Journal of Forestry Research (2012) 23(4): 651–659
DOI 10.1007/s11676-012-0306-9

ORIGINAL PAPER

Virulence and mycotoxic effects of *Metarhizium anisopliae* on Mahogany shoot borer, *Hypsipyla robusta* (Lepidoptera: Pyralidae)

M. Balachander • O.K. Remadevi • T.O. Sasidharan • N. Sapna Bai

Received: 2011-11-15; Accepted: 2011-12-11

Abstract: Developing appropriate control measures for the Mahogany shoot borer, *Hypsipyla robusta* Moore has become increasingly important due to the severe damaging effect of the pest on the establishment of the saplings of *Swietenia mahagoni* Jacq (Sapindales: Meliaceae). Existing management methods are largely limited to silvicultural practices and spraying of chemical insecticides. To identify a potential fungal biocontrol agent, we compared the virulence of six native and two standard ARSEF isolates of *Metarhizium anisopliae* Metsch. against this pest. The average survival time and conidial yield of IWST-Ma7 was higher (6.2 to 7.3 days and 4.9 to 4.7 × 10^5 conidia/ml) than the standards. Significant difference in sporulation on the cadavers between isolates, doses and incubation periods were substantiated for the selection of potential strain. The mycotoxic effects of crude soluble protein extract when incorporated in the artificial diet, the ARSEF 2596 and ARSEF 3603 showed LD_{50} value of 3.7% and 5.6%. However, IWST-Ma7 was highly lethal with significant lowest LD_{50} value of 2.6%. The enzyme activity of IWST-Ma7 was highest for chitinase, CDA, protease and lipase viz., 1.90 U/mg, 1.80 U/mg, 0.98 U/mg and 0.80 U/mg respectively. However the enzyme activity of chitinase and Chitin deacetylase assay for all the isolates was significantly higher than protease and lipase activity. The ITS regions (5.8S rDNA and 28S rDNA) of seven isolates of *M. anisopliae* were amplified using the ITS1 and ITS4 primers which was a unique fragment of approximately 550 bp. Based on ITS regions, phylogenetic tree have been constructed and the isolates have been grouped in to 5 clades. The virulence and mycotoxic effects of different isolates could rationally be used to employ them for the management of the mahogany borer.

The online version is available at http://www.springerlink.com

M. Balachander • O. K. Remadevi (✉)
Wood Biodegradation Division, Institute of Wood Science & Technology, (Indian Council for Forestry Research and Education), Malleswaram P.O., Bangalore -560 003, Karnataka, India.
Email: okremadevi@icfre.org; okremadevi@gmail.com
Phone: 91-080-22190153(O)

T.O Sasidharan. • N. Sapna Bai
Ashoka Trust for Research in Ecology and the Environment, Jakkur P.O., Bangalore 560 064, Karnataka, India

Responsible editor: Chai Ruihai

Keywords: *Metarhizium anisopliae*, mycotoxin, mahogany shoot borer, *Hypsipyla robusta*

Introduction

Mahogany (*Swietenia mahagoni* Jacq.) is a large deciduous high timber value tree used for making furniture, paneling, railway sleepers, industrial and domestic wood wares, traditional medicine, etc. (Wylie 2001; Lopes et al. 2008). Continuous supply of this species is often constrained by low natural regeneration and difficulty in establishment mainly due to the attack by the shoot borer, *Hypsipyla robusta* Moore and *H. grandella* Zeller in the tropical and sub tropical parts of the world (Cunningham et al. 2005; Ofori et al. 2007).

In India, *H. robusta* Moore is a serious pest of meliaceous forest trees such as the exotics, *Swietenia macrophylla*, *Swietenia mahagoni* and native *Toona ciliata*. Although *Swietenia* spp. is grown in plantations in many States, the establishment is difficult because of shoot borer attack during the sapling stage (Varma 2001). There are five generations of *H. robusta* in temperate and sub tropical regions with different generations feeding on flowers, fruits and shoots (Beeson 1941). The borer is reported to cause 29% loss in potential biomass production by attacking about 40% of the saplings in a plantation (Hossain et al., 2004). Despite ready germination of its seeds, the transition from seedlings to saplings is hindered by the pest in natural forests (Lauma-aho 2003). Plants in the age group of 3–6 years and class height 3–5 m were reported to be more susceptible to the pest attack revealing up to 90% infestation, showing the relationship between infestation and age of tree (Cipiao et al. 2009). The most important host response associated with *Hypsipyla* attack is the sprouting of multiple shoots on infested plants which affects the growth and economic value of the timber (Bygrave and Bygrave 2001) Repeated attacks in the early years of plantation eventually lead to death of trees (Lim 2008; Cornelius 2009).

Different Silvicultural practices such as mixed or enrichment plantings, wider spacing, varying tree density (Guimaraes et al. 2004; Perez-Salicrup and Esquivel 2008) provision of shade,

promoting vigorous tree growth, encouraging natural enemies (Newton et al. 1999; Varma 2001; Lopes et al. 2008) and leaving weed rows between young plantations are generally adopted for the management of *Hypsipyla* species. The attack of *H. robusta* varies with the clones and genetic variation for resistance is sometimes exploited as an element for integrated management of mahogany shoot borers (Cornelius and Watt 2003). A few systemic insecticides like carbofuran (Mayhew and Newton 1998) and deltamethrin (Goulet et al. 2005) are occasionally used in the management of shoot borers. These insecticides have limited prospects in today's pest management practices due to environmental concerns. Eco-friendly approaches such as biological control including microbial control assumes greater importance in this context.

Entomopathogens have been used in control of forest pests as alternative to chemical insecticides (Ahmed and Leather 1994). They are considered to be safer than chemical insecticides having little effect on man or other vertebrate and non-targeted invertebrates as a result of which natural control by parasitoids and predator is maintained (Hauxwell et al. 2001). The mitosporic ascomycete (hypocreales) fungus, *Metarhizium anisopliae* has gained significant attention as a biocontrol agent due to its wide geographic distribution, high virulence and vast spectrum of infectivity to a wide range of insect pests (Ypsilos and Magan 2005). The conidia of *M. anisopliae* usually enter into insect mainly through the integument by adhesion, penetration into haemoceol and development of fungal infection (Mycoy et al. 1988). The process of penetration through the insects integument by a hypal germination from a spore involves chemical (enzymatic) and physical forces. In vitro studies indicated that the digestion of the integument follow a sequential lipase-protease-chitins process of digestion (Robert 1969; Coundron et al. 1984; St.Leger et al. 1986; Nahar et al. 2004).

The present study was undertaken to evaluate the pathogenicity and insecticidal activity of *M. anisopliae* isolates against the mahogany shoot borer, *H. robusta* and to determine and correlate the activity of cuticle degrading enzymes produced by these isolates.

Materials and methods

Isolation of *M. anisopliae* from insect cadavers

The *M. anisopliae* isolates used in this study were recovered from infected insects (Table 1). The cadavers were surface disinfected with 5% sodium hypochlorite and placed in an environmental chamber on a water agar medium amended with antibacterial agents, on moistened filter paper in a sealed container and incubated at 25±0.5ºC for seven to fourteen days. The cadavers with hyphae were then transferred to selective medium containing 1% dextrose; 1% peptone; 1.5% oxgall; 3.2% agar; 10µg/mL dodine; 250 µg/mL cyclohexamide & 500µg/mL chloramphenicol for the isolation of *M. anisopliae*. The fungus was then grown on Potato dextrose agar (Hi-Media) fortified with 1% yeast extract at 26±0.5ºC in dark. Slants were prepared from purified culture and stored at 4°C and hyphae were used to identify the genus microscopically for the presence of philades. The isolates were passed through the respective host insects at thirty days of interval to maintain virulence. Theses isolates were maintained on the respective host insects larvae at -20ºC. The morphological features of colonies such as colony color, surface, conidia sizes, length and width were measured.

Rearing of Mahogany shoot borer in laboratory

H. robusta larvae were collected from a mahogany nursery in the South Canara district of Karnataka state and reared in an environmental chamber under a photoperiod of 10:14 h (L: D) at >85% RH and a temperature of 26±0.5ºC. The larvae were reared on a modified semi-synthetic diet (Ramareshiah and Shankaran 1994) packed in young bamboo culms (approx: 15 cm × 8 mm). Before filling the diet, the culms were disinfected by dipping in 3% sodium hypochlorite solution for 24 h. Individual larva was transferred to the culms filled with diet (2 to 4 times) and covered with cotton wool to prevent larva from escaping. After the complete consumption of the diet, the larvae were transferred to new culms filled with fresh diet. This procedure of rearing was adopted considering the boring behavior and cryptic nature of the larva in nature. After pupation cotton plugs were removed and the culms were kept in cages with potted mahogany plants for insect emergence, mating and egg lying. Leaves with eggs were transferred to glass bottles and incubated at 26±0.5ºC and 70%−75% RH to facilitate hatching.

Virulence of *M. anisopliae* against *H. robusta* larvae

M. anisopliae conidia were harvested from seven days old pure cultures in PDAY slants with 0.05% Tween- 80 as a carrier. The germination rate of conidia used in bioassays was ranged from 85% to 90%. The Average Survival Time (AST) for six native isolates and two ARSEF (USDA Agriculture Research Services Entomopathogenic Fungi Culture Collection, Ithaca, NY) strains ARSEF 3603 and ARSEF 2596 as a standard were studied with concentrations of 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia mL^{-1}. Second instar larvae were collected after molting and inoculated by dipping in the conidial suspension for 30 s and transferred to young bamboo culms with artificial diet. The bamboo culms were plugged with cotton wool and maintained at >85% RH and 26±0.5°C. Control larvae were treated with 0.05% Tween-80. Mortality was recorded at 24 h intervals for upto 10 days. Four replicates of twenty larvae each were maintained for each treatment.

Assessment of sporulation on cadavers of *H. robusta*

To assess sporulation, cadavers from each dose was individually transferred to 1.5% water-agar medium and incubated for 7 and 15 days at 25±0.5°C and >85% RH. Following incubation, cadavers from each dose were transferred individually to 10 mL of sterile 0.05% Tween-80 and shaken to facilitate dispersion of conidia. The suspension was then filtered through a sterile Whattman No.1 filter paper to remove larger mycelial particles and serially diluted

and spores were counted under phase contrast optics using an improved Neubauer Haemocytometer. The average conidial yields at different doses and days were estimated.

Extraction and purification of crude soluble protein extract (CSPE)

Conidia of different isolates were harvested from agar medium (PDAY) by flooding 0.08% sterile Tween-80 and the suspensions were filtered through Whattman No.1 filter paper. The filtered suspension were diluted to a concentration of 10^8 conidia/ml using Neubauer Haemocytometer. About 10 mL was inoculated into 1 liter of medium containing dextrose, 40 g/L; yeast extract, 20 g/L and corn steep liquor, 30 g/L & 10% *H. robusta* larval extract and incubated as stationary culture for three days at 26 ±0.5°C and >85% RH in dark. The mycelia were filtered through Whattman No.3 filter paper and the suspension was further filtered through 0.22µm filter (Millipore). The resultant filtrate was subjected to precipitation with 90% saturated ammonium sulphate. The precipitated crude soluble proteins were collected by centrifugation at 10,000 g for 15 min at 4°C. The pellets were re-suspended in 20 mM Tris-HCL buffer (pH-7.5) containing 1 mM phenylmethylsulfonyl flouride (PMSF) and 1 mM EDTA. The solution was desalted in a molecular porous membrane (Spectra Pro®1 Membrane) with a cut off of 6000-8000 Dalton. The desalted fraction was then concentrated at 4°C by embedding the membrane in polyethylene glycol. The proteins are finally purified with pre-packed column of Sephadex™ G-25 gel filtration media (HiPrep™ Desalting 26/10) with typical flow velocity of 150 cm/h. The concentrations of soluble proteins were determined by Lowry's assay using bovine serum albumin as standard at 280 nm absorbance in spectrophotometer.

Mycotoxic activity of *M. anisopliae* against *H. robusta*

The toxicity effects of CSPE were studied by incorporating a standard concentration of 10mg/ml *viz.,* 1%, 2%, 3%, 4% and 5% in to artificial diet packed in bamboo culms. The second instar larvae were transferred to treated artificial diet and maintained at 26±0.5°C and >85% RH. The control diet was incorporated with sterile buffer (20mM Tris-HCL (pH-7.5); 1 mM phenylmethylsulfonylflouride (PMSF) & 1mM EDTA) with different concentration as stated above. Mortality was recorded at 24 h intervals for 10 days. Four replicates of twenty larvae each were maintained for each treatment.

Chitinase assay

Chitinolytic activity was determined by measuring the release of reducing saccharides from colloidal chitin as method described (Tikhonov et al., 2002). A reaction mixture containing 1 mL of crude soluble proteins, 0.3 mL of 1M sodium acetate buffer pH 4.7 and 0.2 mL of colloidal chitin was incubated at 40ºC for 6−24 h and then centrifuged at 12,225 g for 5 min at 6ºC. After centrifugation, an aliquot of 0.75 mL of the supernatant, 0.25 mL of 1% solution of dinitrosalycilic acid in 0.7M NaOH and 0.1 mL of 10M NaOH were mixed in 1.5 mL eppendorf tubes and heated at 100ºC for 5 min. Absorbance of the reaction mixture at 582 nm (A_{582}) was measured after cooling at room temperature. Each tube served as replicate with three replications per treatment. Calibration curves with N-acetyl D-glucosamine as standard were used to determine reducing sugar concentration. One unit of enzyme activity was defined as the amount of enzyme that released 1µmole of N-acetyl D-glucosamine per min.

Chitin deacetylase assay (CDA)

Chitin deacetylase activity was measured using acetylated ethylene glycol chitosan as a substrate. For preparation of the substrate, ethylene glycol chitosan (40 mg) was treated at 48°C with 400 mg of $NaHCO_3$ and 200 µmol of acetic anhydride in a total volume of 4.5 mL and kept at 48°C. After 24 h, 200 µL of acetic anhydride were added and the mixture was allowed to stand for further 24 h at 48°C .After dialysis with molecular porous membrane with a cut off of (6,000−8,000 kDa) the product, acetylated ethylene glycol chitosan (1 mg/mL) was used as a substrate for the assay of CDA. The assay for CDA with 100 mL of 50 mM sodium tetraborate buffer, pH 8.5, 100 µL of 1 mg/mL acetylated ethylene glycol chitosan, and 50 µL of crude soluble protein incubated at 37°C for 30 min. The reaction was terminated with addition of 250 µL of 5% (w/v) $KHSO_4$. For colour development, 250 µL of 5% (w/v) $NaNO_2$ was added and allowed to stand for 15 min, and then 250 µL of 12.5% (w/v) ammonium sulfamate ($N_2H_6SO_3$) was added. After 5 min, 250-µL freshly prepared 0.5% (w/v) 3-methyl-2-benzothiazoline hydrazone (MBTH) was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled under tap water and 250 µL of freshly prepared 0.5% (w/v) $FeCl_3$ was added and estimated spectrophotometrically at 650 nm. One unit of enzyme released 1 µmol of glucosamine from acetylated ethylene glycol chitosan per min (Kulkarani et al. 2008).

Protease assay

Protease activity was assayed according to method described (Hossain et al., 2006) using casein as substrate. The reaction mixture containing 3ml of 1% (w/v) Hammerstein casein in 3ml 0.1M citrate phosphate buffer, pH 7.0 and 3 mL of crude soluble proteins was incubated at 40±1°C for one hour. The reaction was stopped by the addition of 5-mL 20% (w/v) TCA and the absorbance was measured at 650 nm in a spectrophotometer. Each tube served as replicate with three replications per treatment. The amount of amino acids released was calculated from a standard curve plotted against a range of known concentrations of tyrosine. One unit of enzyme was defined as the amount of enzyme that released 1µg of tyrosine mL^{-1} of crude enzyme/h.

Lipase assay

Lipase activity was estimated as detailed (Pignede et al, 2000). The substrate emulsion was prepared with olive oil (50 mL) and

gum arabic (50 ml, 10% w/v). The reaction mixture contained 1 ml crude soluble proteins, 5 ml substrate emulsion, and 2 mL of 50 mM phosphate buffer, pH 6.8, and was incubated at 37°C for one hour under shaking at 80 rpm. The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration with 50 mM NaOH of the release of fatty acids. One unit of lipase is the amount of enzyme that released 1 µmol of fatty acids per min.

DNA extraction

Mycelia and conidia from each isolate were plated on potato dextrose agar (PDA) and single spore colony was grown on potato dextrose broth (PDB), incubated on shaker (150 rpm) at room temperature for 5–7 days. Mycelium was recovered by centrifugation and filtration through Whatman No. 1 filter paper, washed twice with sterilized water, adding liquid nitrogen and ground until a powder mycelium was obtained.

The powder was extracted by DNeasy Plant Mini Kit® (QIAGEN) following the manufacture's instructions and stored genomic DNA at 4°C. For each fungal isolate, about 50 mg of mycelium was homogenized in DNA extraction buffer (100 mM Trizma; 1.4 M NaCl; 20 mM EDTA; 1% polyvinylpyrrolidone; 2% cetyltrimethylammonium bromide (CTAB); 1% mercaptoethanol; 10 mg/mL proteinase K; pH 8.0) and incubated for 45 min at 65°C. After incubation the mixture was extracted twice with 24:1 chloroform:isoamyl alcohol, centrifuged at 3,500 revs min -1 for 15 min and the DNA precipitated with isopropanol and stored at -20°C overnight, after which the DNA was washed with 70% and 95% alcohol, dried at room temperature and resuspended in 150 µL of TE buffer pH 8.0. The DNA concentration was measured with a fluorometer and the DNA stock solutions kept at -80°C until needed

PCR amplification

The ITS1- 5.8s and ITS2 region of rDNA was amplified using the primers ITS-1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplification reactions were performed at a total volume 50 µl, consisted of template DNA, 200 µMol dNTPs, 0.8 *p*Mol each primer, 10X PCR buffer and 1U *Taq* DNA polymerase. The condition of temperature in thermal cycling was one cycle of initial denaturation at 95°C for 5 min, followed by 35 cycles with denaturation at 94°C for 1 minute and 30 seconds, annealing at 55°C for 2 min, and extension at 72°C for 3 min and a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in 1% agarose gels by comparison with 100 bp DNA Ladder.

Phylogenetic analysis

The ITS1 and ITS4 amplified products (about 540 bp) were purified with the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences) and sequenced in an automated system with services provided by Bangalore Gene. The sequences were aligned using the ClustalW (http://www.ebi.ac.uk/clustalw) program and compared with those available in the GenBank data base for *M. anisopliae* and *M. flavoviridae* using the Genetic Data Environment (GDE) software and phylogenetic trees constructed using the Neighbor- joining method (gopher://megasun.bch. umontreal.ca: 70/11/GDE) by 1000 bootstrap resembling. Phylogenetic inferences were performed and exposed using TreeView. The sequences were deposited in NCBI Genbank and Accession number has been received.

Data analysis

Mortality observed in the controls was used to correct mortality in the treated groups using Abbott's formula (P= C-T/C x 100) (Abbott 1925). The cumulative mortality response for each concentration of the isolates across the assessment period was analyzed by Kaplan–Meier survival analysis. The LD_{50} data were subjected to probit analysis. The data from the spore estimation and enzyme activity were subjected to arc sine transformation and analyzed using analysis of variance. The least significant difference test was used to compare the means. All the analyses were carried out using SPSS 11.0 for Windows. The relative potency for each isolates was calculated by dividing the LD_{50} value of each test isolate by that of standard.

Results

Virulence of *M. anisopliae* isolates against *H. robusta*

The time mortality response for six isolates and two standard ARSEF strains was assessed at four different concentration, and the average survival time (AST) for the second instar larvae treated with the isolates varied from 7.3 to 9.6, 7.1 to 9.4, 6.9 to 9.3 and 6.2 to 8.4 days respectively for 1.0×0^4, 1.0×10^5, 1.0×10^6, 1.0×10^7conidia·mL^{-1}. The isolate IWST-Ma7 recorded lowest AST 6.2 to 7.3 days at all the doses tested (Fig. 1a). The AST for two standards isolates ARSEF 3603 and ARSEF 2596 ranged from 7.5 to 9.0 days and to some extent related to that of IWST-Ma13 and IWST-Ma3 respectively. The mean survival time (MST) for ARSEF 2596 (7.8 to 8.1 days) was similar to that of IWST-Ma3. However, the MST remained lowest for the isolate IWST-Ma7 (7.0 to 7.4 days) at all doses tested (Fig 1b).

Assessment of sporulation on cadavers

Incubation periods have significant effect on conidial yield, with cadavers incubated for fifteen days yielding approximately two times as many conidia as those incubated for seven days. For all the isolates the mean sporulation at the inoculation dose, 1×10^7conidia ml^{-1} was high and ranged from $1.1–1.9\times10^5$ conidia·mL^{-1} and $3.0–4.8\times10^5$ conidia·mL^{-1} after 7 and 15 days respectively (Fig 2a). Among the isolate, IWST-Ma7 showed significantly highest conidial yield across the doses ($4.9\text{-}4.7\times10^5$ conidia·mL^{-1} after fifteen DAI (Fig 2b). There are no significant differences in the conidial yield between ARSEF 3603, ARSEF 2596 and IWST-Ma3 across the doses and incubation time. Al-

though IWST-Ma13 demonstrated higher AST (Fig. 1a), sporulation was significantly lesser at seventh and fifteenth day of incubation (Fig. 2a, b).

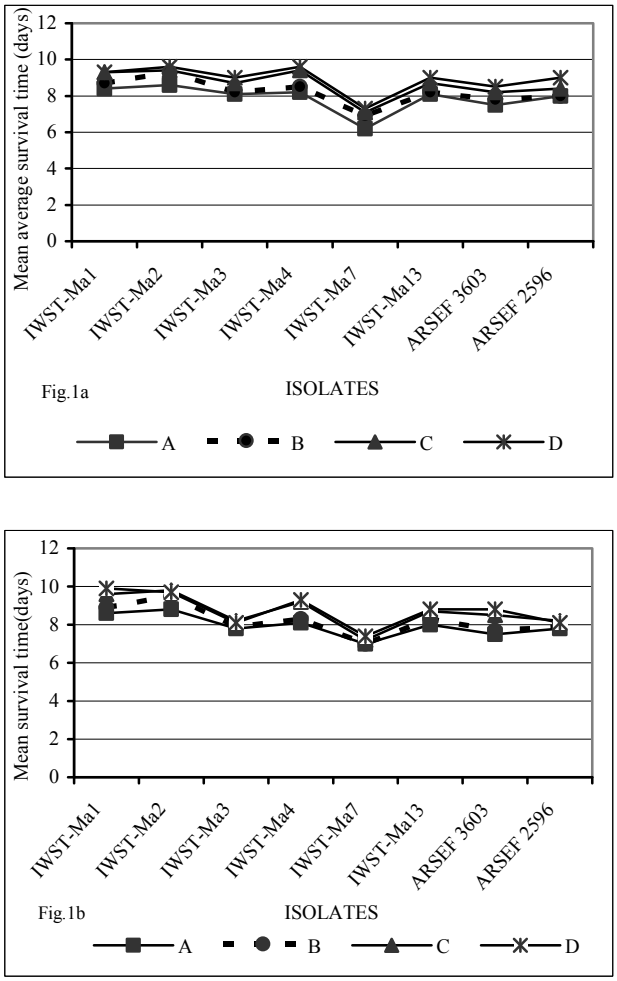


Fig. 1 Kaplan–Meier survival analysis for time–mortality response of *H. robusta* larvae exposed to *M. anisopliae:* a-- Average survival time; b-- mean survival time

Control mortality was corrected and subjected to, Average Survival Time (a) (AST) and Mean Survival Time (b) (MST) evaluation was limited to 10 days α = 0.05% according to the log rank test. The conidial concentration were, A- 1×10^7 conidia ml^{-1}; B- 1×10^6 conidia ml^{-1}; C- 1×10^5 conidia ml^{-1}; D- 1×10^4 conidia ml^{-1}.

Mycotoxic activity of crude protein extract of *M. anisoplaie*

The toxicity effects of crude soluble protein extract of *M. anisopliae* against the second instar larvae of *H. robusta* shows insecticidal activity when administered at five different concentrations. The isolate IWST-Ma7 was highly toxic when compare to standard with significant lowest LD_{50} value of 2.6% (5mg/mL). The two standard isolates ARSEF 2596 & ARSEF 3603 showed LD_{50} value of 3.7% and 5.6% respectively. The toxic effects of crude soluble protein extract to the second instar larvae for the other five isolates were ranged form 5.0 to 7.1 %. The chi-square values were not significant α (0.05), indicating good fitting of the regression line, with regression co-efficient for the isolates varying from 0.5 to 1.7. The potency indices for the six isolates intended with the two standard isolates with a range of 0.76 to 1.91. It indicates that the lower potency indices are correlated with lower LD_{50} values for isolates tested (Table 1). The isolate IWST-Ma7 and IWST-Ma4 showed the lowest potency indices (0.46 and 0.89 when compared with the two standard isolates.

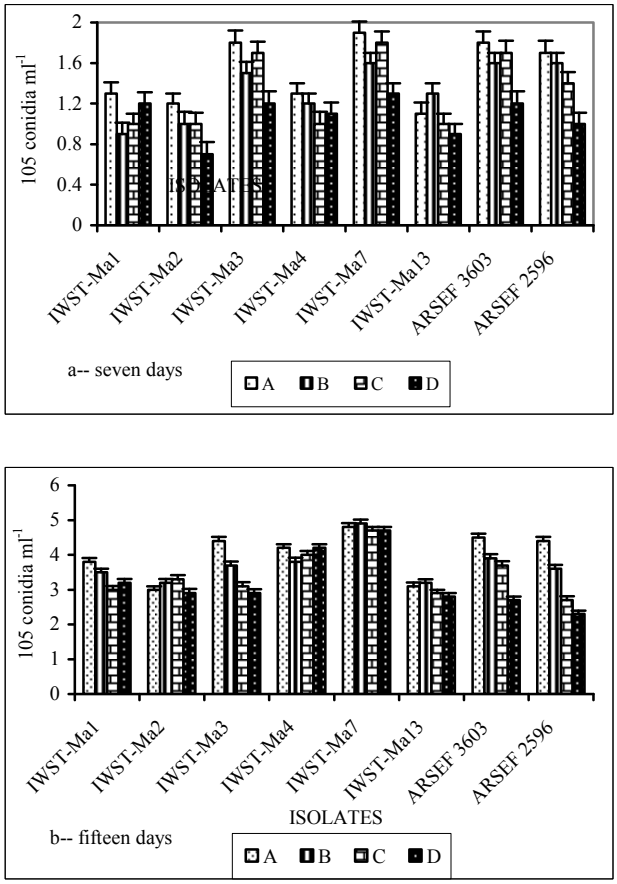
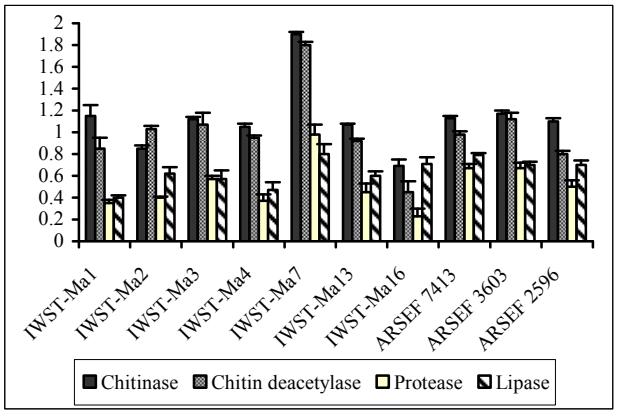


Fig. 2 Sporulation of *M. anisopliae* isolates on cadavers of *H. robusta* (a-- seven days and b-- fifteen day) after incubation

Enzyme activity of *M. anisopliae* isolates

The enzyme activity of CSPE of *M. anisopliae* for chitinase, CDA, protease and lipase ranged from 0.85 to 1.90 U/mg, 0.85 to 1.80 U/mg, 0.35 to 0.98 U/mg and 0.40 to 0.80 respectively (Fig 3). The activity of CSPE of IWST-Ma7 for the four enzymes was highest among the isolates tested. The chitinase activity of ARSEF 3603 (1.17 U/mL) was similar to IWST-Ma3 and IWST-Ma1 and significantly different from ARSEF 2596, IWST-Ma13 and IWST-Ma2 and ranged from 0.85 to 1.10 U/mg. The CDA activity of ARSEF 3603 was highest (1.12 U/mg) when compared to ARSEF 2596, IWST-Ma13 and IWST-Ma1. Though the chitinase activity of IWST-Ma1 was high and equal

to that of ARSEF 3603, the CDA activity was significantly lesser. All the isolates showed a reduction of protease and lipase activity as compared to chitinase and CDA activity. The protease and lipase activity of ARSEF 3603 and ARSEF 2596 were 0.67 & 0.70 U/mg and 0.50 & 0.70 U/mg respectively. The protease activity was lowest for all the isolates tested and ranged from 0.35 to 0.98 U/mg respectively.



Morphology and phylogenetic clade of isolates of *M. anisopliae*

Morphological features of colonies were studied in PDAY medium on the basis of colony color and surface profile. The surface colony is flat and mycelium is smooth in some of the isolates (IWST-Ma3, IWST-Ma4, IWST-Ma7, IWST-Ma13, ARSEF 3603 and ARSEF 2596). The other two isolates showed elevated colony. Mycelium were uplifted as aerial mycelium and showed pale green to magenta green color in isolates, IWST-Ma4, IWST-Ma7 and ARSEF-2596. Colonies of IWST-Ma1, IWST-Ma2, ARSEF-3603 and ARSEF-2596 showed orange/yellow pigmentation (Table 2). The day of formation of aerial conidia and pigmentation differed in different isolates. The aerial conidia of isolates, IWST-Ma4, IWST-Ma7 and ARSEF 2596 were formed within 5 to 7 days and that of the isolates, IWST-Ma13 and ARSEF 3603 formed within 4 to 6 days of incubation. Morphology of conidia in PDAY under bright field light microscope showed cylindrical shapes. Conidia sizes varied in width from 2.56 μm (IWST-Ma13) to 3.90 μm (IWST-Ma7) and length 8.46 μm (IWST-Ma4) to 9.90 μm (IWST-Ma2). Length/width ratio of the conidia was calculated and ranged from 2.34 μm to 3.37 μm. Isolates IWST-Ma13 measured highest length/width ratio followed by ARSEF 3603 and IWST-Ma2.

The ITS regions of Metarhizium were amplified using the ITS1 and ITS4 primers that was a unique fragment of approximately 550 bp for all isolates. Sequencing data conformed that all sampled isolates are *M. anisopliae* (Fig. 4). Genetic distances between isolates are presented by branch length. The isolates within clade A were subdivided into five groups with IWST-Ma1 and IWST-Ma3 forming first group. The isolate IWST-Ma4 is groped with ARSEF442. In the clade B, the isolates were subdivided into two groups where IWST-Ma13 formed a separate group from that of IWST-Ma7 and ARSEF-794. The isolate IWST-Ma2 formed a separate clade C. Though the isolates, IWST-Ma1, IWST-Ma3 and IWST-Ma4 belonging to clade A are from Lepidopteran hosts, they differ in their conidia length/width ratio (2.47 μm to 2.94 μm.)

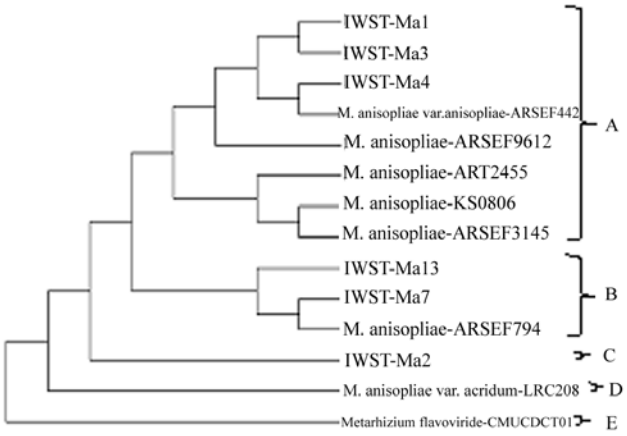


Fig 4: Phylogenetic analysis on ITS region and 5.8S of rDNA using Neighbour-Joining algorithm. Number on branch represent 1000 replicates of Bootstrap values.

Table 1: Insecticidal effects of crude protein extract of *M. anisopliae* against 2nd instar larvae of *H. robusta*

M. anisopliae isolates	Slope ± SEM	LD_{50} Con. (5mg /ml)	95% C.I	χ^2	*P* value	Potency index[a]	Potency index[b]
IWST-Ma1	3.9 ± 1.6	7.1	5.2-11.2	0.69	0.87	1.20	1.91
IWST-Ma2	3.6 ± 1.1	5.9	4.6-14.4	0.11	0.99	1.00	1.59
IWST-Ma3	4.9 ± 1.7	5.8	4.7-14.5	0.42	0.93	1.00	1.56
IWST-Ma4	4.0 ± 1.1	5.0	4.1-8.3	0.24	0.96	0.89	1.35
IWST-Ma7	2.3 ± 0.5	2.6*	1.9-3.5	0.04	0.99	0.46	0.70
IWST-Ma13	3.0 ± 0.9	6.0	4.5-15.6	0.53	0.91	1.07	1.62
ARSEF 3603	2.3 ± 0.7	5.6	4.1-14.7	0.65	0.88	-	-
ARSEF 2596	2.3 ± 0.6	3.7	2.8-5.7	0.60	0.89	-	-

Notes: Control mortality was corrected and subjected to probit analysis; LD_{50} was evaluated by Pearson Chi-Square goodness-of-fit on the probit model (α = 0.05%) according to the log rank test; [a] indicates the potency index calculated with that of ARSEF 3603; [b] indicates the potency index calculated with that of ARSEF 2596.

Table 2: Morphological and phylogenetic clade of *Metarhizium* spp

Isolates	Source (Insect host & stage/ Family)	Geographical orgin & Year of collection	Conidia Size (μm)		Length/ Width ratio	Morphology of conidia in PDAY medium	Clade	Accession number
			Mean Width	Mean Length				
IWST-Ma1	Mummified larvae (Lepidoptera)	Mysore, Karnataka & 2006	3.05	8.78	2.87	Elevated colony, mycelium up righted as aerial mycelium in 5-8 days. Orange to Yellow color pigmentation of medium.	A	JN127776
IWST-Ma2	Mummified larvae (Lepidoptera)	Mysore, Karnataka & 2007	3.30	9.90	3.00	Elevated colony, mycelium up righted as aerial mycelium in 5-8 days. Orange to Yellow color pigmentation of medium.	C	JN127777
IWST-Ma3	Mummified larvae (Lepidoptera)	Madurai, Tamil Nadu & 2007	3.29	9.68	2.94	Flat colony, smooth mycelium conidia become pale green color with in 5 to 8 days.	A	JN127778
IWST-Ma4	*Paliga machoeralis* (Lepidoptera)	Coorg, Karnataka & 2007	3.42	8.46	2.47	Flat colony, smooth mycelium, conidia becomes magenta green with in 5 to 7 days.	A	JN127781
IWST-Ma7	*Paliga machoeralis* (Lepidoptera)	Sullia, Karnataka & 2007	3.90	9.14	2.34	Flat colony, smooth mycelium, conidia becomes magenta green with in 5 to 7 days.	B	JN127784
IWST-Ma13	*Oryctes rhinoceros* (Coleoptera)	Sullia, Karnataka & 2007	2.56	8.65	3.37	Flat colony, smooth mycelium, medium becomes light yellow color with in 4 to 6 days	B	JN127787
ARSEF 3603*	*Myllocerus discolor* (Lepidoptera)	Mudigere, India & 1992	2.92	9.18	3.14	Flat colony, smooth mycelium, medium becomes orange yellow color with in 4 to 6 days	-	-
ARSEF 2596*	*Paliga machoeralis* (Lepidoptera)	India & 1988	3.73	9.64	2.58	Flat colony, smooth mycelium, conidia becomes magenta green with in 5 to 7 days.	-	-

*Agriculture Research Services for Entomopathogenic Fungi

Discussion

Commercial growing of mahogany in the Asia Pacific region has been adversely affected by the attack of shoot borer, *H. robusta.* Economic value of Mahogany is significantly diminished when the damage of terminal shoot leads to multiple shoot production before a reasonable bole height is achieved. Biological control methods using microbes or natural enemies are also very much limited for management of this pest. Effective biological control depends on an understanding of the biology of the insects that might allow application of pathogens to be targeted as susceptible stages. The insecticidal effect of entomopathogenic fungus, *M. anisopliae* against *H. robusta* has not been any time attempted before in the laboratory.

In this investigation, laboratory trials were made to evaluate the potential of a few *M. anisopliae* isolates against this shoot borer. The tested isolates of *M. anisopliae* varied in their virulence ie., speed of kill and sporulation on the cadavers against second instar larvae of *H. robusta*. Salvatierra et al. (1972a) reported 50% mortality of *H. grandella* after infection by *M. anisopliae* at 1.4×10^7 conidia/mL. Salvatierra and Palm (1972b) obtained upto 96% mortality of first instar larvae of *H. grandella* on artificial diet by incorporating *Bacillus thuringiensis*. About 80% mortality of *H. robusta* larvae on *Toona cilita* M. Rome was observed when inoculated with spores of *Beauveria bassiana* (Misra 1993). Remadevi et al. (2010) tested six *M. anisopliae* isolates with different concentration against the third instar larvae of *H. robusta* showed the lowest LC_{50} value of 1.77×10^5 conidia/ml.

It has been hypothesized that most virulent fungal strains are generally isolated from the test organism or a closely related species. However, no apparent relationship between pathogenicity and host of isolates was observed in our study. IWST-Ma7 and IWST-Ma 4 exhibited substantial variation in speed of kill though they have been isolated from the same insect species, *Paliga machoeralis. M. anisopliae* isolated from *Schistocerca. Gregaria* (Orthoptera: Acrididae) caused 100% mortality at 10^8conidia ml^{-1} against second instar larvae of *Spodoptera litura* (Anand et al., 2009).

Inocula dose and days of incubation significantly influenced the sporulation rate of *M. anisopliae* on *H. robusta*. The *in vitro* development rate of this pathogen suggested that sporulation reflected the dose-related vegetative growth of the fungus. However, the estimated sporulation generally tends to be lesser than the actual sporulation rate. Doberski (1981) also reported that spores of entomogenous fungi adhere strongly to the insect cuticle that the number of spores washed off must be regarded as a minimum figure. The amount of conidia produced on individual larvae was variable, even when exposed to same dose of the same isolate under the same conditions. This may be due to the differential fungal colonization and development because of insufficient nutrients on host cuticle, molting, compact integuments and immune response of the individual larva.

The significantly higher production of all the four enzymes by the isolates IWST-Ma7 could have contributed to the higher virulence of these isolates. Extra cellular enzymes produced by *M. anisopliae* are believed to play a key role in cuticle hydrolysis. The in-vitro production of cuticle-degrading enzymes, such as chitinase, proteinase, caseinase, lipase and amylase in fourteen isolates of *M. anisopliae* are studied (Mustafa and Kaur 2009). CDE production, mainly of chitinase and protease, toxic metabolite production, appressorium formation, hydrophobicity of conidia were reported to be significant determinants of virulence

(Murad et al 2007). The toxicity effects of the crude soluble protein extract of *M. anisopliae* were studied (Ortiz-Urquiza et al., 2010) and the electrophoresis analysis suggests that the monomer of 11 kDa plays an important role in the insecticidal effect. Faster cuticle penetration by the fungus decreases the chance of exposure of the fungus to destructive ecological factors such as UV and to constitutive and inducible insect defenses (Fang et al. 2009). Synergistic activity of proteases and chitinases produced by the entomopathogenic fungi aid in the faster penetration of insect cuticle (St. Leger et al. 1996).

The ITS regions of *Metarhizium* were amplified using the ITS1 and ITS4 primers Destéfano et al. (2004) analyzed at the same region with 540 bp fragments for *M. anisopliae var. anisopliae* strain and 600 bp for *M. anisopliae* strain. Our study indicates that the tested isolates belong to different clades and none of our isolate was similar to *M. anisopliae* var. *acridum* sequences as reported by Diver et al. (2000) and Bischoff *et al*. (2009). rDNA sequence data can be used to resolve evolutionary relationships within *M. anisopliae* differing from the others in their biological origin and colony pattern. Our result indicates that the isolates similar in morphological traits belong to different clades. Great diversity in *M. anisopliae* supports those of other workers using both biochemical and molecular markers (Mavridou and Typas, 1998; Diver et al. 2000). *M. anisopliae* was separated into 4 clades by RAPD-PCR method and sequence data from the ITS, 5.8S rDNA and 28S rDNA D3 regions: *M. anisopliae* var. *anisopliae*, var. *major*, var. *acridum* and var. *lepidiotum* (Diver et al. 2000).

From this study, one virulent isolate of *M. anisopliae* that could be exploited for the control of *H. robusta* was identified. Early instars are more susceptible to entomopathogens and targeting the early instars of *Hypsipyla* larvae that feed on the leaf and leaflet axil, infecting and killing them before causing damage is a possible approach for control of this pest. As the use of *M. anisopliae* as a biological control agent increases, a more adequate and accurate identification with reference to its virulence and mycotoxic action on a specific insect host is relevant.

Acknowledgements

The authors are grateful to the Department of Biotechnology, New Delhi for providing financial support to carry out this work. We thank the Directors of Institute of Wood Science and Technology and Ashoka Trust for Research in Ecology and the Environment for the study facilities. The permission granted by the Principal Chief Conservator of Forest of Tamil Nadu & Karnataka Forest Department to undertake surveys in the states. The standards obtained from USDA Agriculture Research Services Entomopathogenic Fungi Culture Collection, Ithaca, NewYork is thankfully acknowledged.

References

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. *Journal Economic Entomology*, **18**: 265–267.

Ahmed SI, Leather SR. 1994. Suitability and potential of entomopathogenic microgranism for forest pest management some points for consideration. *International Journal of Pest Management*, **40**: 287−292.

Anand R, Prasad B, Tiwary BN. 2009. Relative susceptibility of *Spodoptera litura* pupae to selected entomopathogenic fungi. *BioControl* **54**: 85–92 .

Beeson CFC. 1941. *The Ecology and Control of the Forest Insects of India and Neighboring Countries*, Dehra Dun: Vasant Press, p. 678.

Bischoff JF, Rehner SA, Humber RA. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* **101**: 512−530

Bygrave FL, Bygrave PL. 2001. Host preference of the Meliaceae shootborer *Hypsipyla*: further information from grafting *Cedrela odorata* and *Cedrela fissilis* on *Toona ciliata* (Australian red cedar). *Austrialian Forestry* **64**(4): 216−219.

Cipiao L, Bandeira RR, Sitoe SM. 2009. Incidence of *Hypsipyla* sp. (Lepidoptera: Pyralidae) and its population distribution on *Khaya anthoteca* (Meliaceae) stands in the Manica Province, (Central Mozambique). In: Proceedings of XIII World Forestry Congress, Buenos Aires, Argentina, 18 October -23 October.

Cornelius JP, Watt AD. 2003. Genetic variation in a *Hypsipyla*-attacked clonal trial of *Cedrela odorata* under two pruning regimes. *Forest Ecology and Management*, **183**: 341–349.

Cornelius JP. 2009. The utility of the predictive decapitation test as a tool for early genetic selection for *Hypsipyla* tolerance in big-leaf mahogany (*Swietenia macrophylla* King). *Forest Ecology and Management,* **257:** 1815–1821.

Coudron TA, Kroha MJ, Ignoffo CM. 1984. Levels of chitinilytic activity during development of three entomopathogenic fungi. *Comparative Biochemistry and Physics*, **796**: 339–348.

Cunningham SA, Floyd RB, Griffiths MW, Wylie FR. 2005. Patterns of host use by the shoot-borer *Hypsipyla robusta* (Pyralidae: Lepidoptera) comparing five Meliaceae tree species in Asia and Australia. *Forest Ecology and Management,* **205**: 351–357.

Destéfano RHR, Destéfano SAL, Messias CL. 2004. Detection of *Metarhizium anisopliae* var. *anisopliae* within infected sugarcane borer *Diatraea saccharalis* (Lepidoptera, Pyralidae) using specific primers. *Genetics and Molecular Biology,* **27**: 245–252.

Driver F, Milner RJ, Trueman JWH 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. *Mycological Research,* **104**: 134–150.

Doberski JW. 1981. Comparative laboratory studies on three fungal pathogens of the elm bark beetle *Scolytus scolytus*: Effect of temperature and humidity on infection by *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus*. *Journal of Invertebrate Pathology,* **37:** 195–200.

Fang W, Feng J, Fan Y, Zhang Y, Bidochka MJ, St. Leger RJ, Pei Y. 2009. Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **102**: 155–159.

Goulet E, Rueda A, Shelton A. 2005. Management of the mahogany shoot borer, *Hypsipyla grandella* (Zeller) (Lepidoptera:Pyralidae), through weed management and insecticidal sprays in 1- and 2-year-old *Swietenia humilis* Zucc. Plantations. *Crop Protection*, **24**: 821–828.

Guimaraes NAB, Felfili JM, Da Silva FG, Mazzei L, Fagg CW, Nogueira PE. 2004. Evaluation of mahogany homogenous stands, *Swietenia macrophylla* King, compared with mixed stands with *Eucalyptus urophylla* S.T. Blake, 40 months after planting. *Arv Brazilian Journal*, **28**: 777–784.

Hauxwell C, Vargas C, Opuni Frimpong E. 2001. Entomopathogen for control of *Hypsipyla* spp., pp.131−139. In: Proceedings, International Workshop on ACIAR, 20 – 23 August 1996, Canberra, Australia

Hossain MA, Islam MA, Hossain MM. 2004. Rooting ability of cuttings of *Swietenia macrophylla* King and *Chukrasia velutina* Wight *etal* Arn as influenced by exogenous hormone. *International Journal of Agriculture and Biology,* **6**: 560–564.

Hossain MDT, Das F, Marzan LW, Rahman MDS, Anwar MN. 2006. Some properties of protease of the fungal strain *Aspergillus flavus*. *International Journal of Agriculture and Biology,* **8**(2): 162–164.

Kulkarni SA, Ghormade V, Kulkarni G, Kapoor M, Chavan SB, Rajendran A, Patil SK, Shouche Y, Deshpande MV. 2008. Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *Biocontrol Science and Technology,* **18**(8): 809–828.

Lauma-aho T. 2003. Natural regeneration of African mahogany (Khaya ivorensis) in the moist semi-deciduous forest in Ghana. M.Sc. Thesis. University of Helsiki, Helsiki.

Lim GT, Kirton LG, Salom SM, Kok LT, Fell RD, Pfeiffer DG. 2008. Mahogany shoot borer control in Malaysia and prospects for biocontrol using weaver ants. *Journal of Tropical Forest Science,* **20**(3): 147–155.

Lopes J. do. CA, Jennings SB, Matni NM. 2008. Planting mahogany in canopy gaps created by commercial harvesting. *Forest Ecology and Management,* **255**: 300–307.

Mayhew JE, Newton AC. 1998. *The Silviculture of Mahogany*, Oxon: CABI Publishing, p. 226

Mavridou A, Typas MA. 1998. Intraspecific polymorphism in *Metarhizium anisopliae* var. *anisopliae* revealed by analysis of rRNA gene complex and mtDNA RFLPs. *Mycological Research,* **102**: 1233–1241

McCoy CW, Samson RA, Bovcias DG. 1988. *Entomogenous fungi*, CRC Handbook of Natural Pesticides Microbial Insecticides Part-A, Entomogenous Protozoa and Fungi, Vol 5, (Ignoffo, C. M., ed.), BocaRaton, FL. pp. 151–236.

Misra RM. 1993. *Beauveria bassiana* (Balsamo) Vuillemin, a fungal pathogen of *Hypsipyla robusta* Moore (Lepidoptera: Pyralidae). *Indian Journal of Forest,* **16:** 236–238.

Murad AM, Laumann RA, Mehta A, Noronha EF, Franco OL. 2007. Screening and secretomic analysis of entomopatogenic *Beauveria bassiana* isolates in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. *Comparative Biocheistry and Physics*, **145**: 333–338.

Mustafa U, Kaur G. 2009. Extra cellular enzyme production in *Metarhizium anisopliae* isolates**.** *Foliarida Microbiology*, **54**(6): 499–504.

Nahar P, Ghormade V, Deshpande MV. 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 of Pathology*, **85:** 80–88.

Newton AC, Watt AD, Lopez F, Cornelius JP, Mesen JF, Corea EA. 1999. Genetic variation in host susceptibility to attack by the mahogany shoot borer, *Hypsipyla grandella* (Zeller). *Agricultural and Forest Entomology,* **1**: 11–18.

Ofori DA, Opuni-Frimpong E, Cobbinah JR. 2007. Provenance variation in Khaya species for growth and resistance to shoot borer *Hypsipyla robusta*. *Forest Ecology and Management,* **242**: 438–443.

Ortiz-Urquiza A, Garrido-Jurado I., Santiago-Alvarez C, Quesada-Moraga E. 2009. Purification and characterizations of proteins secreted by the entomopathogenic fungus *Metarhizium anisopliae* with insecticidal activity against adults of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Pest Management Sciences,* **65**(10): 1130−1139.

Ortiz-Urquiza A, Garrido-Jurado I, Borrego A, Quesada-Moraga E. 2010. Effects of cultural conditions on fungal biomass, blastospore yields and toxicity of fungal secreted proteins in batch cultures of *Metarhizium anisopliae* (Ascomycota: Hypocreales)**.** *Pest Management Sciences,* **66**(7): 725–35.

Pe´rez-Salicrup DR, Esquivel RT. 2008. Tree infection by *Hypsipyla grandella* in *Swietenia macrophylla* and *Cedrela odorata* (Meliaceae) in Mexico's southern Yucatan Peninsula. *Forest Ecology and Management,* **255**: 324–327.

Pignede G, Wang H, Fudalej F, Gaillardin C, Seman M, Nicaud JM. 2000. Characterization of an Extracellular Lipase Encoded by LIP2 in *Yarrowia lipolytica*. *Journal of Bacteriology,* **182**: 2802–2810.

Ramareshiah G, Shankaran T. 1994. Studies on some parasites of the Meliaceae Shoot borer *Hypsipyla robusta* in South India. *Hexpoda*, **6**(1): 39–46.

Remadevi OK, Sasidharan TO, Balachander M, Sapna Bai N. 2010. *Metarhizium* based mycoinsecticides for forest pest management. *Journal of Biopesticides* **3**: 470–473.

Robert DW. 1969. Toxin from the entomogenous fungus *Metarhizium anisopliae*: isolation of destruxins from submerged culture. *Journal of Invertebrate Pathology*, **14**: 82–88.

Salvatierra HO, Berrios F, Grijipma P. 1972**.** Studies on the shoot borer *Hypsipyla grandella* (Zeller) Lep. Pyralidae. XII. Determination of the LC_{50} of *Metarhizium anisopliae* (Metsch.) Sorokin spores on fifth instar larvae, *Turrialbacia,* **22**: 431-438.

Salvatierra HO, Palm JD. 1972. Studies on the shoot borer *Hypipyla grandella* (Zeller) Lep. Pyralidae. XIV. Susceptibility of first instar larvae to *Bacillus thuringinesis*. *Turrialbacia,* **22**: 467–468.

St-Leger RJ, Cooper RM, Charnley AK. 1986. Cuticle-degrading enzyme of entomopathogenic fungi: cuticle degradation in-vitro by enzymes from entomopathogens. *Journal of Invertebrate Pathology,* **47:** 167–177.

St-Leger RJ, Joshi L, Bidochka MJ, Roberts DW. 1996. Characterization of chitinases from *Metarhizium anisopliae*, *M. flavoviridae* and *Beauveria bassiana*, and ultrastructural localization of chitinase production during invasion of insect cuticle. *Applied Environmental Microbiology,* **62**: 907−912.

Tikhonov VE, Lopez-Llorea LV, Salinas J, Jansson HB 2002. Purification and characterization of chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *Verticillium suchlasporium*. *Fungal Genetics and Biology,* **35**: 67–78.

Varma RV. 2001. *Hypsipyla* shoot borers of Meliaceae in India,. In: R.B. Floyd and C. Hauxwell (eds.), Proceedings, International Workshop ACIAR, 20 -23 August 1996, Kandy, Sri Lanka. p.189.

Wylie FR. 2001. Control of *Hypsipyla* spp. Shoot borers with chemical pesticides: a review in *Hypsipyla* Shoot Borers in Meliaceae.. In: R.B. Floyd and C. Hauxwell (eds.), Proceedings, International Workshop ACIAR, 20-23 August 1996, Kandy, Sri Lanka, pp. 109–115

Ypsilos IK, Magnan N. 2005. Characterization of optimum cultural environmental conditions for the production of high numbers of *Metarhizium anisopliae* blastospores with enhanced ecological fitness. *Biocontrol Science and Technology,* **15**(7): 683–699.