ABSTRACT

The entomopathogenic fungi have excellent promise for use as selective bio-control agents as compared to bacteria and viruses as they are effective by contact, host specific, least effective against non-target and beneficial organisms and their mass production is easy. However, the entomopathogenic fungus to be used as a biocontrol agent should be virulent against the target host and the biochemical and molecular aspects of fungus-insect interaction should be known for the development of effective biocontrol agents. Understanding the mechanism of fungal pathogenesis in insects will help in the production of more efficient mycoinsecticides, either by identifying fungal virulence determinants or by identifying genes that could be upregulated or otherwise manipulated to enhance virulence. The entomopathogenic fungi enter through the host cuticle both by enzymatic degradation and mechanical pressure. The cuticle-degrading enzymes (CDEs) like chitinase (EC 3.2.1.14), protease (EC 3.4.21.62), lipase (EC 3.1.1.3), chitin deacetylase (CDA) (EC 3.5.1.41) and chitosanase (EC 3.2.1.132) actively destroy or modify the structural integrity of the host cuticle. Many studies regarding the role of CDEs viz. protease, chitinase and lipase in virulence of entomopathogenic fungi has been previously carried out. The possible role of CDA in the penetration process and self defense of the entomopathogenic fungus *Metarhizium* against insect chitinases was proposed by Nahar et al. (2004). Therefore, the present study was initiated to determine the importance of CDEs in the virulence of *Metarhizium* isolates against *Helicoverpa armigera* and further biochemical and molecular studies of CDA to understand its role in fungus-insect interaction.

Chapter 1: Introduction

This chapter is comprised of the literature survey on the mechanism of entomopathogenesis and the killing components responsible for virulence of the entomopathogenic fungi giving special emphasis on CDEs. Further, a comprehensive account of sources of CDAs, biochemical and molecular studies of CDAs as well as significance and applications of CDAs in different fields has been discussed. (The part of the literature survey has been published in Ghormade et al. (2010).
Chapter 2: Materials and methods
This chapter describes the sources of chemicals and kits used as well as the media used for the growth and enzyme production in the present study. The details of isolation, maintenance, growth, conidial and vegetative transfers of *Metarhizium* isolates and; preparation and maintenance of *Escherichia coli* JM109 competent cells have been described. The microbial techniques like germ tube formation and appressorium formation are outlined. Further, the methods used for the estimation of CDEs viz. chitinase, protease, lipase, CDA and chitosanase have been described. The rearing of *H. armigera* along with the procedure of bioassay is briefly described. The production of conidia of *Metarhizium* isolates using solid state fermentation has been included. The details of the molecular methods used including DNA extraction, RNA extraction, cDNA synthesis, gene amplification by PCR, qRT-PCR, cloning and transformation of desired amplicons, plasmid extraction, DNA sequencing and phylogenetic analysis have been described. The statistical methods used for data analysis have been mentioned.

Chapter 3: Screening of *Metarhizium* isolates for the control of *Helicoverpa armigera*
In this chapter, screening of sixty eight *Metarhizium* isolates obtained from the soil samples of different crop fields and the insect hosts was carried out based on the extracellular *in vitro* production of CDEs and virulence against 3rd instar larvae of *H. armigera*. The twelve out of sixty eight *Metarhizium* isolates exhibiting >90% mortality and higher CDE activities were used for the determination of LT$_{50}$. Further, on the basis of LT$_{50}$ values, the five isolates (M34311, M34412, M81123, M91427 and M91629) with lowest LT$_{50}$ values (3.3-4.1 d) were selected for the evaluation of LC$_{50}$. As the LC$_{50}$ values of the three isolates (M34311, M34412, M81123) were lower (1.4×10$^3$-5.7×10$^3$ conidia/ml), they were further studied for conidia production on a solid substrate, viability and settling time of conidia. The *Metarhizium* isolate M34412 produced 67 g/kg rice conidia, exhibited higher conidial germination (97%) and faster sedimentation time (ST$_{50}$-2.3 h) in 0.1% (w/v) Tween 80 than the other two isolates; M34311 and M81123. These three isolates were identified as *Metarhizium anisopliae* based on ITS1-5.8S-ITS4 sequencing. On the basis of above results, *M. anisopliae* M34412 was considered as the most effective isolate among sixty eight *Metarhizium* isolates.
Chapter 4: Cuticle degrading enzymes as biochemical and molecular markers of *Metarhizium* isolates

A. Molecular characterization of *Metarhizium* isolates based on polymorphism in protease (Pr1A) gene

The present chapter describes the correlation between the polymorphism in Pr1A gene of sixty eight *Metarhizium* isolates, *in vitro* protease activity and mortality of *H. armigera*. Initially, a positive correlation was observed between the protease activities and mortality of *H. armigera* as the protease activities for the highly pathogenic (>85.6%), moderately pathogenic (67.7-85.6%) and less pathogenic (<67.7%) groups ranged between 2.1-3.38 U/ml, 1-2.4 U/ml and <1 U/ml, respectively. The Pr1A gene (1.2 kb) of *Metarhizium* isolates was amplified. The digestion of Pr1A amplicons using restriction endonucleases namely RsaI, MspI and DdeI showed multiple polymorphisms with 7, 2 and 5 restriction patterns designated as A-G, H-I and J-N, respectively. After combining the restriction digestion patterns of sixty six *Metarhizium* isolates, 15 cumulative profile types were produced. The cumulative profile type I (D-I-M pattern) was most prevalent as out of sixty six *Metarhizium* isolates, thirty three (50%) isolates exhibited this profile and eleven isolates among these were from highly pathogenic group possessing high protease activity (>2.0 U/ml) and >90% mortality. These results supported the selection of *M. anisopliae* M34412 as the most effective isolate among sixty eight *Metarhizium* isolates.

B. Effect of repeated *in vitro* sub-culturing and *in vivo* passage on cuticle degrading enzyme production and virulence of *Metarhizium anisopliae* against *Helicoverpa armigera*

The effect of repeated *in vitro* sub-culturing and *in vivo* passage of the most effective isolate *M. anisopliae* M34412 on different aspects considered as parameters of virulence has been described in this chapter. The morphological, cultural characteristics and germination efficiency of *M. anisopliae* was not significantly affected whereas ~20% reduction in the appressorium formation from 1st to 40th sub-culture due to repeated *in vitro* sub-culturing on PDA was observed which subsequently increased after passage in *H. armigera*. Further, a gradual decrease in the constitutive and induced production of chitinase, chitosanase, CDA and protease was observed due to repeated *in vitro* sub-culturing when conidial inoculum was used and these activities increased subsequently after passage in *H. armigera*. However,
there was no significant effect on lipase production. When mycelial inoculum from the 10\textsuperscript{th} serial vegetative transfer was used, significant decrease in CDA activity was observed in both YPG (19.8\%) and chitin containing (23.2\%) medium as compared to the 1\textsuperscript{st} vegetatively transferred mycelium inoculum. However, chitinase, protease, lipase and chitosanase activities in YPG and chitin containing medium were not significantly affected. Similar trend for the effect of repeated in vitro sub-culturing on mortality of H. armigera and LT\textsubscript{50} and LC\textsubscript{50} values was observed. The genetic analysis of in vitro and in vivo sub-cultures of M. anisopliae showed correlation with the biochemical data.

**Chapter 5: Biochemical and molecular studies of chitin deacetylase**

**A. Evaluation of Metarhizium isolates based on chitin deacetylase activity**

The chapter describes the screening of sixty eight Metarhizium isolates based on cluster analysis using in vitro extracellular CDA activity in YPG medium and corrected mortality against 3\textsuperscript{rd} instar larvae of H. armigera. The Metarhizium isolate M161063 showing 82\% mortality, high CDA (2.26 U/ml) and chitosanase (13.22 U/ml) activity and; low chitinase activity (1.01 U/ml) was selected for further studies. The Metarhizium isolate M161063 was identified to be M. anisopliae based on ITS1-5.8S-ITS4 sequencing. The intracellular CDA activity in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h) and blastospores (24 h) of M. anisopliae M161063 was determined and the trend of intracellular CDA activity was observed to be differentiation specific.

**B. Sequencing of chitin deacetylase gene from Metarhizium anisopliae M161063**

In this chapter, the molecular characterization of CDA gene from M. anisopliae M161063 was carried out for its further use in confirming the role of CDA in fungus-insect interaction. The CDA gene from M. anisopliae M161063 was amplified using degenerate primers (CDAF1-CDAR2) designed towards the conserved polysaccharide deacetylase domain of previously reported fungal CDA genes from the NCBI database. The partial CDA gene sequence (594 bp) was obtained that shared maximum identity with deacetylases of ascomycetous fungi. The deduced amino acid sequence of CDA displayed the presence of five conserved catalytic domains. Further, the phylogenetic tree constructed using the deduced amino acid sequence of M. anisopliae CDA with amino acid sequences of other fungal and bacterial deacetylases coincided with the
taxonomic classification of fungi. The partial sequence of CDA gene from *M. anisopliae* M161063 was further used for studying the differential expression of CDA.

**C. Expression studies of chitin deacetylase from *Metarhizium anisopliae* M161063**

The present chapter aimed at analyzing the change in expression of CDA gene in different morphological forms viz. conidia from PDA slants (7 d), germinating conidia (12 h), appressoria (24 h), mycelia (24 h, 48 h, 72 h) and blastospores (24 h, 48 h, 72 h) of *M. anisopliae* M161063 using qRT-PCR. Initially, the housekeeping gene showing constant expression in above mentioned morphological forms was identified for normalization of CDA gene expression data obtained after qRT-PCR. The five housekeeping genes namely 18S RNA, GAPDH, Ubc, Tub-a and Tub-b were evaluated by qRT-PCR for the expression stability. Out of five housekeeping genes studied, genes encoding 18S RNA displayed relatively low CP values, indicating high expression of this gene in *M. anisopliae* as compared to other studied housekeeping genes. On the basis of gene expression stability of housekeeping genes determined using the software; *Bestkeeper*, 18S RNA was identified as the most consistently expressed housekeeping gene as it exhibited SD (±CP) values <1. Subsequently, 18S RNA was used as a reference gene for studying the expression of CDA gene in different morphological forms of *M. anisopliae* M161063. The primers for qRT-PCR studies of CDA gene were designed using the partial CDA sequence of *M. anisopliae* M161063. The expression of CDA increased in germinating conidia (12 h), appressoria (24 h) and mycelia (24 h) by 0.3-fold, 0.4-fold and 1.3 fold, respectively whereas the mycelia (48 and 72 h) and blastospores (24 h) showed a lower expression level of CDA. Further, the CDA gene was repressed in conidia from PDA slants (7 d) and blastospores (48 h and 72 h). Thus, the results of the qRT-PCR studies supported the trend of the intracellular CDA activity in different morphological forms of *M. anisopliae* M161063 suggesting the differentiation specific regulation of CDA in *Metarhizium* and its role in fungus-insect interaction.

**Chapter 6: Summary and conclusions**

All the results and findings of the present investigation are summarized in this chapter.