Journal of Biological Control, 25 (3): 223-228, 2011

Research Article

Chitinase activity and virulence of different isolates of *Beauveria bassiana, Metarhizium anisopliae* and *Lecanicillium* spp.

B. RAMANUJAM*, M. BALACHANDER, G. ROOPA, R. RANGESHWARAN and PRITAM KARMAKAR

National Bureau of Agriculturally Important Insects, H. A. Farm Post, Bellary Road, Bangalore 560 024. Karnataka, India
**Corresponding author email:* bonamramanujam58@gmail.com

ABSTRACT: In order to identify promising isolates with higher chitinase activity, 122 entomofungal isolates comprising of *Beauveria bassiana* (58), *Metarhizium anisopliae* (33), *Lecanicillium lecanii* (15), *Lecanicillium attenuatum* (11), *L. longisporum* (3) and *L. muscarium* (2) isolated from different insect hosts/soils of India were studied. The partially purified proteins from the isolates were subjected to chitinase activity and were estimated by measuring the release of reducing saccharides (one NAGA Unit) from colloidal chitin spectrophotometrically at 582 nm (A_{582}). Forty nine isolates of *B. bassiana* showed chitinase activity ranging from 21 to 182 µg/ml, with the highest enzyme activity by the isolate PDBC-Bb-5a. Thirty three isolates of *M. anisopliae* exhibited chitinase activity ranging from 23 to 144 µg/ml and the highest (144 µg/ml) was by the isolate Ma-4. Among the 15 isolates of *L. lecanii* tested, three isolates viz., Vl-7, Vl-24a, Vl-25a had high chitinase activities ranging between 100 and 126µg/ml. Vl-22 isolate of *L. attenuatum*, Vl-24 of *L. longisporum* and Vl-8 of *L. muscarium* showed higher activities (90, 110 and 117µg/ml respectively). Bioassay studies with these isolates on cowpea aphid, *Aphis craccivora* in glass house indicated higher nymphal mycosis ranging from 72.3 – 83.0%.

KEY WORDS: Entomopathogenic fungi, chitinase activity, NAGA unit, cowpea aphid, *Aphis craccivora*, virulence

(Article chronicle: Received: 18.08.2011; Sent for revision: 27.08.2011; Accepted: 02.07.2011)

INTRODUCTION

Chitinase activity has been considered as one of the most important factors for the pathogenicity of *Metarhizium anisopliae, Beauveria bassiana* and *Lecanicillium* spp. Insect cuticle is composed mainly of chitin embedded in a protein matrix (Hepburn, 1985). The cuticle represents the main barrier for the penetration of fungal germ tube through the cuticle and the fungi produces chitinase, protease and lipase commonly referred to as Cuticle Degrading Enzymes (Krieger de Moraes *et al.,* 2003). The entry of entomopathogenic fungi through the insect cuticle is considered to occur by a combination of mechanical pressure and enzymatic degradation (Charnley, 1984). Highly pathogenic strains showed detectable amounts of extracellular chitinase, lipase and protease activities in the culture (Samuels *et al.*, 1989). Among the exochitinases, the most prominent one is N-acetyl glucosaminidase releasing acetylglucosamine from the reducing ends of the chitin chains.

In the present study, 122 isolates of entomopathogenic fungi were studied for chitinase activity. Some of the promising isolates were tested for their virulence against cowpea aphid, *Aphis craccivora* Koch.

MATERIALS AND METHODS

Isolates and Culture Conditions

The 122 selected isolates of entomopathogenic fungi used in the enzymatic and bioassay studies were isolated from different insects and soil samples from various agro-climatic zones of India. The identity of these fungi was established through ITS-1 and ITS-2 sequences analysis and by blast confirmation. The sequences have been deposited in GenBank (Table 1). The cultures of these fungi were preserved in mineral oil slants of Sabouraud dextrose agar in the repository of National Bureau of Agriculturally Important Insects, Bangalore.

Preparation of Colloidal Chitin

The colloidal chitin was obtained as per the procedure of Nahar *et al*. (2004). Chitin (20 g) was dissolved in 200 ml of concentrated HCl with stirring for 3 min at 40°C.

The chitin was precipitated as a colloidal suspension by slowly adding water (2 L) at 5°C.

Table 1. Isolates of entomopathogenic fungi used in the chitinase enzyme assay

Fungi (NBAII isolates)	Insect derived isolates	Soil isolates	Total	GenBank Accessions
Beauveria bassiana	39	19	58	JF742994, JF742995, JF750391 to JF750398, JF837082 to JF837101, JF837118 to JF837125, JF837128 to JF837141, JF837143, JF837144
Metarhizium anisopliae	12	21	33	JF837102, JF837104 to JF837116, JF837145 to JF837159, JF837161 to JF837163
Lecanicillium lecanii	15	–	15	JF718693 to JF718707
L. attenuatum	11	–	11	JF718708 to JF718718
L. longisporum	3	–	3	JF718719 to JF718721
L. muscarium	2	–	2	JF718722, JF718723
Total	82	40	122	–

Colloidal suspension was collected by filtering through coarse filter paper; washed with tap water until the pH of the suspension was about 4.0.

Extraction of exo-chitinase

The fungal isolates were grown in one liter of culture medium containing salts (0.05% $MgSO_4$ and 0.5% NaH_2PO_4) and colloidal chitin (2% w/v) at 26°C for 4 days with an inoculum of $1x10^8$ conidia per liter of culture medium. Extraction of exo-chitinase was done according to Yanai *et al.* (1992). Fungal cultures were centrifuged at 3000g for 15 min and the supernatant was filtered through Whatman filter paper No. 2 and No. 5 and then through a 0.22-ì m-pore-size membrane filter (Millipore). Ammonium sulfate was added to the filtrate to achieve 85% saturation (608 g/L). After the mixture was allowed to stand overnight, the precipitate was collected by centrifugation (15,000g, 15 min) and dissolved in 40 ml of 20 mM Tris–HCl buffer (pH 7.5) containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM EDTA. The solution was dialyzed with 20mM Tris-HCl buffer for three times each for 5 hours at 4°C and then centrifuged at 15,000g for 15 min. The precipitates were discarded and the supernatant was used for the enzyme assays.

Enzyme Assay

Reaction mixture containing 1 ml of culture supernatant, 0.3ml of 1M sodium acetate buffer (pH-4.7) and 0.2ml of colloidal chitin was incubated at 40° C for 6-24h and then centrifuged at 12225g for 5min at 6°C. After centrifugation, an aliquot of 0.75ml of the supernatant, 0.25ml of 1% solution of dinitrosalicylic acid in 0.7M NaOH was mixed in 1.5ml micro centrifuge tube and heated at 100°C for 5 min. The chitinase activity was estimated by measuring the release of reducing saccharides (one NAGA Unit) from colloidal chitin spectrophotometrically at 582 nm (A_{582}).

Bioassay on *Aphis craccivora*

Isolates of *B. bassiana, M. anisopliae* and *Lecanicillum* spp. with varied degree of chitinase activity were selected (Table 2) for bioassay studies against *A. craccivora.* The entomopathogenic fungi were grown on Sabouraud dextrose agar with yeast extract (1% w/v) for 10 days. The spores of each isolate was harvested by flooding the plate with sterile distilled water containing 0.01 percent Tween 80 and scraping the surface with sterile spatula. Then, the suspension was passed through a double-layered muslin cloth and the filtrate was diluted with known quantity of 0.01 percent Tween 80 emulsion to get spore concentration of $1x10^7$ spores/ml. The required spore concentration was adjusted with the help of a Neubauer's improved haemocytometer. Nymphs of *A. craccivora* reared on cowpea seedlings were used in the bioassay. Three ml of spore suspension containing $1x10^7$ spores/ml was sprayed on the leaves of 10-day old healthy cow pea seedling. After the inoculum on the leaves dried, ten nymphs of *A. craccivora* were released on each cowpea seedling. Three replications were maintained for each fungal isolate. Sprayed plants were kept in glass house for 10 days and observations on the mortality of nymphs were recorded at an interval of

24 hrs. Dead nymphs were transferred to humid chambers for development of mycosis. The data obtained were analysed in ANOVA single factor using AgRes statistical software with significant different (P≤0.01). Percentage mycosis were angular transformed before analysis.

RESULTS AND DISCUSSION

Chitinase activity

Among the 58 isolates of *B. bassiana* tested, chitinase activity was observed in 49 isolates, ranging from 21 to 182 µg/ml. Highest activity was observed with PDBC-Bb-5a strain (182 µg/ml) (Fig. 1), followed by PDBC-Bb-59 (107µg/ml). All the 33 isolates of *M. anisopliae* showed chitinase activity ranging between 23 to 144 µg/ml and the highest activity was observed with PDBC-Ma-4 (144 µg/ml), followed by PDBC-Ma-2 (118 µg/ml) (Fig. 2). All 31 isolates of *Lecanicillium* spp., except Vl-20 of *L. lecanii* showed chitinase activity. The 15 isolates of *L. lecanii* exhibited chitinase activity ranging from 19 to 123 µg/ml (Fig. 3) with significantly high enzyme activities detected in Vl-7, Vl-24a, and Vl-25a isolates (123, 102 and 100 µg/ml respectively). The 11 isolates of *L. attenuatum* showed chitinase activity ranging from 23 to 90 µg/ml, with the highest activity detected in Vl-22 strain (Fig. 4). The Vl-24 isolate of *L. longisporum* and the Vl-8 isolate of *L. muscarium* showed chitinase activity of 110 µg/ml and 117 µg/ml respectively (Fig. 5). In terms of pathogenicity, although proteases play a very important role, chitinase plays a

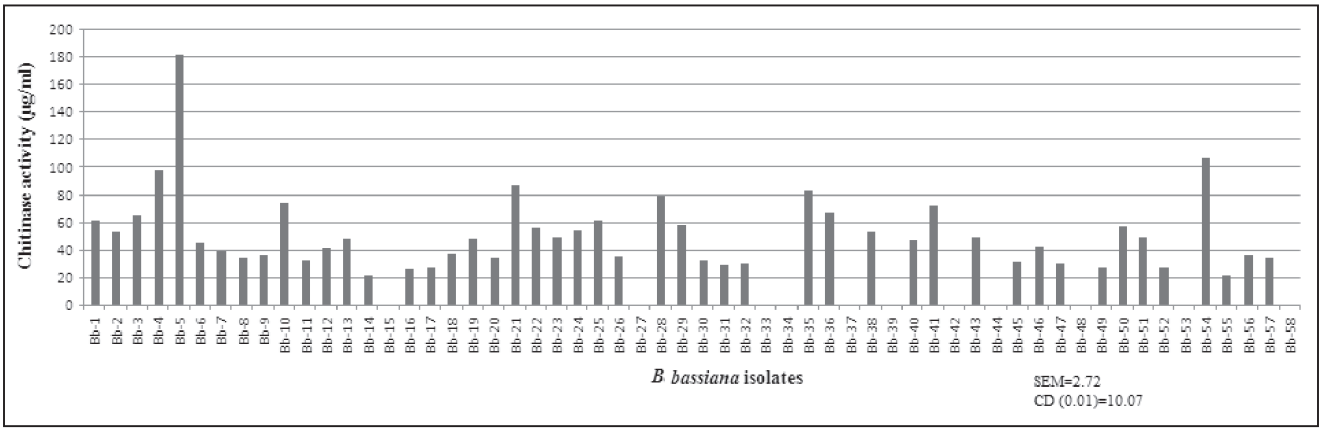

Fig. 1. Chitinase activity of *Beauveria bassiana* isolates (*P*≤0.01)

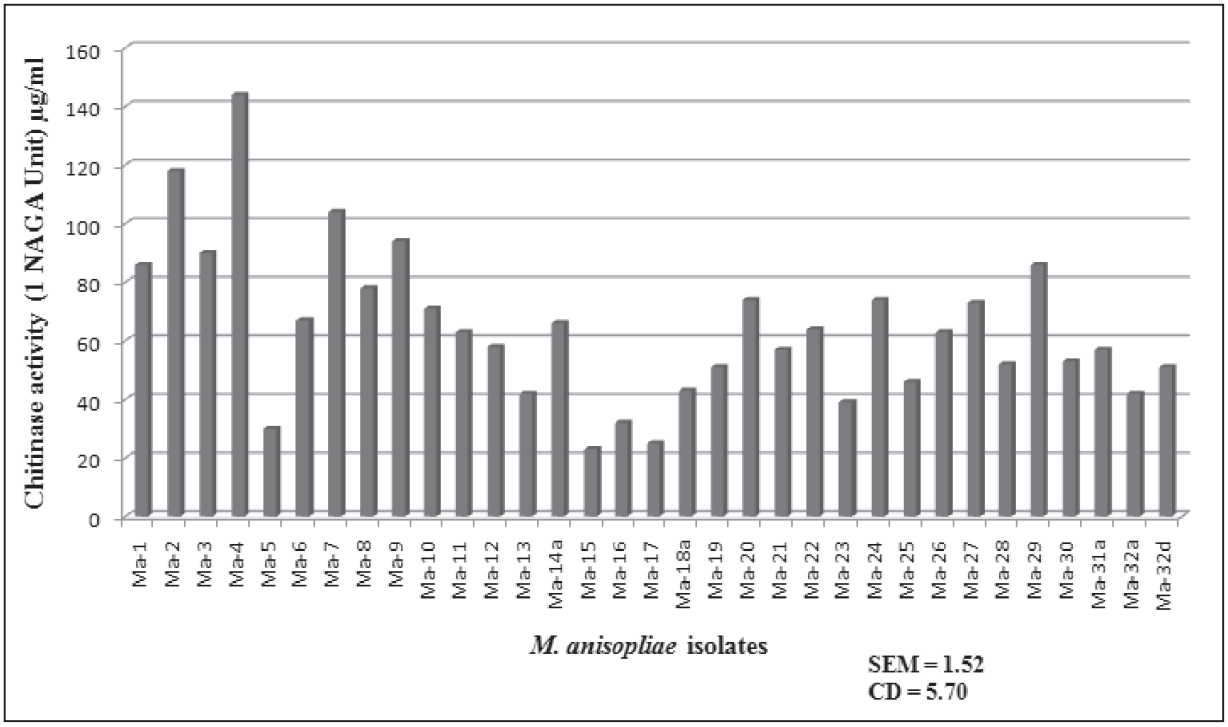

Fig. 2. Chitinase activity of *Metarhizium anisopliae* isolates (*P*≤0.01)

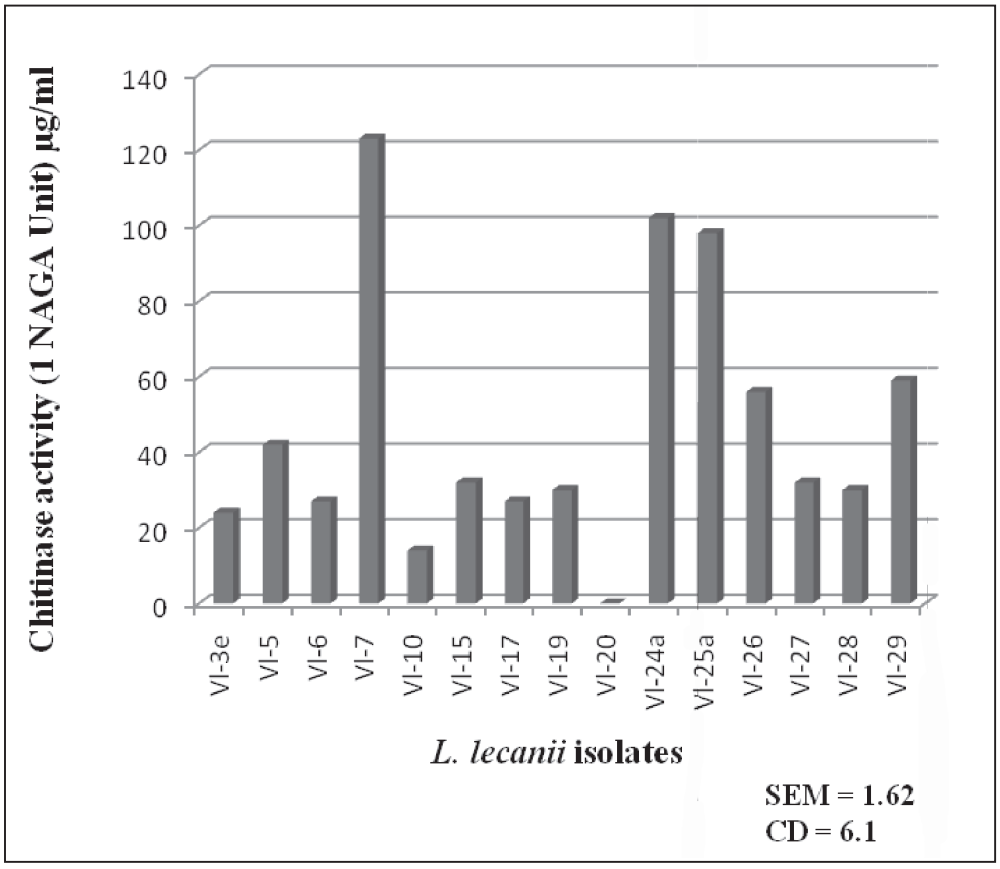

Fig. 3. Chitinase activity of *Lecanicillium lecanii* isolates ($P \leq 0.01$)

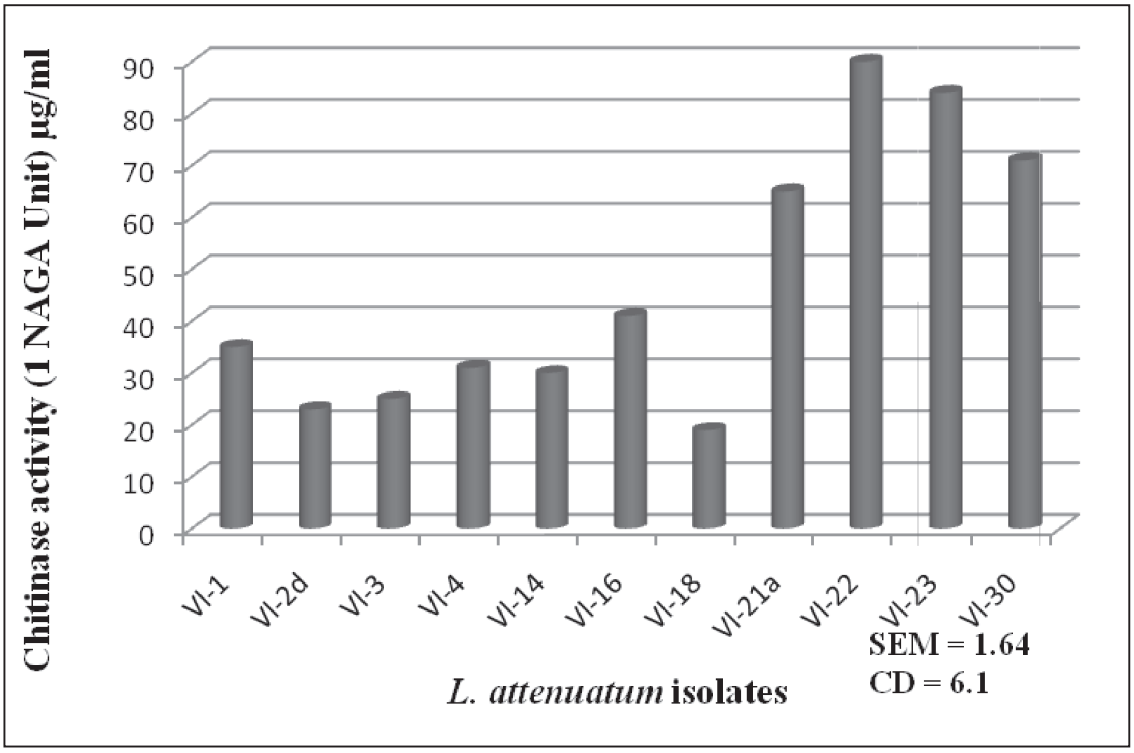

Fig. 4. Chitinase activity of *Lecanicillium attenuatum* isolates ($P \leq 0.01$)

major role in fungal virulence including *M. anisopliae* (St. Leger *et al*., 1991), *B. bassiana* (Havukkala *et al*., 1993) and also *Lecanicillium* spp. Samuels *et al*. (1989) proved that highly pathogenic strains showed detectable amounts of extracellular chitinase, lipase and protease activities in the culture. Though we did not test them for protease activity, it is understood that the data on their chitinase activity will help in segregating the virulent ones. Chitinases secreted by *Lecanicillium.* have considerable importance in the biological control of some insect pests like aphids and white flies. Insects produce chitinase to degrade old cuticle during moulting, which can also act on fungal cell wall chitin. To combat insect chitinases, entomopathogenic fungi can also adopt this as a defence mechanism. In the present study, among the 122 fungi tested some of them exhibited higher chitinase activity which could make them to be more virulent or pathogenic. In order to study their virulence, the isolates with higher/lesser chitinase activity were tested for their infectivity on the nymphs of cowpea aphid, *A. craccivora*.

Bioassay against *A. craccivora*

It was observed that fungi with higher chitinase activity exhibited increased mycosis (Table 2). The fungi showing maximum chitinase activity namely *B. bassiana* (Bb-5a), *M. anisopliae* (Ma-4), *L. lecanii* (Vl-7) and *L. muscarium* (Vl-8) showed >80% nymphal mycosis of *A. craccivora* as compared to the 30-40% mycosis exhibited by non chitinase producing isolates of *B. bassiana* (Bb-15), *L. lecanii* (Vl-20) and *M. anisopliae* isolate with lesser chitinase activity (Ma-15). The bioassay results clearly establish the fact that entomopathogenic fungi with higher chitinolytic activity also showed higher virulence. Such isolates will be more suitable for field application or commercial use. In the present study among the 122 fungi tested, some isolates like Bb-5a, Ma-4 Vl-7 and Vl-8 were able to exhibit higher chitinase activity which could make them more virulent. The present study has brought out a comprehensive analysis of the chitinase activity of the entomopathogenic fungal isolates of India and its role in eliciting enhanced virulence.

Table 2. Chitinase activity and virulence of selected isolates of entomopathogenic fungi against *Aphis craccivora*

Entomopathogenic fungi		Chitinase activity (µg/ml)	Per cent mycosis
Beauveria bassiana	Bb-5a	182	81.0 (64.54^{abc})
	Bb-59	107	79.0 (63.09^{bcd})
	Bb-4	98	75.6 (60.81^{def})
	Bb-25	87	76.6 (61.47^{cde})
	Bb-15	0.00	30.0 (33.52^{h})
Metarhizium anisopliae	Ma-4	144	82.3 (65.49^{ab})
	Ma-2	118	72.3 (58.59^{f})
	Ma-7	104	77.3 (61.93^{de})
	Ma-9	94	73.7 (59.45^{ef})
	Ma-15	23	32.8 (34.42^{g})
Lecanicillium lecanii	Vl-7	123	83.0 (66.03^{a})
	Vl-24a	102	76.3 (61.22^{de})
	Vl-20	0.00	40.0 (39.52^{g})
Lecanicillium muscarium	Vl-8	117	82.0 (65.27^{ab})
Lecanicillium longisporum	Vl-24	110	78.3 (62.61^{cd})
	SEM	0.739	
		C.D (P = 0.01)	2.04

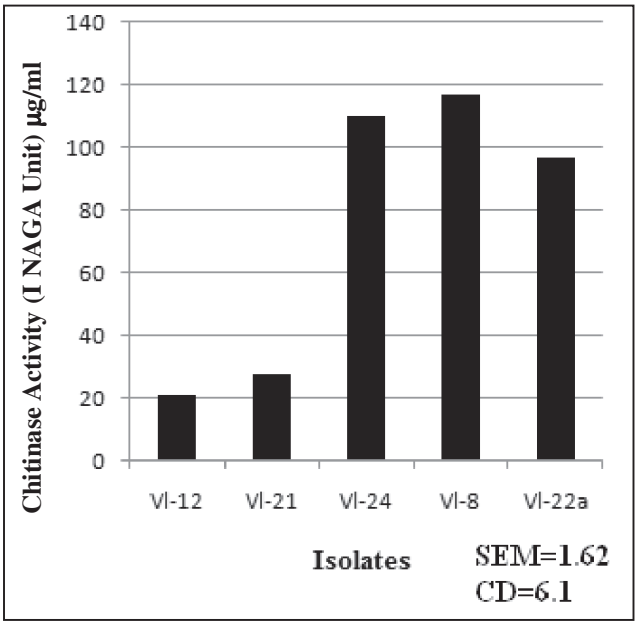

Fig. 5. Chitinase activity of *Lecanicillium longisporum* (Vl-12, 21 & 24) and *L. muscarium* (Vl-8 & 22a)

REFERENCES

Charnley, A. K. 1984. Physiological aspects of destructive pathogenesis in insects by fungi; a speculative review. In: J. M. Anderson, A. D. M. Rayner and D. W. H. Walton (eds) *Invertebrate-Microbial Interactions*, pp. 229–270. Cambridge University Press.

Havukkala, I., Mitamura, C., Hara, S., Hirayae, K., Nishizawa, Y. and Hibi, T. 1993. Induction and purification of *Beauveria bassiana* chitinolytic enzymes. *Journal Invertebrate Pathology*, **61**: 97–102.

Hepburn, H. R. 1985. Structure of the integument. In Kerbut, G.A. and Gilbert, L.I, (eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* Volume, 3, pp. 1–58. Pergamon Press, Oxford, UK.

Krieger de Moraes, C., Schrank, A. and Vainstein, M. H., 2003. Regulation of extracellular chitinase and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. *Current Microbiology*, **46**: 205–210.

Nahar, P., Ghormade, V. and Deshpande, M.V. 2004. The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: Possible edge to entomopathogenic fungi in the biological control of insect pests. *Journal of Invertebrate Pathology,* **85**: 80–88

Samuels, K. D. Z., Heale, J. B. and Llewellyn, M. 1989. Characteristics relating to the pathogenicity of *Metarhizium anisopliae* toward *Nilaparvata lugens. Journal of Invertebrate Pathology,* **53**: 25–31.

St. Leger, R. J., Cooper, R. M. and Charnley, A. K. 1991. Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, **58**:415–426.

Yanai, K., Takaya, N., Kojima, M., Horiuchi, H., Ohta, A. and Takaki, M. 1992. Purication of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. *Journal of Bacteriology*, **174**: 7398–7406.