymenoptera: Ichneumonidae), a parasitoid of *Plutella xylostella* (Lepidoptera: Plutellidae), *Biocont. Sci. Technol.*, **14**: 713-723.

(Received 8 April 2011, revised 16 May 2011)