ABSTRACT

Title of Document: GENETIC DIVERSIFICATION, SAPROPHYTIC COMPETENCE AND GENETIC ENHANCEMENT OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM*

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Entomopathogenic fungi are being investigated as alternatives to chemical insecticides. This study explored the versatility of the entomopathogenic fungus *Metarhizium anisopliae* by examining its diversification, saprophytic competence and potential for genetic enhancement.

M. anisopliae is a radiating species containing both generalist and specialized lineages with broad and narrow host ranges and as such provides an excellent model system to study the evolution of pathogenesis. Using 18S RNA and protease sequences, I demonstrated that strains can be selected representing evolutionary distances ranging from <1 to 8 MY and their natural molecular variation allows analysis of processes of adaptive change.

M. anisopliae is particularly abundant in the rhizosphere. Germination of *M. anisopliae* strain 2575 was >96% in 1 mg/ml root exudate (RE) and growth in RE resulted in 29 (58%) genes being up-regulated and 21 (42%) down-regulated. The identity of these genes is helping to define the physiological requirements for rhizosphere competence. Hypothetical and orphans proteins (41.4%) were also actively expressed indicating that many previously uncharacterized genes may have functions related to survival at the soil-root interphase.

Using the fungus as a delivery vehicle for foreign toxins presents a powerful approach for increasing virulence. *M. anisopliae* was modified to express a scorpion toxin (AaIT) in insect haemolymph and bioassayed against the coffee berry borer *Hypothenemus hampei.* AaIT increased mortality up to 56.6%, and reduced the medial lethal concentration (LC_{50}) by 15.7-fold and the average survival time (AST) by 20.1%. The *AaIT* gene and the *M. anisopliae* esterase gene (*Mest1*) were inserted into three strains of *Beauveria bassiana* (ARSEF 252, 8998 and 9184) with high, medium and low mortality, respectively, against the Colorado potato beetle (CPB) *Leptinotarsa decemlineata*. Mortality rates were strain- and dose- dependant and increased from 16.1 to 36.7% in single transformants (*AaIT* or *Mest1*) and from 7.1 to 33.5% in double transformants (*AaIT-Mest1*). The AST was reduced up to 33% and the LC_{50} up to 5.9-fold. Although singly both *AaIT* and *Mest1* increased the killing power of *B. bassiana* against second instar CPB, combining *AaIT* and *Mest1* together did not produce synergistic effects.

GENETIC DIVERSIFICATION, SAPROPHYTIC COMPETENCE AND GENETIC ENHANCEMENT OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM*

By

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Dedication

To my family whose love is the center of my life.

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Chapter 1: Divergence of protease gene families in the entomopathogenic fungus *Metarhizium* spp.

1.1 ABSTRACT

Pathogen biodiversity is an under-exploited source of inference regarding disease processes and the evolution of pathogens and pathogenesis. However, the entomopathogenic fungus *Metarhizium anisopliae* provides an excellent model system for applying this approach. It is a radiating species, and contains both generalist and specialized lineages with broad and narrow host ranges. Thus, their natural molecular variation could allow analysis of processes of both adaptive change and phylectic differentiation still in operation, even in intermediate states. To initiate this study we established divergence times for entomopathogenic representatives of the Clavicipitacean family using 18S ribosomal RNA sequences. We then used protease sequences to establish the divergence times for individual *Metarhizium* species, subspecies and strains. At the genus level strains can be selected representing evolutionary distances ranging from <1 to 57 MY, and at the species level between <1 to 8MY. Divergence of *Metarhizium* was also related to host range and geography area of origin. Finally, we investigated the evolutionary process that drives amino acid substitutions within protease gene families to investigate diversification within the species of *M. anisopliae* and to infer the nature of the evolutionary forces that operate during this process.

1.2 INTRODUCTION

Clavicipetaceaus fungi (Ascomycota, Hypocreales) have important industrial and agricultural applications. Members of this family produce active metabolites or alkaloids (i.e. *Cordyceps subsessilis* and *Claviceps purpurea* are sources of the drug cyclosporine), they live as endophytes or pathogens of plants (i.e. *Epichloë*), and can be used for biological control of insect pests (i.e. *Metarhizium* and *Beauveria*) (Yokoyama et al. 2006, Sung et al. 2007b). The importance of the Clavicipetacean family has made it the target of several phylogenetic analyses (Yokoyama et al. 2006, Spatafora et al. 2007, Sung et al. 2007b, Sung et al. 2007a) and fossils have allowed divergence times to be estimated for several species based on phylogenies using multiple gene sequences (Sung et al. 2008).

While a great deal of biodiversity can be explored at the level of species, these taxa may be generally too divergent to be useful in evaluating many important evolutionary processes which occur on a much shorter time scale. To date, timelines have not been produced for adaptive radiation within a fungal species, and it will be necessary to use molecular sequences with a high rate of change to provide the sensitivity and resolution to measure such relatively recent occurrences. We have employed the proteases of *Metarhizium anisopliae* to investigate diversification within this species and to infer the nature of the evolutionary forces that operate during this process (Rao et al. 1998, Bagga et al. 2004). *M. anisopliae* provides a novel model system to study evolutionary processes as it contains biologically distinct subtypes with wide host ranges (e.g. *M. anisopliae* var. *anisopliae* strain 2575), and subtypes that like var. *acridum* (used for locust control) show specificity for certain locusts, beetles, crickets, hemipterans, etc. and

do not infect other insects (St. Leger et al. 1992b, Driver et al. 2000, Bidochka et al. 2001). This kind of pathogen diversity is a rich source of inference that can be used to develop models of the evolution of pathogens and pathogenicity and should, for example, provide a novel perspective on the evolution and strategies of highly specialized fungi. We expect to address a number of questions that span much of molecular evolution including: (1)what roles do changes in gene complement or expression profiles play in generating intraspecific differences? (2) How do these differences correlate with metabolic and biosynthetic adaptations to specific hosts? (3) What are the relative rates of different kind of mutations and do these vary between strains? (4) What are the mechanisms by which novel pathogens emerge with either wide or narrow host ranges? (5) What variables drive the functional divergence of gene variants between strains e.g., does phylogenetic or host range diversity predict genic novelty?

The eleven *M. anisopliae* subtilisin endoproteases (Pr1s) identified from ESTs in strain 2575 (Bagga et al. 2004) can degrade a wide variety of substrates such as casein, albumin, insect cuticle, collagen and root exudates. High level expression of each protease and extensive conservation of sequence suggests that they are not pseudogenes and conversely that each protease makes a selectable contribution to pathogen fitness (St. Leger et al. 1992b, Bagga et al. 2004). Some of the amino acid substitutions distinguishing the subtilisins predict that they will differ in their interactions with cuticular components including the protease inhibitors produced by the host in response to fungal invasion. This is consistent with functional differences that allow the proteases to act synergistically to more efficiently hydrolyze the insect cuticle, increase adaptability

and host range or have different functions in survival in various ecological habitats outside the host (Boucias and Pendland 1987, Bagga et al. 2004).

The most convincing evidence of adaptive molecular evolution derives from comparison of synonymous (silent) and non-synonymous (amino acid changing) substitutions rates in protein-coding genes (Yang 2003). Using the number of synonymous nucleotide substitutions per synonymous site (d_S) and the number of non-synonymous substitutions per non-synonymous site (d_N) it is possible to obtain the ratio of substitutions between multigene families ($\omega = d_N / d_S$). If selection has no effect on fitness, then $d_N = d_S$ and $\omega = 1$. If non-synonymous mutations are deleterious, purifying (negative) selection will reduce their fixation rate so that $d_N < d_S$ and $\omega < 1$. However, non-synonymous mutations are favored by positive selection and they will be fixed at a higher rate than synonymous mutations resulting in $d_N > d_S$ and $\omega > 1$. Changes in gene sequences occur at different rates and thus have different utilities in evolutionary studies. In this paper we established a divergence time for entomopathogenic representatives of the Clavicipitacean family using the 18S ribosomal RNA sequence. We then established the divergence times for individual *Metarhizium* species, subspecies and strains using orthologous sequences of two different subtilisins. Divergence of *Metarhizium* was also related to host range and geographic area of origin. Finally, we investigated the evolutionary process that drives amino acid substitutions within protease gene families.

1.3 MATERIALS AND METHODS

1.3.1 Phylogeny of the family Clavicipitaceae using the 18S rRNA gene

To calibrate divergence times within the *Metarhizium* clade with previous phylogenetic studies done on clavicipetaceaus fungi (Sung et al. 2007a), thirty-three 18S rRNA nucleotide sequences from Clavicipitaceaus fungi were retrieved from GenBank (NCBI). The sequences include fourteen strains of *Metarhizium* (accession numbers are included in Figure 1-1). Sequences were aligned using the multi sequence alignment software CLUSTAL X version 2.0 and edited by visual inspection. The phylogenetic tree with 1000 bootstraps was produced using the neighbor joining (NJ) method embedded in MEGA version 4.1 (Kumar et al. 2004).

1.3.2 Phylogenetic analysis of protease (Pr1) sequences from *Metarhizium* species, subspecies and strains

To examine the dynamics of change in genome content over an evolutionary time scale, we selected strains of *Metarhizium* that differ in host range and geographical area. *Metarhizium* nucleotide and amino acid sequences orthologous to subtilisin endoprotease (Pr1H) and protease A (Pr1A) from strain 2575 were retrieved from GenBank. In addition, Pr1H orthologous amino acid sequences of ten taxa belonging to well characterized Ascomycetes were also retrieved from GenBank. All accession numbers are included in Figure 1-2. Pr1H orthologs appear to exist as a single copy in all fungal genomes and encode intracellular proteases. Pr1H orthologs are comparatively conserved i.e., have changed more slowly than the Pr1A sequences, which encode one of many secreted proteases with possibly redundant, overlapping functions. Both,

nucleotide and amino acid sequences of each Pr1 group were aligned using the CLUSTAL X version 2.0 as described before.

Additional *Metarhizium* strains were obtained from the U.S. Department of Agriculture Entomopathogenic Fungus Collection in Ithaca, NY (ARSEF collection). The generalist multi-host strains were 549, 818, 1009, and 794. The specialist strains were 297, specific to scarabs, 1943 (*M. album*) specific to leaf and plant hoppers and 2023 that is specific to acridids and due to its high degree of genetic homogeneity with *M. anisopliae* var. *acridium* has been reclassified by Driver et al. (2000) as such. Fungal strains were grown at 27°C for 48 hrs in potato dextrose broth (PDB) and mycelial biomass was then removed through vacuum filtration. DNA extraction of fresh mycelia was done following the FastDNA spin kit manufacturer instructions. Amplification of genomic DNA using Pr1A and Pr1H primers and sequencing were performed as previously described (Freimoser et al. 2003, Bagga et al. 2004). The Pr1H and Pr1A sequences generated *de novo* in this study were deposited on GenBank and accessions numbers are included in the corresponding figures.

1.3.3 Calibration of the Molecular clock

In order to establish the divergence time of *Metarhizium* species within the family Clavicipitacea, a NJ tree was constructed using the aligned amino acid sequences of the Pr1H orthologs. The nonparametric relative-rate test of Tajima (1993) in MEGA 4.1, was used to determine if sequences showed significant differences in rates of divergence. Using DNA sequences from five genes (*nsSSU*, *nrLSU*, elongation factor 1α *TEF*, *RPB1* and *RPB2*) Sung et al. (2008) estimated from fossil evidence that the age of divergence of the Clavicipetacean family was 117 million years ago (MYA) in the Early Cretaceous.

They included a 95% credibility interval to address the uncertainty of the time estimates (CI: 95-144). We used the 117 (95-144) MYA divergence time to calibrate the 18S RNA and Pr1H trees.

A NJ tree constructed with Pr1A sequences was used to fine-tune the molecular clock and estimate divergence times for isolates of *M. anisopliae*. This tree was calibrated with the estimated times of divergence between plant pathogens/endophytes and *Metarhizium* spp that we obtained from both the Pr1H tree (102 MYA with a 95% CI of 84-126 MYA) and the 18S rRNA tree (88.5 with a 95% CI of 72-109 MYA).

1.3.4 Ancestral reconstruction of insect host and geographic area

Ancestral reconstruction using parsimony was performed on insect host and geographic area of origin using the NJ bootstrap consensus tree topology of the Pr1A amino acid dataset of *Metarhizium* strains. Ancestral character state reconstruction was performed using Mesquite (Maddison and Maddison 2009). Character states of insect host were either the insect order or substrate (soil) where fungal strains were originally reported. Character states of geographic area of origin included the continent (Asia, Europe, Africa, Australia and America: North America and South America) where the strains were originally found (Table 1-1).

1.3.5 Evolution of Subtilisin Sequences in *Metarhizium spp*.

Evolutionary pressures that contribute to genetic variation were quantified through the identification of rapidly evolving or unusually conserved sites in regions of protein coding sequences. We used likelihood- and parsimony-based counting methods

integrated in the phylogenetic package HYPHY (**Hy**pothesis Testing Using **Phy**logenies) version 0.99 (Pond et al. 2005).

Before starting analysis using HYPHY, a NJ tree for each group of Pr1A and Pr1H codon sequences was inferred using MEGA 4.1. With the starting trees, we used MODELTEST (Posada and Crandall 1998) to estimate the best-fit model of nucleotide sequence evolution. The best models for Pr1H and Pr1A sequences were selected according to the lowest Akaike information criterion (AIC) which is a measure that rewards models for good fit, but imposes penalties for unnecessary parameters in the most complex models. The best nucleotide model for Pr1A sequences was TrN+G (Tamura-Nei Model + gamma distribution) and for Pr1H sequences it was TIM+G (Transition Model + gamma distribution). AIC criteria were also used to select the best codon model for each data set of amino acid sequences of both Pr1H and Pr1A. Model selection was performed using the codon Model Compare analysis from HYPHY, which selects from 203 evolutionary models the one most appropriate for each alignment (Pond et al. 2005).

We used the genetic algorithm of HYPHY to detect which sites in the Pr1A and Pr1H alignments evolve adaptively and which sites are functionally constrained. To detect positively and negatively selected sites we ran a single likelihood ancestor counting (SLAC) analysis for the codon sequences of the ten Pr1H gene and fixed effects likelihood (FEL) analysis for the twenty-nine coding sequences of the Pr1A gene. SLAC and FEL are the preferred methods for detection of selective pressure on individual sites of alignments of < 20 and < 50, respectively (Pond et al. 2005). To test whether a site *s* is under selection, the likelihood ratio test (LRT) was performed to Pr1H and Pr1A amino

acid sequences by fitting a single parameter (H_0: $d_N = d_S$) versus two parameter H_A: $d_N \neq d_S$ and employing chi square distribution of the LRT to asses significance. Because of the conservative nature of the test a significance level of 0.2 was used for both Pr1H and Pr1A coding sequences as recommended (Pond et al. 2005).

1.4 RESULTS

Tajima's (1993) relative rate test showed mutation rate consistency in all NJ trees constructed in this study demonstrating that 18S rRNA nucleotide sequences and Pr1 amino acid sequences have evolved at a constant rate in all lineages consistent with the molecular clock hypothesis. Thus, subtilisin proteases provide a new set of ubiquitous fungal sequences for phylogenetic analysis.

The 18S rRNA tree (Figure 1-1) shows that the *Metarhizium* clade clusters with Clavicipetacean endophytes and pathogens of grasses, an interesting observation given that *M. anisopliae* is most abundant around grass roots (Bidochka et al. 1998, Quesada-Moraga et al. 2007, St. Leger 2008). It also demonstrates that *M. album* is basal to *M. anisopliae*, and the locust specialist *M. anisopliae* var. *acridum* is basal to *M. anisopliae* var. *anisopliae.*

The 18S rRNA NJ tree was calibrated using 117 MYA (CI: 95-144) for the split between the Clavicipitacea and the non-clavicipetacean *Cordyceps* spp. (Sung et al. 2008) (Figure 1-1, node 1). Major lineages within the Cordyceps group of insect pathogens including *Cordyceps militaris* and *Beauveria bassiana* diverged from each other approximately 44 (36-54) MYA (Figure 1-1, node 16). *B. bassiana* and *B. caledonica* are estimated to have split 17 (14-21) MYA (Figure 1-1, node 20). The

Metarhizium spp cluster with clavicipetacean plant pathogens and endophytes e.g., *Claviceps* and *Epichloë*, that split from clavicipetacean arthropod pathogens in the genera *Hirsutella* and *Paecilomyces* about 112 (91-139) MYA i.e., soon after the Clavicipitacea/*Cordyceps* split (Figure 1-1, node 2). The *Metarhizium* clade split from the *Claviceps* and *Epichloë* plant pathogens/endophytes about 88.5 (72-109) MYA (Figure 1-1, node 3). *M. album* split from other *Metarhizium* lineages about 70.3 (57-87) MYA (Figure 1-1, node 4). According to our tree, *Metarhizium anisopliae* var. *acridum* diverged from other var. *anisopliae* lineages about 21 (17-26) MYA (Figure 1-1, node 5). The distribution of *Metarhizium* lineages shown in Figure 1-1 is congruent with the new majority rule consensus phylogram for *Metarhizium* produced by Bischoff et al. (2009). Clade I in our Figure 1-1 corresponds to their MGT clade (that includes *M. majus* and *M. guizhoenses*) whereas Clade II corresponds to the PARB clade (that includes *M. pingshaense, M. anisopliae*, *M. robertsii* and *M. bruneum*). Figure 1-1 indicates that these two clades of *Metarhizium* diverged about 6.2 (5-8) MYA (Figure 1-1, node 7) during the Miocene.

Figure 1-2 presents the NJ tree constructed using amino acid sequences of Pr1H. This tree includes six *de novo* sequences from *Metarhizium* strains ARSEF 549, 818, 1009, 794, 297 and 1943 (GenBank accession numbers are included in Figure 1-2). The Pr1H tree is also totally consistent with known relationships within the Clavicipitacean clades (Sung et al. 2007a) and was calibrated using the 117 (95-144) MYA divergence time of the Clavicipitacean family (Sung et al. 2008). As an outgroup, the tree includes several species of the genus *Aspergillus* and *Penicillium* that belong to the class Eurotomycetes of the division Ascomycota. *Aspergillus* and *Penicillium* are calculated to

have diverged about 88.7 (72-109) MYA (Figure 1-2, node 15) whereas divergence among *Aspergillus* species (*A. fumigatus*. *A. niger* and *A. nidulans*) is calculated to have occurred about 64.6 (52.5-79.6) MYA (Figure 1-2, node 16). This divergence time among sister species of *Aspergillus* is similar to the estimate between 43.9 and 73.6 MY provided by Kasuga et al. (2002) for *A. fumigatus* and *A. niger*. They calculated this divergence time range using the Sordariomycetes divergence time of 400 MY and 670 MY as calibration points, respectively. They also used the *pyrG* gene sequence and the nonparametric rate-smoothing (NPRS) algorithm (Kasuga et al. 2002).

Consistent with 18S RNA data, Figure 1-2 shows that the clade of clavicipetacean plant pathogens/endophytes diverged from *Metarhizium* spp. about 102 (84-126) MYA (Figure 1-2, node 2), during the Cretaceous and also places *M. album* as basal to the *Metarhizium* clade. The estimated divergence time between *M. album* and other lineages of *Metarhizium* is about 47 (38-58) MYA (Figure 1-2, node 4) which coincides with the range reported by Sung et al. (2008). Splits between other *M. anisopliae* lineages are also estimated to be more recent with Pr1H than with 18S RNA data. Thus, *M. acridum* separated from other lineages of *Metarhizium* 9.1 (7.4-14.3) MYA (Figure 1-2, node 5) or 21 (17-26) MYA (Figure 1-1, node 5) using Pr1H and 18S RNA date, respectively. The *M. anisopliae* clade split about 6.8 (5.5-11.2) MYA (Figure 1-2, node 6) and other *Metarhizium* lineages diverged about 5.1 (4.1-8.4) MYA (Figure 1-2, node 7) during the Pliocene and the Pleistocene epochs.

Figure 1-3 shows the NJ tree constructed using Pr1A sequences from twenty-eight *Metarhizium* strains including five sequences that were obtained *de novo* (ARSEF strains 549, 818, 1009, 2023 and 794). GenBank accession numbers are included in Figure 1-3.

The divergence time of 102 (84-126) MYA between plant pathogens/endophytes and the *Metarhizium* clade estimated from the Pr1H tree was used for calibration of this tree (Figure 1-3, node 1). Because the 18S rRNA gave us an earlier time estimate between these two clades, we additionally calibrated the Pr1A tree using the divergence time of 88.5 (72-109) MYA estimated from the 18S rRNA tree (Figure 1-1, node 3). The difference between the 18S and Pr1H calibration estimates had little impact, particularly on the shallower branches of the tree representing diversification within major clades of *M. anisopliae*. Our Pr1A tree contains three clades and these are in accord with the *Metarhizium* phylogeny reported by Bischoff et al. (2009): *M. acridium*, *M. robertsii* and *M. anisopliae*. The Pr1A tree includes good bootstrap support Using the Pr1H calibration point suggests that the clade of *M. acridum* diverged from other *Metarhizium* species about 14.9 (12.3-18.5) MYA (Figure 1-3, node 3), a date similar to the one estimated using our 18S RNA data (Figure 1-1, node 5). The clade of *M. robertsii* and the major lineages within *M. anisopliae* clade diverged about 6.0 (4.9-7.5) MYA (Figure 1-3, node 4). *M. robertsii* (ARSEF 2575) and members of this clade diverged 0.3 (0.2-0.4) MYA (Figure 1-3, node 15). The *M. anisopliae* complex contains five major subclades: Subclades 1 and 2 are sister groups of subclades 3 and 4 and diverged about 5.6 (4.6-7.0) MYA (Figure 1-3, node 5) whereas subclade 5 has evolved independently. Divergence of subclades 1 and 2 took place more recently, 1.5 (1.2-1.9) MYA (Figure 1-3, node 6), than the split between subclades 3 and 4 that took place 1.9 (1.6-2.4) MYA (Figure 1-3, node 10). Subclade 2 includes two *M. anisopliae* var. *acridium* strains (ARSEF 3341 and CA22) that split from other *M. anisopliae* subspecies very recently e.g. 0.9 (0.8-1.2) MYA (Figure1-3, node 9).

Figure 1-4 shows the ancestral reconstruction states of host and geographic area of origin of *Metarhizium* strains using the Pr1A amino acid data. Ancestral reconstruction suggests that specialist *M. anisopliae* var. *acridium* adapted to orthopteran hosts evolved independently in both Australia (ARSEF 324) and South America (ARSEF 2023). The *M. robertsii* clade evolved in the America's from a soil dwelling ancestor with orthopteran and coleopteran hosts and dispersed worldwide as broad host range strains. Figure 1-4 also shows that some more recently evolved *M. anisopliae* strains still have a localized geographical distribution, particularly in Asia (subclade 1) and the Americas (subclade 2), but do not have localized hosts since they are found on soil and a variety of insect orders such as Blattodea, Lepidoptera, Hemiptera and Orthoptera. Subclades 3, 4 and 5 of *M. anisopliae* have a world-wide distribution (Africa, Europe, Asia and North America) and broad host range that includes orders of Coleoptera, Orthoptera, Lepidoptera and Acari.

To detect nucleotide sites under positive and negative selection, we ran the SLAC analysis included in the phylogenetic package HYPHY v. 0.99 for Pr1H codon sequences and performed FEL analysis for Pr1A codon sequences. No amino acid sites under positive selection were found in the Pr1H codon sequences and 16 sites were found to be under negative selection with a P-value lower than 0.2 (Table 1-2). Among the twenty-eight Pr1A coding sequences, four positively selected sites and fifty-two negatively selected sites were found with P-values lower than 0.2 (Table 1-3). The four codon sites under positive selective pressure are 163, 173, 190 and 294 with p-values of 0.163, 0.117, 0.178 and 0.072, respectively (Figure 1-5).

1.5 DISCUSSION

The phylogenetic trees for18S rRNA sequences (Figure 1-1), subtilisin endoproteases Pr1H (Figure 1-2) and Pr1A (Figure 1-3) all confirmed that *Metarhizium* is a monophyletic group as described previously by Driver et al. (2000), Spatafora et al. (2007) and Bischoff et al. (2009).

Although the molecular clock hypothesis have been the focus of controversy, mainly because of the incompleteness of fossil data and the phylogenetic topology (Bromham and Penny 2003, Donoghue and Benton 2007) it has been widely used to provide age constraints of evolutionary splitting events among organisms, including fungi (Remy et al. 1994, Redecker et al. 2000, Berbee and Taylor 2001, Kasuga et al. 2002, Sung et al. 2008).

A global molecular clock is in effect if every subtree of the entire tree obeys the clock. The existence of local molecular clocks among closely related species is more probable than the existence of global clocks. However, global molecular clocks for the fungal 18S rRNA sequences and proteases were significantly accepted using a molecular clock test and we therefore used them to determine divergence times within the clavicipitacean family.

The divergence time of the *Metarhizium* clade from plant pathogens/endophytes was about 88.5 (72-109) MYA (Figure 1-1, node 3) using 18S rRNA data and about 102 (84-126) MYA (Figure 1-2, node 2) using Pr1H data. Both dates coincide with the range reported by Sung et al. (2008) and belong to the early Cretaceous period (145.5 – 65.5 MYA). The Cretaceous was characterized by a relatively warm climate and the appearance of new groups of flowering plants and phytophagous insects (Jarzembowski

1995). It is, therefore, not surprising that new plant/insect interactions during this period led to the divergence of fungal plant pathogens/endophytes/entomopathogens, a dynamic process of inter-kingdom host jumping that has been characteristic of the clavicipitacean family (Nikoh and Fukatsu 2000, Spatafora et al. 2007).

Using the Pr1A amino acid sequences, we determined that an initial divergence of *M. robertsii* and *M. anisopliae* lineages ranged from 4.3 to 7.5 MYA (Figure 1-3, node 4) during the Cenozoic era. The Pleistocene epoch (2 MYA - 10,000 YA) shows extensive radiation of *M. anisopliae* (Figure 1-3, nodes 6, 7, 8, 9, 11, 12, 15 and 16). Therefore, strains of *M. anisopliae* can be selected representing evolutionary distances ranging from <1 to 8 MY and their natural molecular variation could allow analysis of processes of both adaptive change and phylectic differentiation still in operation, possibly even in intermediate states. Climate change during the late Cenozoic era (the past 10 MY) was critical for the evolution of many species and the diversification of modern animals, flowering plants, trees, and associated insects (Hay et al. 2002). The evolution of large grazing animals in the Cenozoic also contributed to the spread of grasses (Deenihan et al. 2009). If only large contiguous chunks of grasslands are counted, grasslands cover 31% of the planet's land (Attenborough 1984). This may have favored the diversification of *M. anisopliae*, which is most abundant in undisturbed pasture soils, 2-6 cm deep, where it can reach 10^6 propagules/g (Milner 1992). Presumably a large population of insect hosts could contribute to these *Metarhizium* populations. However, microbial soil populations as large as those characteristic of *M. anisopliae* are normally the result of organic substrates in rhizospheres of the upper layers of the soil (Dix and Webster 1995). Given that rooting density is particularly high in grasses i.e., < 3mm spaces between roots

(Barley 1970), the world-wide *Metarhizium* community must be living in overlapping rhizospheres.

Most strains classified as *M. acridium* are basal to the *M. anisopliae* clade in phylogenies using 18S rRNA sequences (Figure 1-1), Pr1H and Pr1A amino acid sequences (Figures 1-2 and 1-3, respectively). The divergence of *M. acridium* clade from the other clades of *Metarhizium* is estimated to be 21 (17-26) MYA using 18S rRNA data (Figure 1-1, node 5), 9.1 (7.4-14.3) MYA using the Pr1H amino acid sequence (Figure 1-2, node 5) and from 10.5 to 18.5 MYA using Pr1A amino acid data (Figure 1-3, node 3). Although the divergence time estimated using Pr1H is more recent, it is still contained within the Miocene epoch (24 – 5 MYA) when woodland environments were replaced by savanna grasslands. Grass-feeding acridids probably became abundant during this epoch (Stidham and Stidham 2000).

Figure 1-3 shows a subclade of *M. anisopliae* (subclade 3) that contains two strains of *M. anisopliae* var. *acridium*: strain ARSEF 3341 isolated from an orthopteran host in Niger, Africa and strain CA22 isolated from soil in USA. This finding demonstrates that *M. anisopliae* var. *acridium* is a paraphyletic clade and specificity to orthoptera has arisen more than once.

Host-Pathogen interactions are important for shaping diversity among populations and cuticle-degrading enzymes that disrupt structural barriers and provide nutrients will have a strong selective advantage to the pathogen. Gene divergence could be very important for the adaptation of different strains of *M. anisopliae* to different environments and habitats, including new hosts (Bagga et al. 2004). Sites in a multiple alignment of codon sequences do not evolve homogeneously due to selective pressures

caused by the genetic code, the physicochemical properties of encoded amino acids and protein function. Analyzing the rates of synonymous and non-synonymous nucleotide substitutions in the protein-coding sequences of Pr1H and Pr1A could potentially unite form, function and phylogeny to glean insight into the process of molecular adaptation (Bagga et al. 2004). Using Tajima's neutrality test, we calculated mutations rates in both Pr1H and Pr1A protein-coding sequences in 8 *Metarhizium* strains (Ma 818, 549, 794, 1009, 2023, 2575, 820 and 324) (data not shown). Estimates of sequence divergence confirmed lower number of segregating sites (polymorphisms) in Pr1H indicating that less genetic variation is tolerated in this gene than in the *Pr1A* gene. We used Proteinase K from *Tritirachium album* (accession number X14689) to align Pr1A sequences from *M. anisopliae* strains (Figure 1-5). The analysis of synonymous and non-synonymous nucleotide substitutions showed significant purifying selection effecting only 52 amino acids from the 28 *Metarhizium* strains. This was clearly an underestimate as most amino acid variation is concentrated in regions of the gene not directly concerned with catalysis. The specificities of subtilisins are primarily determined by the S1 and S4 active site pockets (Gron et al. 1992, Siezen and Leunissen 1997). The hydrophobic S4 pocket formed by Leu96, Tyr104, Ile107, Leu126, Gly127 and Gly128 (McPhalen and James 1988) is completely conserved in the Pr1A sequences. Residue 104 at the pocket entrance varies considerably between subtilisin paralogs in *M. anisopliae* (Bagga et al. 2004), but all the Pr1A orthologs have Tyr104, which would preclude entrance of large amino acids into the S4 pocket. Residues of the catalytic triad Asp32, His64 and Ser221 were completely conserved in the Pr1 subtilisins. Gly166 is also conserved which is of particular significance as it is a characteristic of subtilisins with a primary specificity for

cleaving after large hydrophobic amino acids, e.g., Phe and Leu (Siezen and Leunissen 1997), the principal substrates for the Pr1A of strain 2575(St. Leger et al. 1992a). These amino acids at active site domains were not recognized by the evolutionary programs as being under purifying selection although they were highly conserved suggesting that mutations in these sites are disadvantageous. Evidently, inspite of degeneracy, each of these proteases must have a definable non-substitutable role contributing to the fitness of the fungus otherwise there would be less purifying selection.

In addition, positive Darwinian selection was identified in four sites of the Pr1A coding sequences from the 28 *Metarhizium* strains (ω = infinity) (Table 1-3 and Figure 1-5). This indicates that nonsynonymous mutations at these particular sites (amino acids 48, 58, 75 and 169) become fixed at a greater rate than synonymous mutations. Fortunately, site directed mutagenesis and protein engineering have provided intimate knowledge of structure–function relationships in subtilisins. It is possible by comparing subtilisins to distinguish from a myriad of possible amino-acid replacements a large number already known to cause functional differences. For example, site 58 is in a calcium binding loop (Thr51 to Gly61 in proteinase K) that makes a major contribution to protease stability (Muller et al. 1994), and changing charge distribution in this loop alters thermostability (Narhi et al. 1991). The Arg58 in proteinase K and two of the Pr1A sequences was replaced by polar amino acids (Ser, Thr). Eijsink et al. (1992) increased the stability of a neutral protease by removing charged residues at the N-terminus of alpha helices. The negatively charged Asp169 in Proteinase K is at the N terminus of an alpha helix and is replaced by Ala in six of the Pr1A's. Pantoliano et al. (1989) increased the stability of subtilisin BPN by replacing Gly169 with Ala. However, Volkl et al.

(1994) found that a subtilisin from a hyperthermophilic bacteria had a mixture of Ala and Asp at the N-terminus of surface loops, and suggested Asp increases thermostability because of interactions with the helix dipole. Instead of thermostability, the charge transitions may be related to pI and interactions with negatively charged residues on insect cuticle which subtilisins encounter (St. Leger et al. 1992a). The high pI of the strain 2575 Pr1A is responsible for Pr1a being much more resistant than proteinase K to autodigestion at high pH (St. Leger et al. 1992a). However, it is notable that asparagines residues adjacent to glycines in Proteinase K (Asn 62 and Asn99) are substituted for other amino acids in the Pr1A's. This should prevent cyclization with glycines and increase thermostability (Narhi et al. 1991). Site 48 has not been mutated in bacterial subtilisins to test for significance (Bryan 2000). However, while proteinase K has Met48 (large hydrophobic side group), the Pr1A's have small apolar (Gly), hydrophilic (Ser) and large hydrophobic side chain (Phe) amino acids at this site. Site 48 is predicted to be on an outer loop and these substitutions presumably impact interactions with the subtilisins environment. In contrast, site 75 which is predicted to be on the inside of the protein has a conservative substitution of Ser for another polar amino acid Thr. Although detected as an example of Darwinian evolution such a change would not be expected to radically alter conformation of peptide chains, mechanism of action or other properties in a selectable fashion. In fact, conservative substitution is also consistent with purifying selection, Thr and Ser may be the only two amino acids which retain function at site 48. Other variable regions of the proteases were not reported as being significantly variant for Darwinian evolution. For example, the surface loop region of subtilisins from 200 to 220 faces the substrate when the substrate bond is in a position to be cleaved. It is

sensitive to amino acid replacement and variants affect surface adsorption, alkaline stability and thermostability. This is a variable region among the paralogous *M. anisopliae* subtilisins (Bagga et al. 2004). The current study shows that it is also variable among the Pr1A orthologs even though this variation did not result in statistically significant examples of Darwinian evolution. The interactions of these residues are complex. However, The Pr1A orthologs had Arg (6 examples), Ser (23 examples) or Gly (1 example) at 213. Arg should facilitate ionic binding to negatively charged groups on insoluble subsrates(St. Leger et al. 1986) (Brode et al. 1996), a prerequisite for the activity of strain 2575 Pr1A activity against insoluble insect cuticle (St. Leger et al. 1986). Nonpolar or hydrophobic residues at 213 (Gly) should compromise alkaline stability (Cunningham and Wells 1987), but like Ser increase activity against soluble substrates at the expense of insoluble ones (Brode et al. 1996). With the exception of strain 2023, Pr1A proteases with Arg residues at 213 also had a small polar amino acid (Ser or Thr) at 203, which would also be expected to increase surface adsorption to charged substrates.

Homology modeling thus predicts differences between the Pr1 orthologs is adsorption properties and stability, indicative of functional differences. These assumptions will eventually need to be supported by purification and biochemical analysis of individual Pr1 orthologs.

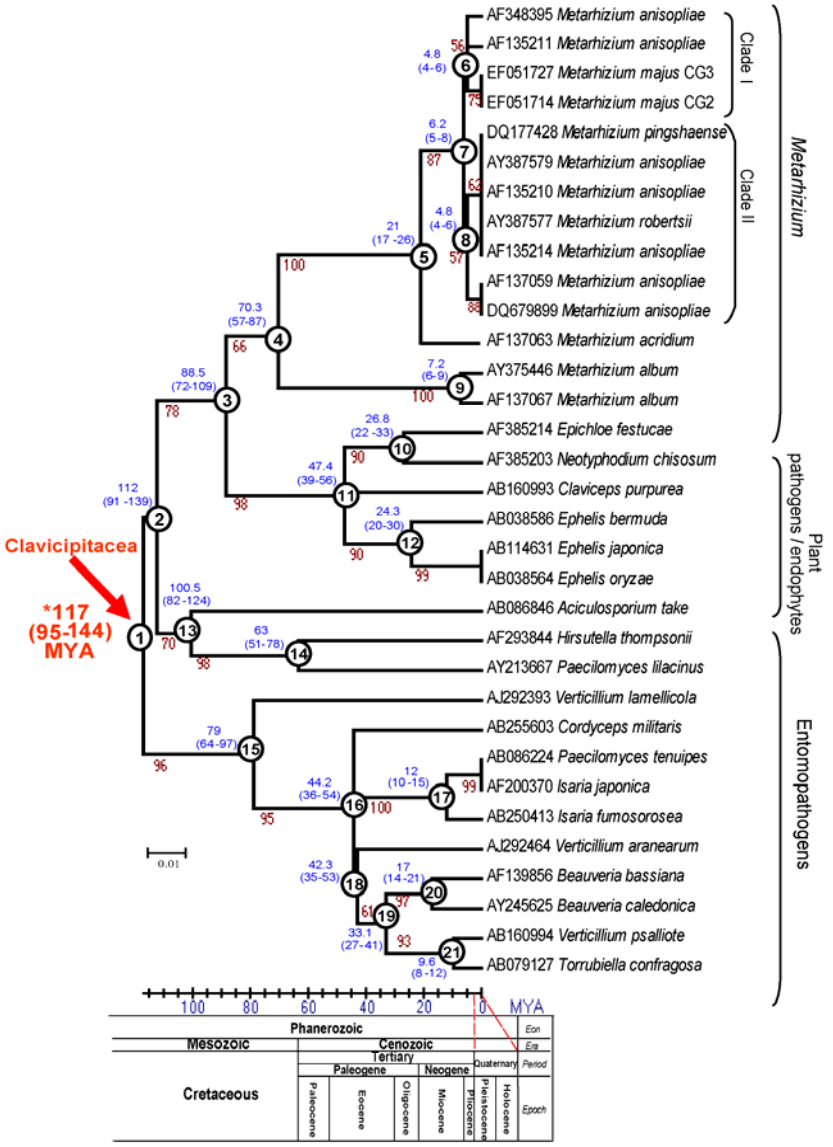

Figure 1-1. Neighbor-Joining tree using the 18S rRNA sequence of 33 Clavicipitacean species (GenBank accession numbers are included in the tree). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The molecular clock was calibrated based on the 117 (95% CI: 95-144) MYA divergence point of the Clavicipetacean family reported by Sung et al. 2008 (node 1). Phylogenetic analyses were conducted using MEGA 4.1.

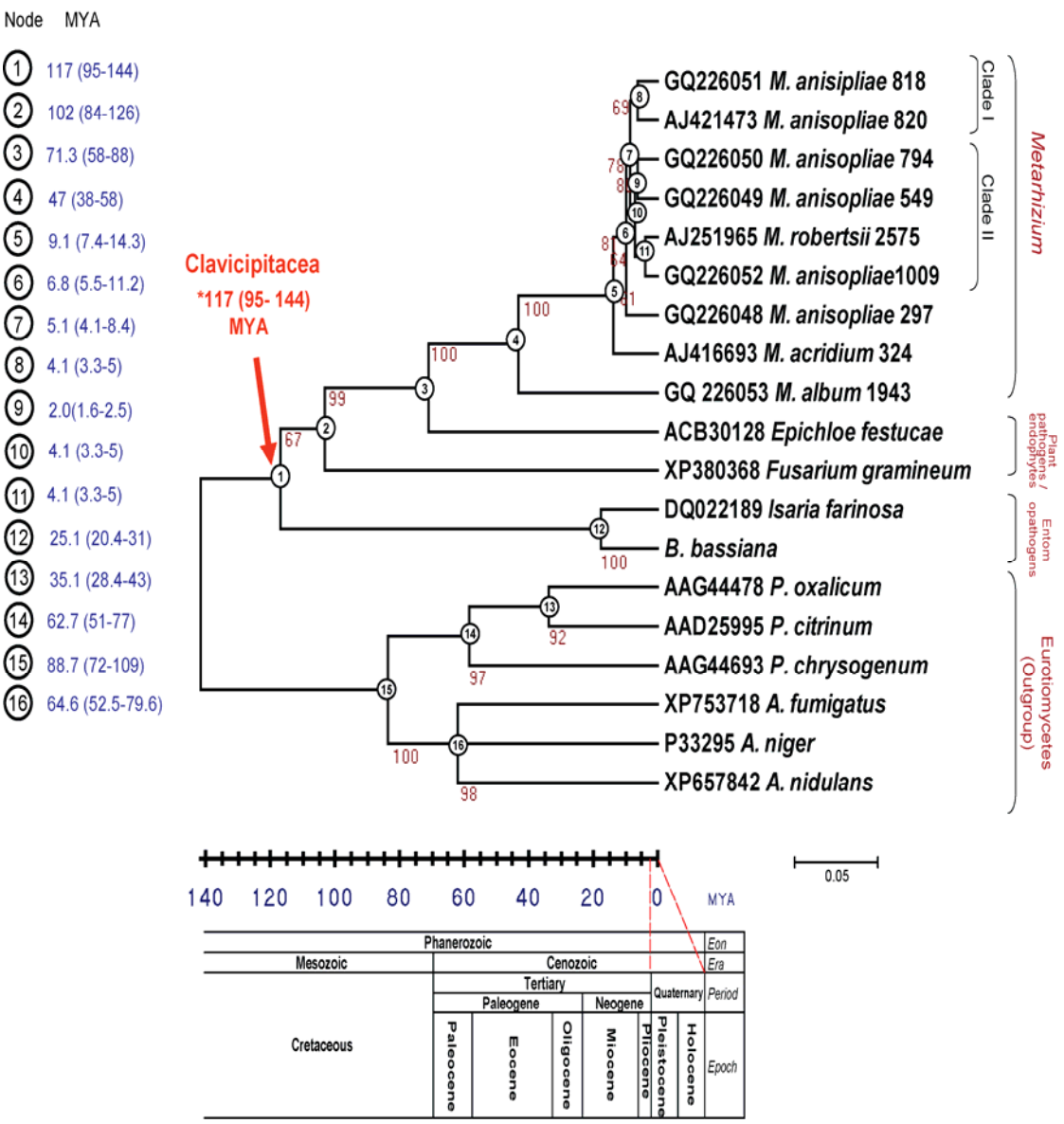

Figure 1-2. Neighbor-Joining tree using the subtilisin endoprotease, Pr1H amino acid sequence of 19 taxa including nine *Metarhizium* species, six of them obtained *de novo* in this study (GenBank accession numbers are included in the tree). Several species of *Penicillium* and *Aspergillus* that belong to the Eurotomycetes are included as outgroups. The molecular clock was calibrated based on the 117 (95% CI: 95-144) MYA divergence point of the Clavicipetacean family reported by Sung et al. (2008) (node 1). Phylogenetic analyses were conducted using MEGA 4.1.

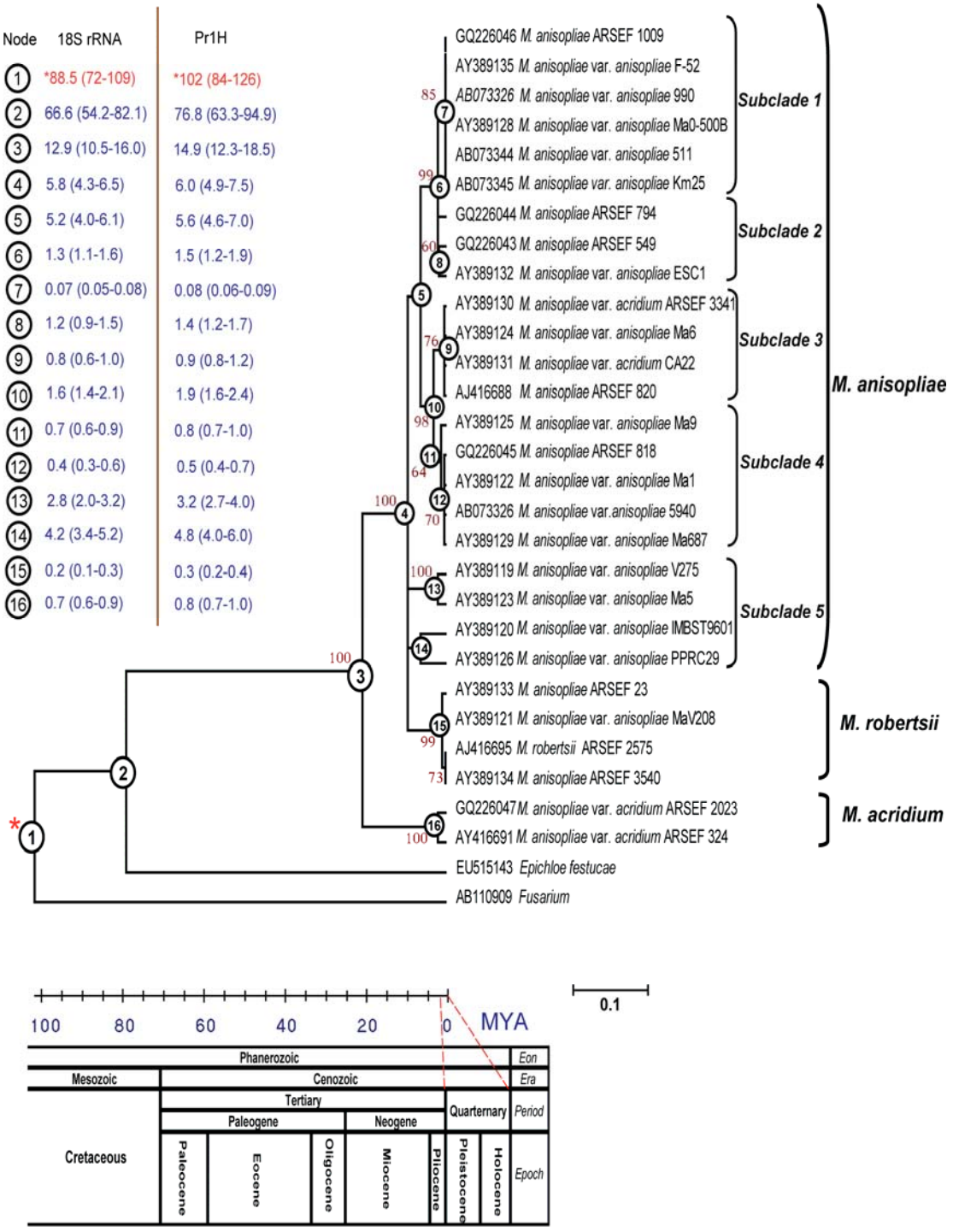

Figure 1-3. Neighbor-Joining tree using the subtilisin protease, Pr1A amino acid sequence of 28 *Metarhizium* strains, five of them obtained *de novo* in this study

(GenBank accession numbers are included in the tree). The molecular clock was calibrated in node 1 based on the divergence points of the clade of plant pathogens/endophytes and *Metarhizium* strains obtained from the 18S rRNA tree (88.5 (72-109) MYA) (Figure 1, node 3) and also from the Pr1H amino acid tree (102 (84-126) MYA) (Figure 2, node 2). Phylogenetic analyses were conducted using MEGA 4.1.

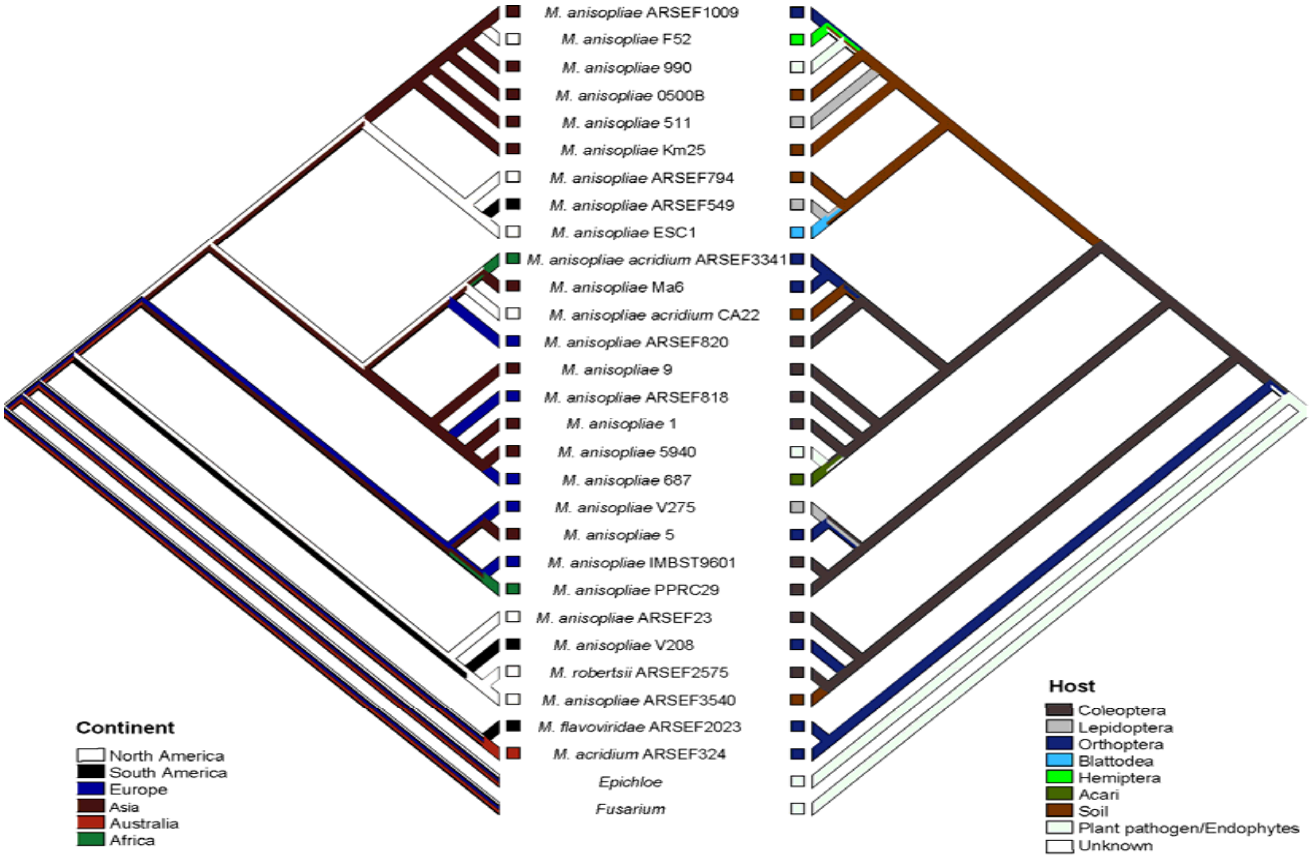

Figure 1-4. Ancestral reconstruction of geographic area of origin (left) and host (right) of Pr1A amino acid data of *Metarhizium* species.

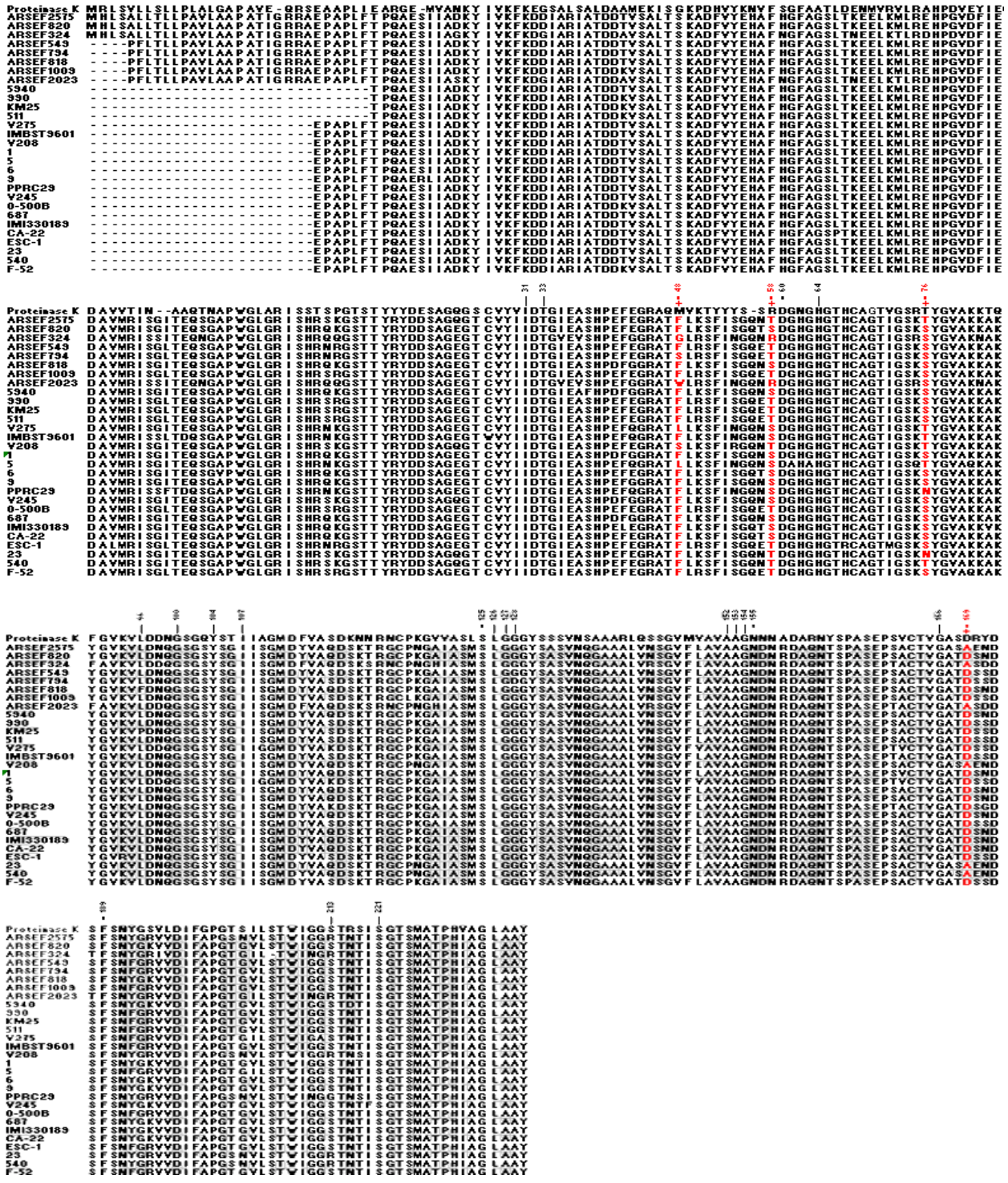

Figure 1-5. Alignment of Pr1A subtilisins from all strains of *M. anisopliae* with proteinase K from *Tritirachium album* using Clustal X. The regions highlighted in dark represent positively selected sites and blue color represents negatively selected sites.

Sites under selective pressure were selected using fixed effects likelihood (FEL) analysis of the HYPHY v 0.99 software. A p-value <0.2 was considered significant.

Table 1-1. List of *Metarhizium* taxa used to construct the protease A (Pr1A) tree.

Taxon		Isolate	GenBank Accession no.	Collection*	Host/ Substrate	Country	Continent
Metarhizium anisopliae	var. *acridium*	ARSEF 324	AJ416691	ARSEF, USA	Orthoptera	Australia	Australia
Metarhizium anisopliae		ARSEF 1009	GQ226046	ARSEF, USA	Orthoptera	Japan	Asia
Metarhizium anisopliae		ARSEF 23	AY389133	ARSEF, USA	Coleoptera	USA	North America
Metarhizium anisopliae		ARSEF 3540	AY389134	ARSEF, USA	Soil	USA	North America
Metarhizium anisopliae		ARSEF 549	GQ226043	ARSEF, USA	Lepidoptera	Brazil	South America
Metarhizium anisopliae		ARSEF 794	GQ226044	ARSEF, USA	Soil	USA	North America
Metarhizium anisopliae		ARSEF 818	GQ226045	ARSEF, USA	Coleoptera	France	Europe
Metarhizium anisopliae		ARSEF 820	AJ416688	ARSEF, USA	Coleoptera	France	Europe
Metarhizium anisopliae	var. *acridium*	IMI 330189/ ARSEF 3341	AY389130	IMI, UK/ARSEF, USA	Orthoptera	Niger	Africa
Metarhizium anisopliae	var. *acridium*	CA-22 / ERL845	AY389131	NK/UVM ERL, USA	Soil	USA	North America
Metarhizium anisopliae	var. *anisopliae*	511	AB073344	RCEF, China	Lepidoptera	Japan	Asia
Metarhizium anisopliae	var. *anisopliae*	687	AY389129	RCEF, China	Acari	UK	Europe
Metarhizium anisopliae	var. *anisopliae*	990	AB073326	RCEF, China	Unknown	Japan	Asia
Metarhizium anisopliae	var. *anisopliae*	5940	AB073327	RCEF, China	Unknown	Japan	Asia
Metarhizium anisopliae	var. *anisopliae*	0-500B / ERL259	AY389128	NK / UVM ERL, USA	Soil	Taiwan	Asia
Metarhizium anisopliae	var. *anisopliae*	ESC-1 / ERL73	AY389132	NK / UVM ERL, USA	Blattodea	USA	North America
Metarhizium anisopliae	var. *anisopliae*	F-52 / ERL1320	AY389135	NK / UVM ERL, USA	Hemoptera	USA	North America
Metarhizium anisopliae	var. *anisopliae*	IMBST 9601	AY389120	IMBST, Austria	Coleoptera	Austria	Europe
Metarhizium anisopliae	var. *anisopliae*	KM25	AB073345		Soil	Japan	Asia
Metarhizium anisopliae	var. *anisopliae*	Ma1	AY389122	Ma, Germany	Coleoptera	China	Asia
Metarhizium anisopliae	var. *anisopliae*	Ma5	AY389123	Ma, Germany	Orthoptera	China	Asia
Metarhizium anisopliae	var. *anisopliae*	Ma6	AY389124	Ma, Germany	Orthoptera	China	Asia
Metarhizium anisopliae	var. *anisopliae*	Ma9	AY389125	Ma, Germany	Coleoptera	China	Asia
Metarhizium anisopliae	var. *anisopliae*	PPRC-29	AY389126	PPRC, Ethiopia	Coleoptera	Ethiopia	Africa

Taxon		Isolate	GenBank Accession no.	Collection*	Host/ Substrate	Country	Continent
Metarhizium anisopliae	var. *anisopliae*	V208	AY389121	V, UK	Orthoptera	Brazil	South America
Metarhizium anisopliae	var. *anisopliae*	V275	AY389119	V, UK	Lepidoptera	Austria	Europe
Metarhizium anisopliae	var. *acridium*	ARSEF 2023	GQ226047	ARSEF, USA	Orthoptera	Ecuador	South America
Metarhizium anisopliae		ARSEF 2575	AJ416695	ARSEF, USA	Coleoptera	USA	North America

***IMBST**: Institut fur Mikrobiologie Leopold-Franzens-Universität Innsbruck, Austria; **IMI**: CABI Bioscience - UK Centre, Egham, UK. (Formerly International Mycological Institute); **ARSEF**: Agriculture Research Service Entomopathogenic Fungal Collection; **RCEF**: Research Center of Entomopathogenic Fungi Collection, Anhui Agricultural University, Hefei, China; **KVL**: Royal Veterinary and Agricultural University, Frederiksberg, Denmark; **V**: School of Biological Sciences, Swansea, UK (Dr. Butt); **Ma**: Biologische Bundesanstalt, Darmstadt, Germany (Dr. Zimmermann); **PPRC**: Plant Protection Research Center and Alemaya University entompathogenic fungal collections, Ethiopia; **UVM ERL**: University of Vermont WorldWide Collection of Entomopathogenic Fungi, Burlington, VT; **NK**: Not Known.

Table 1-2. Negatively selected codon sites of the subtilisin endoprotease Pr1H of ten strains of the entomopathogenic fungus *Metarhizium*. Significantly different codon sites under selective pressure were selected using the single likelihood ancestor counting (SLAC) analysis of the HYPHY v 0.99 software. A p-value <0.2 is considered significant.

Codon Site	dN-dS	p-value
14	-2.00000	0.11111
23	-2.35660	0.08003
85	-0.04167	0.01931
105	-2.00000	0.11111
107	-2.00000	0.11785
131	-2.00000	0.11111
188	-2.00000	0.11111
226	-2.91159	0.05243
230	-0.01435	0.00768
256	-2.81153	0.05623
312	-2.81176	0.05622
338	-2.00000	0.11111
340	-3.00000	0.03704
400	-2.00000	0.11111
403	-2.00000	0.11111
431	-2.34498	0.08082

Table 1-3. Negatively and positively selected codon sites of the subtilisin endoprotease Pr1A of twenty-eight strains of the entomopathogenic fungus *Metarhizium*. Significantly different codon sites under selective pressure were selected using fixed effects likelihood (FEL) analysis of the HYPHY v 0.99 software. A p-value <0.2 is considered significant.

Codon Site	dN	dS	dN/dS	dS=dN	LRT	p-value	Selective Pressure
39	0	11.8791	0	2.98057	9.42681	0.002138	Negative
57	0	17.2411	0	1.5638	8.68819	0.003203	Negative
59	0	9.32668	0	2.5105	7.18119	0.007367	Negative
62	0	3.22938	0	0.772908	2.84127	0.091872	Negative
63	0	2.8847	0	0.693086	2.80969	0.093697	Negative
70	0	6.22209	0	0.81582	3.88071	0.048844	Negative
75	0.817577	7.40832	0.110359	1.52891	2.12587	0.144831	Negative
86	0	2.12653	0	0.72066	2.15334	0.14226	Negative
95	0	3.59965	0	0.868585	2.7323	0.098337	Negative
102	0	17.2792	0	2.64794	9.66482	0.001878	Negative
120	0	2.51464	0	0.740935	2.44146	0.118166	Negative
123	0	6.04944	0	1.63482	5.14757	0.023279	Negative
132	0	4.62497	0	0.791718	3.49011	0.061737	Negative
138	0	3.20714	0	0.802527	2.70265	0.100182	Negative
140	1.50337	33.725	0.044577	2.25644	3.42941	0.064045	Negative
146	0	3.74194	0	0.727617	3.22101	0.072699	Negative
150	0	3.29082	0	0.78623	2.78805	0.09497	Negative
161	0	3.46951	0	0.716068	3.05291	0.080593	Negative
164	**6.82446**	**0**	**inf**	**5.73382**	**1.94851**	**0.162748**	**Positive**
165	0	6.76657	0	1.55991	5.74489	0.016537	Negative
166	1.40791	13.8546	0.101621	2.02151	2.24408	0.134127	Negative
174	**5.21793**	**0**	**inf**	**4.02132**	**2.45066**	**0.117476**	**Positive**
175	0	10.9125	0	1.55284	7.56891	0.005938	Negative
178	1.07932	8.30523	0.129956	2.5435	2.59729	0.107047	Negative
185	0	5.38485	0	1.56087	4.91042	0.026695	Negative
186	0	8.95001	0	2.15237	8.37642	0.003801	Negative
190	1.41574	28.917	0.048959	4.03462	8.83425	0.002956	Negative
191	**4.88034**	**0**	**inf**	**3.92056**	**1.80799**	**0.17875**	**Positive**

Codon Site	dN	dS	dN/dS	dS=dN	LRT	p-value	Selective Pressure
193	0	3.13765	0	0.77812	2.72326	0.098896	Negative
207	0	5.36174	0	0.758449	3.86057	0.049434	Negative
209	0	36.5985	0	0.653566	7.73393	0.005419	Negative
212	0	3.12763	0	0.77748	2.7188	0.099173	Negative
225	0	2.661	0	0.765788	2.45247	0.11734	Negative
228	0	3.48533	0	0.800305	2.83374	0.092303	Negative
231	0	3.39502	0	0.722644	3.06677	0.079908	Negative
235	1.27791	11.5023	0.1111	1.84908	2.39168	0.121983	Negative
242	0	5.36171	0	0.706949	3.99594	0.04561	Negative
253	0	8.10208	0	0.584571	5.23743	0.022106	Negative
255	0	3.24189	0	0.812666	2.74731	0.097418	Negative
257	0	3.14554	0	0.799069	2.67696	0.10181	Negative
259	0	3.36462	0	0.855694	2.64379	0.103955	Negative
261	0	3.15431	0	0.792835	2.68951	0.101011	Negative
265	1.04534	8.22533	0.127088	2.46821	2.79	0.094855	Negative
271	0	6.27301	0	0.667862	4.28843	0.038373	Negative
277	0	17.9206	0	0.652147	6.49995	0.010788	Negative
289	0	3.19807	0	0.706812	2.94655	0.086061	Negative
295	**3.48271**	**0**	**inf**	**1.52963**	**3.23651**	**0.072014**	**Positive**
302	0	5.3613	0	0.855305	3.60956	0.057448	Negative
311	0	4.18593	0	0.752011	3.3989	0.06524	Negative
319	0	4.64967	0	0.836038	3.43823	0.063704	Negative
323	0	4.18593	0	0.752011	3.3989	0.06524	Negative
327	0	9.44674	0	1.58121	7.18975	0.007332	Negative
332	0	16.5001	0	2.55577	9.72361	0.001819	Negative
341	0	3.43715	0	0.823444	2.82898	0.092577	Negative
342	0	7.29433	0	1.60242	5.67055	0.017252	Negative
343	0	2.12653	0	0.72066	2.15334	0.14226	Negative

Chapter 2: Time-course of gene expression by *Metarhizium anisopliae* growing in plant root exudate

2.1 ABSTRACT

The entomopathogenic fungus *Metarhizium anisopliae* is a common inhabitant of soils World-wide. It is particularly abundant in rhizospheric soils. Our experiments evaluated the germination of spores from eleven strains of entomopathogenic fungi (genera *Beauveria* and *Metarhizium*), two soil saprophytic fungi (the non-rhizospheric fungus *Aspergillus niger* and the best studied rhizosphere competent fungus *Trichoderma harzianum*) at several concentrations of bean root exudates (RE) (1, 2.5, 5, 10 and 20 mg/ml). At concentrations of RE <1 mg/ml *M. anisopliae* strain ARSEF 2575 showed higher germination rates than *T. harzianum*. Using microarrays, we identified the subset of genes that *M. anisopliae* expressed after 1, 4, 8 and 12 hours of growth in 5 mg/ml of RE. Results showed that fifty genes (2.9%) were differentially expressed (DE) at all time points and they were organized into functionally related groups. Twenty-nine DE genes (58%) were up-regulated and twenty-one (42%) were down-regulated. Up-regulated genes were involved in carbohydrate metabolism (10.4%), lipid metabolism, cofactor and vitamins (6.9%) energy metabolism (6.9%), proteolysis (3.4%), extra cellular matrix/cell wall proteins (13.9%), transport proteins (6.9%), DNA synthesis (3.4%), sexual cycle (3.4%) and stress response (3.4%). A large number of hypothetical and orphan proteins (41.4%) were also up-regulated indicating that many previously uncharacterized genes may have functions related to saprophytic survival.

2.2 INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* is a natural inhabitant of the soil and is has been found almost everywhere, even in sub-Antarctic soils (Roddam and Rath 1997, Zimmermann 2007). The occurrence and geographic distribution of different genotypes of *M. anisopliae* in the soil depends upon environmental factors, such as temperature and humidity, and soil conditions, such as pH and organic matter content (Quesada-Moraga et al. 2007, Zimmermann 2007). Different strains of the fungus are found in forest and grassland soils (Bidochka et al. 1998, Quesada-Moraga et al. 2007, St. Leger 2008) and in grasslands it can reach up to 10^6 propagules/g at 2-6 cm deep (Milner 1992). In recent studies, *M. anisopliae* has been shown to be a rhizosphere competent organism (Hu and St. Leger 2002b), with possible roles in plant protection and plant growth (St. Leger 2008). This may help to explain the persistence of the conidia of *M. anisopliae* in soils (unlike other entomopathogenic fungi) for long periods of time in the absence of an insect host (Bidochka et al. 2001) and at the same time suggests that this fungus may be subject to two different selective pressures: one for insect colonization, the other for soil survivability (Prior 1992, Wang et al. 2005, St. Leger 2008).

The presence of genetic groups of *M. anisopliae* in soils is shaped by their adaptability to specific soils and habitat types rather than for their pathogenicity to insects (Bidochka et al. 1998). However, the large populations of insects in soils (i.e. white grubs) may also influence the presence of *M. anisopliae* populations (St. Leger 2008). As a result, the soil/root interface is a place where insects, plants and microbial populations will interact to effect *Metarhizium* populations. Similar interactions have been studied with the best known rhizosphere competent fungus *Trichoderma* spp. This

fungus is also used as biocontrol agent because it can parasitize other plant fungal pathogens and establishes symbiotic relationship with the roots of some plants increasing plant growth and productivity (Harman 2006). The complex processes between multiple strains of *Trichoderma*, the roots of plants and other fungal pathogens demonstrates that their exists an established molecular cross-talk between them (Woo et al. 2006). However, the genetic and physiological factors controlling rhizosphere competence on *Trichoderma* are little understood (St. Leger et al. 1986).

Likewise, while a lot of research has been performed on the entomopathogenic lifestyle of *M. anisopliae*, its saprophytic lifestyle has received little consideration. While a set of functionally related genes could be commonly expressed as this fungus adapts to different environments (insect cuticle, insect blood and root exudates), there might also exist different subset of genes active in each environment (Wang et al. 2005).

Increasing knowledge of the genes that are involved in the rhizospheric life style of *M. anisopliae* will be the first step in elucidating pathways that are used by this fungus for soil survivability and adaptability. In this study we evaluated gene expression by *M. anisopliae* growing on plant root exudates in laboratory conditions over a time course. A deeper understanding of the mechanistic basis of rhizosphere competency could enable us to identify genes that we could use to develop *M. anisopliae* as a comprehensive plant symbiont or at least improve persistence and consequently provide greater long term protection against insect pests.

2.3 MATERIALS AND METHODS

2.3.1 Bean root exudates

In order to simulate rhizospheric conditions in the lab, *M. anisopliae* was grown on bean root exudates (RE) from black pea seeds (*Vigna unguiculata* subsp. *unguiculata*). To obtain the RE, bean seeds were disinfected and placed on wet, sterile paper for initial germination. Seedlings were subsequently transferred to containers with sterile distillated water and kept there connected to an aquarium air pump until roots were formed (about one week) (Figure 2-1). RE was then collected, freeze dried, prepared in a stock solution of 40 mg/ml, and filtered sterilized before storing it at -20°C until use.

2.3.2 Fungal strains and spore germination in root exudates

In order to determine the optimal bean RE concentration for germination of the fungal spores, an experiment with a completely randomized design (CRD) was performed. This experiment evaluated 13 fungal strains (11 entomopathogenic fungi from the genera *Beauveria* and *Metarhizium*, and 2 soil saprophytic fungi: *Aspergillus niger* (non-rhizospheric) and *Trichoderma harzianum* strain T22 (rhizosphere competent), at 5 concentrations of bean RE (1, 2.5, 5, 10 and 20 mg/ml). Information about the geographic area and insect host (if entomopathogen) of all the fungal strains used is shown in Table 2-1. Fungal spores were also added to 0.1% of yeast extract (Fisher Scientific, Pittsburgh, PA) and sterile distilled water as positive and negative controls, respectively. Three replicates were performed for each treatment combination (fungal strains and RE concentrations).

Each fungal strain was grown on Sabouraud dextrose agar (SDA) (Fisher Scientific, Pittsburgh, PA) for two weeks at 27°C. Fungal spores were collected and suspended in 0.01% Tween 20 (Sigma Chemical Co., St Louis, MO) and vortexed for 2 minutes. The concentration of spores was determined using a haemocytometer. A final concentration of 10^4 spores/ml was prepared and 100 µl were added to sterile tubes containing 900 µl ml of the selected concentration of RE. The percentage spore germination of entomopathogenic and saprophytic fungi was determined after 24 hours of incubation at 27°C with shaking at 250 rpm. After the incubation time, one hundred spores were counted on glass slides using light microscopy and the number of germinated spores was recorded (Whalley and Taylor 1973).

In order to see differences in germination between *T. harzianum* and *M. anisopliae*, we evaluated germination of their fungal spores at concentrations lower than 1 mg/ml (0.01, 0.05, 0.1 and 0.5 mg/ml).

Data were analyzed using one-way analysis of variance (ANOVA) (SAS Institute Inc. 2006). A P value ≤ 0.01 determined the significance level and biologically important results were evaluated as well.

2.3.3 Culture conditions and total RNA extraction

To proceed with microarray analyses, *M. anisopliae* mycelia grown for 30 h on 100 ml of Sabouraud dextrose broth (SDB) were collected through vacuum filtration and washed three times with sterile distilled water. A small portion of the filtered mycelium was collected for further RNA extraction and hybridization (time zero). The remaining mycelium was suspended in 20 ml of sterile distilled water. Five ml of the mycelium

suspension were transferred to four 50-ml flask, each containing five ml of 10 mg/ml of RE for a final concentration of 5 mg/ml of RE. Samples were incubated in a shaker/incubator at 27°C / 220 rpm (New Brunswick Scientific, Edison, NJ) and mycelium was collected from individual flasks after 1, 4, 8 and 12 hours of incubation. Total RNA was immediately extracted from the samples using the Qiagene Rneasy Mini kit (Valencia, CA, USA) according to manufacturer's instructions. RNA samples were treated with Qiagene DNase I. Two biological replicates were performed.

2.3.4 cDNA Microarray

For microarray analysis, we used cDNAs isolated from three libraries that were previously constructed using mycelium of *M. anisopliae* (ARSEF 2575) growing on insect cuticle (Freimoser et al. 2003) and from insect blood and plant root exudates (Wang et al. 2005). PCR amplified ESTs were spotted on glass slides following the standard protocol developed at University of Maryland Biotechnology Institute (http://www.umbi.umd.edu/carb/core-facilities/microarray-services/facility.php). Gamma amino propyl II-coated glass slides (Corning Incorporated, Corning, N.Y.) were used. A total set of 1748 unigenes (870 from cuticle, 276 from hemolymph and 602 from the root library) were printed per triplicate per slide. Blanks using water and SSC buffer were also included in the slides as negative controls. The genes were classified in nine groups of functionally related genes and this classification was also used to initiate comparisons of expressed genes (Wang et al. 2005).

2.3.5 Microarray design and RNA hybridization

To detect expression profiles for genes in RE in a time-course, we applied the loop design experiment where every time point is compared to the previous. The contrast between adjacent time points contains information about the expression pattern of a gene over time.

Total RNA from fungal mycelia grown in SDB (time 0) and RE at each time point (1, 4, 8 and 12 hours) was amplified and directly labeled with Cy3 (green) and Cy5 (red) cyanine dyes using the Ovation™ Aminoallyl system (Nugen, San Carlos, CA). Five slides were hybridized per replicate (time 0-1h; 1h-4h; 4h-8h; 8h-12h and 12h-time 0). Competitive hybridization of the second biological replicate was performed using a reverse dye-assignment. Applying dye-swap to our second biological replicate allowed us to eliminate bias on the efficiency of dye incorporation.

2.3.6 Microarray data analysis

Hybridized slides were scanned at the University of Maryland Biotechnology Institute (http://www.umbi.umd.edu/carb/core-facilities/microarray-services/facility.php) using the Axon 4200 microarray scanner (Molecular Devices). To obtain differential signal expressions, the TIFF images generated (2 image files per hybridized slide) were analyzed using the Spotfinder program (http://www.pfgrc.tigr.org/tools.shtml, JCVI, Rockville, MD). Data was then normalized using LOWESS (LOcally WEighted Scatterplot Smoothing) technique using the software MIDAS (http://www.pfgrc.tigr.org/tools.shtml, JCVI, Rockville, MD). MIDAS output files project included standard deviation regularization, low intensity filter and in-slide

replicate analysis. Array data has been deposited in the Gene Expression Omnibus (GEO) database with accession number **GSE16848**.

To detect differentially expressed genes in our time-course microarray experiment, the Log 2 expression ratios (Cy5/Cy3 for replicate 1 and Cy3/Cy5 for replicate 2) were calculated per gene at each time point in the two replicates. Normalized data were inputted to the Bayesian Analysis of Time Series microarray experiments (BATS) software (version 1.0) (http://www.na.iac.cnr.it/bats/) (Angelini et al. 2008). In order to account for the limited number of time instants we chose as parameter of the analysis the maximum expected degree of polynomial equal to 3, the parameter lambda for the truncated Poisson prior probability equal to 4 (corresponding to an expected polynomial degree about 2) and we accounted for multiple comparisons using the Bayesian Multiple Testing Procedure with the binomial prior. Default values were used for the other parameters.

2.3.7 Validation of differentially expressed genes through Reverse Transcription RT-PCR

Total RNA extracted for microarray analysis was used for RT-PCR using the Verso™ cDNA Kit (Thermo Fisher Scientific Inc. Waltham, MA) according to manufacturer's recommended protocol using oligo-dT primers. The cDNA product was subsequently amplified through PCR reaction using gene specific primers. As a control, RT-PCRs also were carried out for the *gpda* mRNA, encoding glyceraldehyde-3-phosphate dehydrogenase.

2.4 RESULTS

2.4.1 Fungal strains and spore germination on bean root exudates

Germination of fungal spores from all fungal strains evaluated was observed at the lowest concentration of RE (1 mg/ml). *T. harzianum* and *M. anisopliae* strain 2105 showed the highest germination rates at the five concentrations of RE that were evaluated (Figure 2-2a). These germination rates ranged from 96.8 to 99.3 % and they were not significantly different from each other. Generalist entomopathogenic *M. anisopliae* strains 2575, 549 and 1080 showed germination rates ranging from 76.6 to 93.9% at 1 mg/ml of RE, from 91.6 to 95.2% at 2.5 mg/ml and from 87 to 93% at 5 mg/ml RE. These germination rates were not significantly different from those of the rhizospheric fungus *T. harzianum* at the same concentrations of RE. However, strains 2575 and 549 showed significantly lower germination rates compared to *T. harzianum* at concentrations greater than 5 mg/ml RE (Figure 2-2a).

Specialist entomopathogenic *M. anisopliae* subtypes show specificity for certain insects and are unable to infect other insects i.e. *M. anisopliae* var. *acridum* strain 324 is specific to locusts and related grasshoppers (Orthoptera: Acrididae). Other specialist strains such as *M. anisopliae* strains 4600 and 4620 can infect the Tasmanian soldier fly (Diptera: Stratiomyidae) and *M. anisopliae* strain 2974 can infect mosquitoes (Diptera: Culicidae). *M. anisopliae* var. *acridum* 324 showed its highest germination rate (47.3%) at a concentration of 1 mg/ml of RE and its lowest germination rate (24.7%) at the highest concentration of RE we evaluated (20 mg/ml). This rate is significantly different at all concentrations of RE from both the rhizospheric fungus *T. harzianium* and the non-rhizospheric fungus *A. niger* (Figure 2-2a). At concentrations of 1 mg/ml of RE, *M.*

anisopliae strains 4620, 2974 and 4600 presented the lowest germination rate of all fungal strains evaluated (22.5, 12.5 and 6.5%, respectively) and these rates were not significantly different from the germination rate of *A. niger* (19.3%) at that same concentration. However, at higher concentrations of RE these germination rates increased (except at 20 mg/ml when strains 4620 and 2974 showed slightly reduced germination rates) and became significantly different from the germination rate of *A. niger* that declined with increasing concentration of RE (germination rates of *A. niger* were 19, 14, 13, 2 and 0% at concentrations of 1, 2.5, 5, 10 and 20 mg/ml of RE, respectively) (Figure 2-2a).

Three *B. bassiana* strains were included among the fungal strains tested. Two of those strains were isolated from Lepidopteran hosts (Bb 9205 was isolated from a Pyralid and Bb 9112 from a Geometrid). *B. bassiana* strain 3113 was isolated from soil and can enter and move in corn plants as a fungal endophyte providing protection against lepidopteran pests such as *Ostrinia nubilalis* (Pingel and Lewis 1996, Wagner and Lewis 2000). At a concentration of 1 mg/ml of RE germination rates of *B. bassiana* strains 9205, 9112 and 3113 were 73, 50.5 and 64%, respectively. These rates of germination were not significantly different from each other but they were significantly higher than *Metarhizium* specialist strains (4620, 2974 and 4600) and the non-rhizospheric fungus *A. niger* (Figure 2-2a). Germination rates of *B. bassiana* strains 9112 and 3113 were significantly lower than *M. anisopliae* generalist strains (2105, 2575, 549 and 1080) and *T. harzianum* strain T22. *B. bassiana* strains 9112 and 9205 slightly increased their germination rate at concentrations of 2.5 and 5 mg/ml RE but germination decreased at higher concentrations (10 and 20 mg/ml) and diverged significantly at 20 mg/ml.

Germination rates of *B. bassiana* strain 3113 gradually declined at concentrations >1 mg/ml and had diverged significantly from the other fungal strains at 10 mg/ml RE (Figure 2-2a).

In order to detect any differences between *T. harzianum* and *M. anisopliae* strains 2105 and 2575 we evaluated concentrations of RE lower than 1 mg/ml (0.01, 0.05, 0.1 and 0.5 mg/ml). Figure 2-2b shows that at concentrations ≤0.5 mg/ml the germination rate of *T. harzianum* is significantly lower than *M. anisopliae* 2575. Even though *M. anisopliae* strains 2105 and 2575 are both generalist strains, their germination rates differed significantly at concentrations of RE <0.5 mg/ml. The percentage germination of Ma 2105 is 1, 12.5 and 42% at concentrations of 0.01, 0.05 and 0.5 mg/ml RE, respectively whereas germination rates of Ma 2575 are 88.6, 98 and 93% at the same concentrations. This indicates that strain 2575 of *M. anisopliae* is hypersensitive to RE.

2.4.2 Microarray data analysis

Out of 1748 genes included in the cDNA microarrays, we identified 50 genes (2.9%) that were differentially expressed (DE) across the time instants evaluated. DE genes were automatically detected using BATS and ranked according to their Bayes factors (BF <0.006). Nucleotide query sequence of these genes were subsequently compared against the already existing sequences in the NCBI non-redundant protein database (BLASTX) (http://blast.ncbi.nlm.nih.gov/) and re-organized into previously classified groups of functionally related genes (Wang et al. 2005) (Figure 2-3). *M. anisopliae* genes that were differentially expressed under RE conditions over this time course belong to the groups of hypothetical proteins (predicted sequences that lack experimental evidence of *in vivo* expression) (13/413, 3.1%), unknown proteins (orphan

sequences with no homologous in data bases) (1/236, 0.4%), cell metabolism (7/335, 2.1%), energy metabolism (2/64, 3.1%), protein metabolism (11/172, 6.4%), cell structure and function (10/231, 4.3%), cell cycle, division and growth (3/93, 3.2%) and stress response and defense (3/107, 2.8%) (Figure 2-3).

Overall, 27 (54%) of these genes were originally isolated from the EST root library, 18 (36%) from the cuticle library and 5 (10%) from the insect blood library of *M. anisopliae* 2575. Thus, many genes expressed during the cuticle degradation and growth in insect blood are also involved in root colonization.

Identification of individual genes, their Log 2 expression ratio (fold change, FC) at specific time points and information about functional groups are included in Table 2-2. Figure 2-4a shows the linear expression map (LEM) with the average of the Log 2 ratio of DE genes classified by functional groups at each time point. Twenty-nine DE genes (58%) were actively involved under RE conditions. These genes were identified because they were down- regulated while the fungus was growing on SDB (time 0) and were up-regulated at a later time point. They are involved in carbohydrate metabolism (10.4%), lipid metabolism, cofactor and vitamins (6.9%) energy metabolism (6.9%), proteolysis (3.4%), extra cellular matrix/cell wall proteins (13.9%), transport proteins (6.9%), DNA synthesis (3.4%), sexual cycle (3.4%), stress response (3.4%), hypothetical proteins (37.9%) and unknown proteins (3.4%) (Figure 2-4a). The level of expression of individual genes, the maximum fold-change (FC) and the time (hours) when the maximum FC occurred is presented in figure 2-4b. From the twenty-nine genes that were up-regulated under RE conditions, 9 genes (31%) obtained their maximum FC after 4 h, 14 (48%) after 8 hours and 6 genes (21%) after 12 h (Figure 2-4b). As expected, there is

also a set of DE genes (21 genes, 42%) that were up-regulated while the fungus was growing on SDB (time 0) and that were shut down at a later time presumably because these genes were not required by *Metarhizium* to grow well in RE. These genes were in the groups of ribosomal proteins, translation, and transposable elements (Figure 2-4a).

Figure 2-5a-j shows the threshold of expression ratios through time of the fifty genes of *M. anisopliae* 2575 that were DE under RE conditions. Figure 2-5a-f shows the twenty-nine genes that were up-regulated and figure 2-5 g-j shows the twenty-one genes that were down-regulated. Up-regulated genes showing the greatest fold change through time belong to the group of hypothetical proteins: AJ273764 (FC of 3.7 at 8h), CN808927 (FC of 3.3 at 4h) and CN809209, CN808884, CN809514 and AJ274093 that reached the maximum FC of 3.3, 3.0, 2.5 and 2.5, respectively after 8 h of being in RE (Figure 2-5a and 2-4b). The expression of the subtilisin-like serine protease (Pr1A) gene (CN808958) increased through time under RE conditions having the maximum expression ratio (1.5) after 8 hours; this is a 3.3 FC increase (Figure 2-5b). Genes involved in energy metabolism: Dihydrolipoyl dehydrogenase (AJ273762) and oxidoreductase (CN808777) were also up-regulated and they acquired their maximum FC of 2.9 and 2.0 after 8 of and 12 h, respectively (Figure 2-5b and 2-4b). Genes involved in stress response such as the heat shock protein (CN808235) reached the maximum FC of 2.6 after 4 h of being under RE substrate (Figure 2-5c and 2-4b). Up-regulated transport proteins include the ABC transporter ATP-binding protein (CN809103) and the GABA (gamma-amino-n-butyrate) permease (CN808046) with maximum FC of 2.4 and 0.8, respectively after 12 h in RE (Figure 2-5d and 2-4b). Genes required for the metabolism of carbohydrates such as Glycosyl hydrolase (CN808813), beta-glucosidase (AJ273623)

and ferulic acid esterase A (*faeA*) (AJ273114) were also up-regulated under RE conditions showing the maximum FC of 2.2, 1.9 and 1.3, respectively after 8 (for CN808813) and 4 hours (for AJ273623 and AJ273114) (figure 2-5d and 2-4b). Interactions with the RE environment also increased the regulation of genes associated with the extra cellular matrix and cell wall proteins such as the Hydrophobin-like protein precursor (AJ274156) and the cell wall protein (AJ273845) that reached the maximum FC of 2.1 after 8 h and 12 h of being in RE, respectively (Figure 2-5e and 2-4b). Also, the plant cell wall adhesin MAD2 proteins (CN809626 and CN809322) reached their maximum FC values of 1.9 and 1.6, respectively, after 4 h of being under RE substrate (Figure 2-5c and 2-4b). Other genes that are involved in the synthesis of DNA (mediator of replication checkpoint 1, CN809288), lipid metabolism (Diacylglycerol O-acyltransferase – *DgaT*, CN808018), sexual development (EsdC protein, CN809127) and cofactor and vitamins (amidase protein, AJ273042) were also up-regulated. These genes showed a FC increase between 2.0 and 0.8 after 4 and 8 h or being in contact with the RE (Figure 2-5f and 2-4b).

Twenty-one DE genes (42%) were up-regulated while the fungus was growing on SDB (time 0). These genes were not actively involved under RE conditions since they gradually decreased the expression ratio and obtained the maximum decreased in FC at a later time point: 2 genes (10%) obtained the maximum FC decreased at 4 hours (FC of 1.0 and 1.2), 5 genes (24%) at 8 h (FC from 1.0 to 1.6) and 14 genes (46%) at 12 h (FC from 0.8 to 2.4) (Figure 2-4b). Genes that gradually decreased in expression ratio after 1 h of incubation under RE conditions included ribosomal proteins (Figure 2-5g), transposable elements and modulators of translation (Figure 2-5h).

Correspondingly, genes belonging to other functional groups such as stress response (cold acclimation induced protein 2-1, CN808997), transport proteins (carrier protein ADP/ATP translocase, CN809461), sexual cycle (sexual development EsdC protein, CN808640), hypothetical protein AJ272794 and extra cellular matrix/cell wall proteins (hydrophobin, CN809178 and AJ273847) gradually decreased in expression under RE conditions (Figure 2-5i). However, the FC of the majority of those genes increased (between 0.1 to 0.7) after 8 h of incubation in RE and then decreased again at 12 h. Hydrophobin genes, AJ273847 and CN809178, had a higher FC increase at this particular time (FC increased of 1.5 and 1.9, respectively) (Figures 2-5i). This is an indication that genes belonging to functional group of the extra cellular matrix/cell wall proteins are required by the fungus to adapt to RE.

Contrary to the expression pattern described above, three genes (the C-3 sterol dehydrogenase protein - AJ274219, the potassium channel protein – CN808889 and the hypothetical protein CN808746) slightly increased their expression ratio during the initial 4 h of incubation in RE (FC increases of 0.4, 0.5 and 0.7, respectively) before starting to decrease at later time points (Figure 2-5j). This indicates that these genes were required by the fungus for a longer period of time probably for degradation of the lipids present in the RE and for intake of nutrients into the cells.

2.4.3 Reverse Transcriptase polymerase chain reaction (RT-PCR) verification of differentially expressed genes

Five genes (predicted protein-AJ273764, subtilisin-like serine protease PR1A-CN808958, Hydrophobin-like protein-AJ274156, *Metarhizium* adhesin protein Mad2-CN809626 and a ribosomal protein-CN809270) were selected for validation of

microarray analysis through RT-PCR. The results indicated that expression patterns of these genes were consistent with microarray results (Figure 2-6).

2.5 DISCUSSION

The symbiotic association between fungi and plant roots (mycorrhiza) is essential for the survivability of both parts. While the fungus can access carbohydrates such as glucose and sucrose (final products of photosynthesis), the plant benefits from the increased uptake of water and nutrients such as nitrogen and phosphorus that induce plant growth (Harman and Shoresh 2007). Root exudates play a key role in stimulating the germination and hyphal branching of arbuscular mycorrhizal (AM) fungal spores (Bucking et al. 2008) so it is not surprising if they also play an important role for entomopathogenic fungi adapting to soil environments.

Our results show that generalist strains of *M. anisopliae* (ARSEF 2105, 2575, 549 and 1080) can use RE as well as the rhizosphere competent fungus *T. harzianum*. Spores of specialist strains of *M. anisopliae* behaved differently at different concentrations of RE. Although some specialist strains of *Metarhizium* are scattered among clades of generalist strains in phylogenies (Figure 1-3), they have clearly evolved independently to adapt to host pathogenicity and nutrient conditions. Thus, some specialist strains of *M. anisopliae* need higher concentration of the nutrients present in root exudates to germinate (4620, 2974 and 4600). However, high concentrations of RE inhibit growth of diverse phylogenetically distant specialist (Ma 324) and generalist strains (2105, 2575, 549 and 1080) (Figure 2-2a) (Wang et al. 2009a) (See also Chapter 1) indicating that RE contains a mixture of inhibitory as well as stimulatory chemicals that effect strains differently.

M. anisopliae is commonly found in natural soils, particularly in cultivated grassland soils where is able to survive for long periods of time without an insect host (Milner 1992, Bidochka et al. 1998, Quesada-Moraga et al. 2007, St. Leger 2008). Our results show that this fungus contains a set of DE genes that can rapidly adapt to nutrients present in RE. A previous study (Wang et al. 2005) showed that the overall pattern of gene expression in RE was mainly down-regulated after 24 h at low concentration of RE (0.1 mg/ml). Here we demonstrate that the up-regulation of genes under RE conditions occurs at earlier time points (mainly 4 and 8 hours post-inoculation into RE) and that these up-regulated genes have FC increases between 0.8 and 3.7 (Figure 2-4b).

Root exudates are composed of a complex mixture of soluble compounds such as carbohydrates, lipids, amino acids, organic acids, proteins, polysaccharides, nucleotides, flavonoids, growth factors, enzymes, growth inhibitors and repellants. The presence and concentration of these compounds will depend upon the plant species, growth conditions and state of plant development (Rovira 1965, Hale et al. 1978). RE is also known to regulate the microbial community in the rhizosphere, help plants withstand hervibory and encourage beneficial symbioses (Walker et al. 2003, Bais et al. 2004). Our results are consistent with RE containing repressors as well as stimulators of fungal growth.

The highest percentage of up-regulated genes (41.4%) by *Metarhizium* under RE conditions belongs to the category of hypothetical proteins or unknown proteins, indicating that many previously uncharacterized *Metarhizium* genes may have functions related to saprophytic survival. Some of these genes have orthologs in plant pathogens and other soil fungi so whatever role they play in adapting to soil conditions may be

conserved. New genome analysis of *Metarhizium* will elucidate the function of these hypothetical or orphan proteins that were also up-regulated under RE conditions.

The presence of at least ten sugars (i.e. glucose, fructose, sucrose, xylose, maltose and rhamnose, among others) has been identified in the RE of a wide variety of plants (Rovira 1965). *M. anisopliae* genes involved in carbohydrate metabolism (Glycosyl hydrolase-CN808813, beta-glucosidase-AJ273623 and Ferulic acid esterase-AJ273114) were up regulated during the first 8 hours under RE conditions. Genes involved in proteolysis (Pr1A- CN808958) were also up-regulated in the first 4 h of incubation documenting the rapid adaptation of this fungus to new environments. Wang et al. (2005) also reported the up-regulation of the *pr1A* gene (CN808958) after 24 h of incubation in RE indicating the long-term involvement of this protease in utilizing RE. A formidable array of hydrolytic enzymes is secreted during the pathogenic life cycle of *M. anisopliae* that included Pr1A (Bagga et al. 2004, Wang et al. 2009a) and its rapid and high expression in RE implies an important role for proteolysis in utilizing RE.

Cell wall proteins (e.g., adhesin protein MAD2-CN809626-CN809322) were also up-regulated in the first 4 h of incubation in RE. The up-regulation of gene CN809626 after 24 h of incubation in RE was also reported by Wang et al. (2005). CN809626 was later designated as *Mad2* (for *Metarhizium* adhesin-like protein 2) and it was found to be responsible for the attachment of the fungus to plant surfaces and is crucial for them to effectively persist and colonize roots (Wang and St Leger 2007b). Mutant strains of *Metarhizium* with the deletion of *Mad2* gene were constructed and they showed 90% reduction in adherence to plant cells suggesting that there is little redundancy in adhesion molecules for plant surfaces (Wang and St Leger 2007b). However, we found that a

fragment (261/630, 42%) of the EST sequence of the root library CN809322, originally designated as an unknown protein, presented 98% similarity (E value 2e-136) with the MAD2 transcript (CN809626) and although expressed at lower levels than MAD2 showed an identical pattern of gene expression through time under RE conditions (Figure 2-4c). This suggests that this transcript is also actively involved in the attachment of the cells to plant surfaces and it is perhaps responsible for the 10% of *Mad*2 mutant spores that are still adherent.

Another fragment (147/817, 18%) of the EST root library (CN809103) that was originally designated as an unknown protein, showed 46% similarity (E value 3e-02) to a partial sequence of the putative ABC transporter (ATP-binding protein) of the symbiotic nitrogen-fixing actinobacterium *Frankia alni* (Table 2-2). ABC transporter proteins are ubiquitous in all organisms and are primarily involved in the active transport of a wide array of different molecules across plasma membranes. Some ABC proteins serve as a cellular toxic defense mechanism by transporting deleterious compounds out of the cell. These may include xenobiotics, anthropogenic contaminants, natural product toxins and endogenous metabolites (Wolfger et al. 2001). The ABC transporter gene (CN809103) of *M. anisopliae* was up-regulated after 1h under RE conditions and presented a maximum FC increase of 2.4 after 12 h (Figures 2-4b and 2-5d). This indicates that this protein may be required to facilitate the efflux of compounds present in RE that may be toxic to the fungus, thus preventing the intracellular accumulation of these substrates.

Rhizosphere competent organisms such as *Trichoderma* spp. are also effective biological control agents of plant diseases caused by soil-borne fungi such as *Rhizoctonia solani*, *Phytium* spp. (Harman et al. 1980, Aziz et al. 1997) and *Sclerotium rolfsii* (Hadar

et al. 1979, Elad et al. 1980). Likewise, *M. anisopliae* has been shown to have antagonistic effects on plant pathogenic fungi, including *Fusarium oxysporum*, *Botrytis cinerea* and *Alternaria solani* (Chul et al. 1996). Thus, *M. anisopliae* may have multiple roles in plant protection and the symbiotic relationships that they can establish with plant communities above and below ground could create a positive feedback in plant productivity.

The rhizosphere competence of *M. anisopliae* and knowledge of the genes involved as it adapts to soil environments, particularly at early time points is important when considering the potential commercial use of biological control agents toward root herbivores and soil-borne plant pathogens. The long term genetic adaptations and evolution of this fungus in cultivated soils (turf plots) is also being studied in our lab (O'Brien 2008). The comprehensive knowledge of the short and long term below-ground interactions between *Metarhizium*, insects, plants and microbial soil populations will reveal ecological links that will help us to understand the molecular cross-talk between them that could ultimately be exploited to benefit plant growth and productivity. Further research is also needed to discern the function of the array of hypothetical/orphan proteins that were differentially expressed in RE in order to elucidate their roles in *M. anisopliae*, and possibly other fungi.

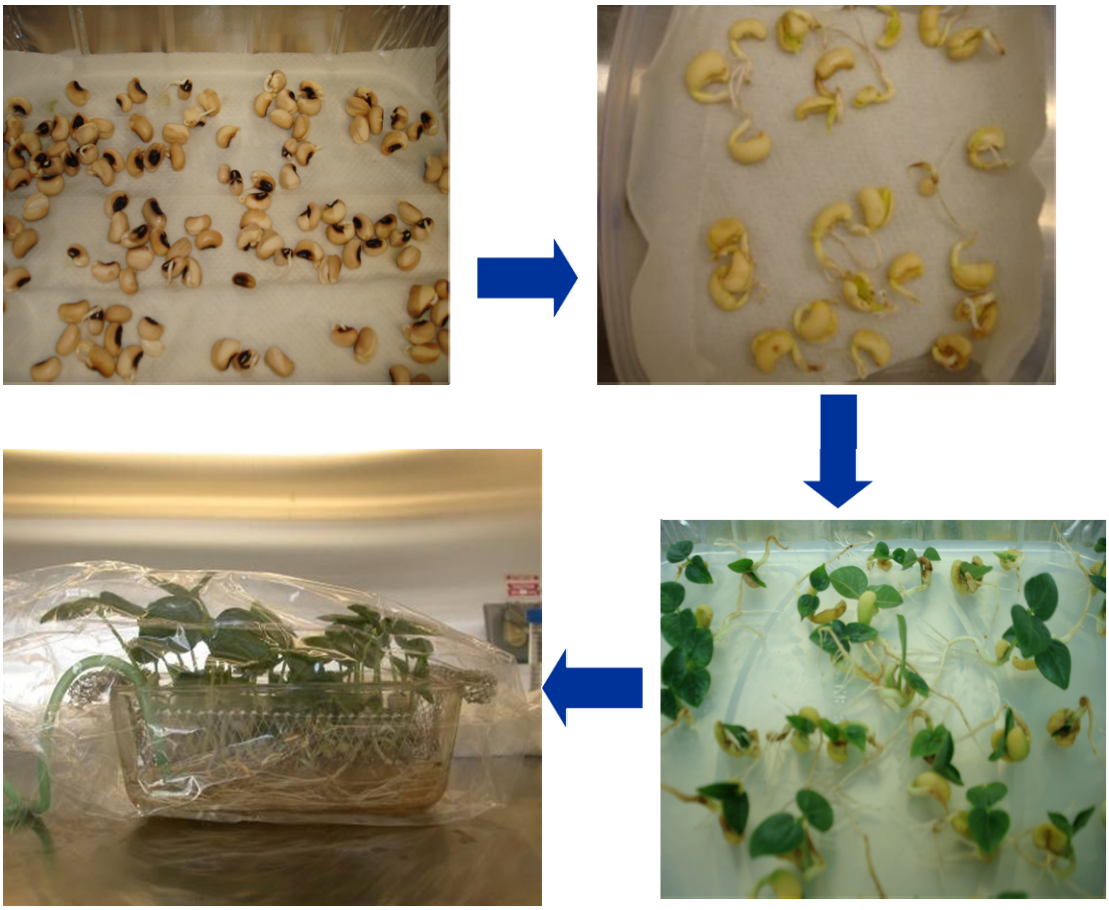

Figure 2-1. Obtention of bean root exudates (RE) from black pea seeds (*Vigna unguiculata* subsp. *unguiculata*) in the lab.

Table 2-1. List of taxa used in this study, strain identification, collection, the host/substrate affiliation and geographic area of origin are included.

Fungi	Strain	Collection*	Host/Substrate	Geographic Origin
Metarhizium anisopliae	ARSEF 2105	ARSEF	Diptera:Ephydridae	Java, Indonesia
Metarhizium anisopliae	ARSEF 2575	ARSEF	Coleoptera: Curculionidae	South Caroline, USA
Metarhizium anisopliae	ARSEF 549	ARSEF	Lepidoptera:Galacticidae	Brazil
Metarhizium anisopliae	ARSEF 1080	ARSEF	Lepidoptera: Noctuidae	Florida, USA
Metarhizium acridium	ARSEF 324	ARSEF	Orthoptera: Acrididae	Queensland, Australia
Metarhizium anisopliae	ARSEF 4600	ARSEF	Diptera: Stratiomyida	Tasmania, Australia
Metarhizium anisopliae	ARSEF 4620	ARSEF	Diptera: Stratiomyida	Tasmania, Australia
Metarhizium anisopliae	ARSEF 2974	ARSEF	Diptera: Culicidae	Buenos Aires, Argentina
Beauveria bassiana	ARSEF 3113	ARSEF	Soil	Iowa, USA
Beauveria bassiana	CENICAFE 9205	CENICAFE	Lepidoptera: Pyralidae	Valle, Colombia
Beauveria bassiana	CENICAFE 9112	CENICAFE	Lepidoptera: Geometridae	Caldas, Colombia
Aspergillus niger	ATCC 10574	ATCC	Culture plate contaminant	Egham, England
Trichoderma harzianum	T22 - ATCC 20847	ATCC	Soil	Not known

***ARSEF**: Agriculture Research Service Entomopathogenic Fungal Collection**; CENICAFE:** National Centre of Coffee Research**;**

ATCC: American Type Culture Collection**.**

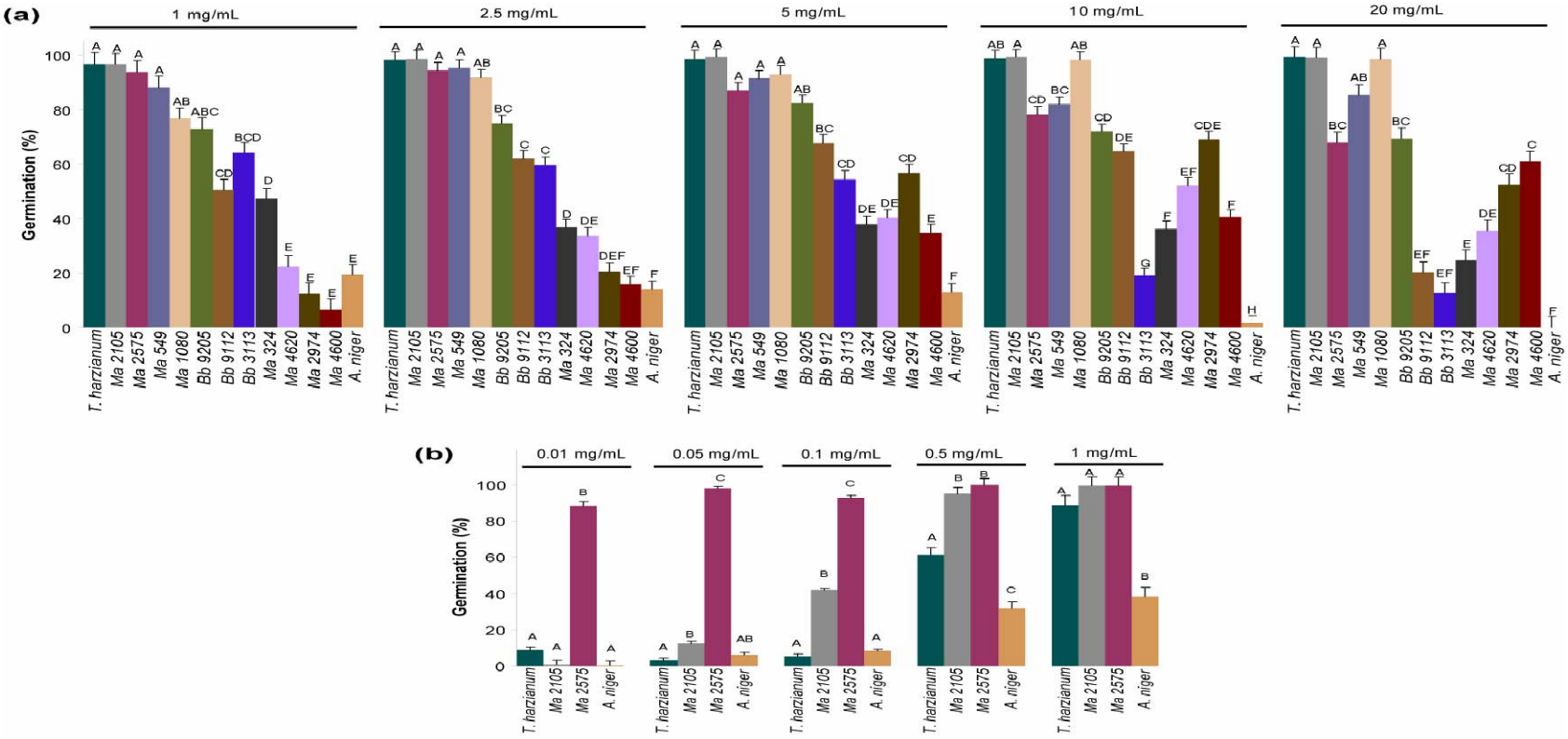

Figure 2-2. Germination of spores of entomopathogenic fungi (genus *Metarhizium,* Ma and *Beauveria,* Bb), *Trichoderma harzianum* (rhizhospheric) and *Aspergillus niger* (non-rhizhospheric) at different concentrations of root exudates (RE). **(a)** Concentrations of RE from 1 to 20 mg/ml. **(b)** concentrations of RE less than 1 mg/ml. Average with the same letter are not significantly different (P<0.01).

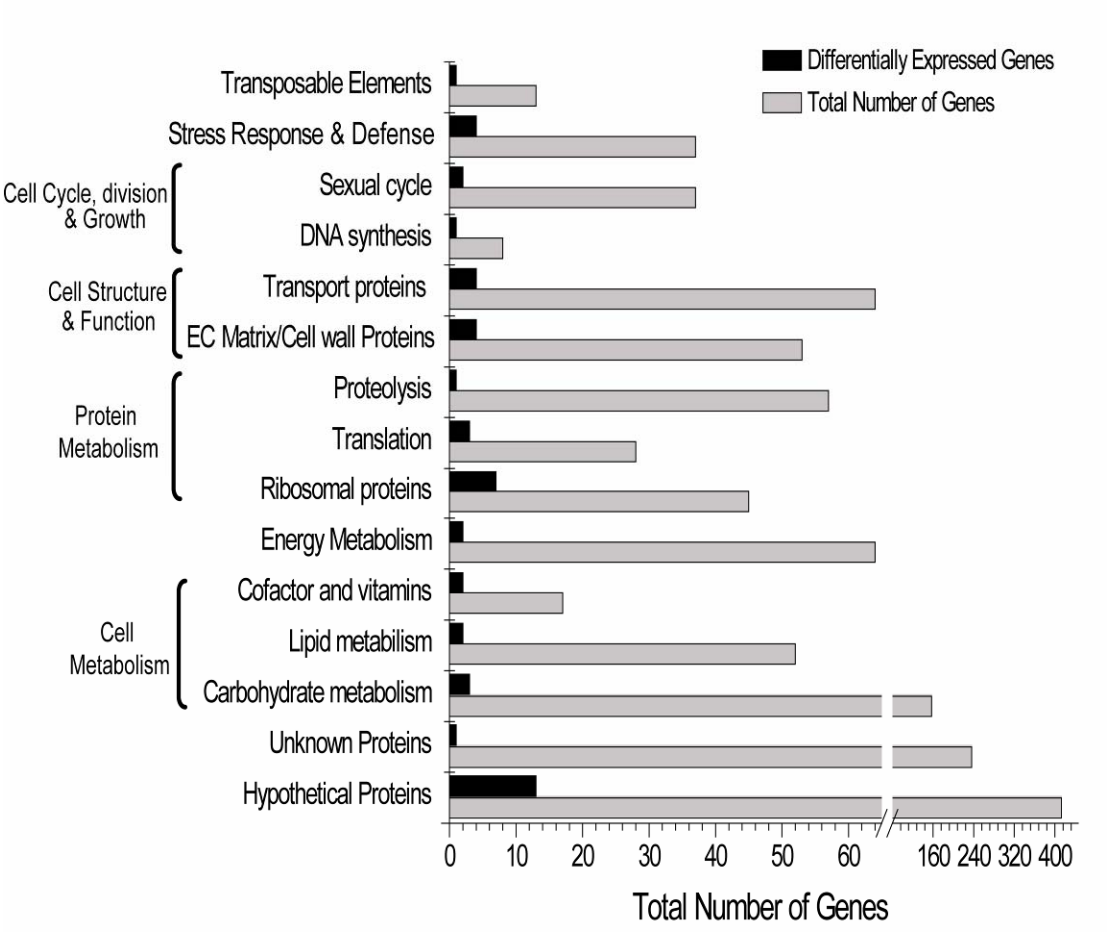

Figure 2-3. Differentially expressed (DE) genes of the entomopathogenic fungus *M. anisopliae* 2575 during growth on RE across all time points evaluated (0, 1, 4, 8 and 12 hours). DE genes are compared to the total number of genes present in each functionally related group.

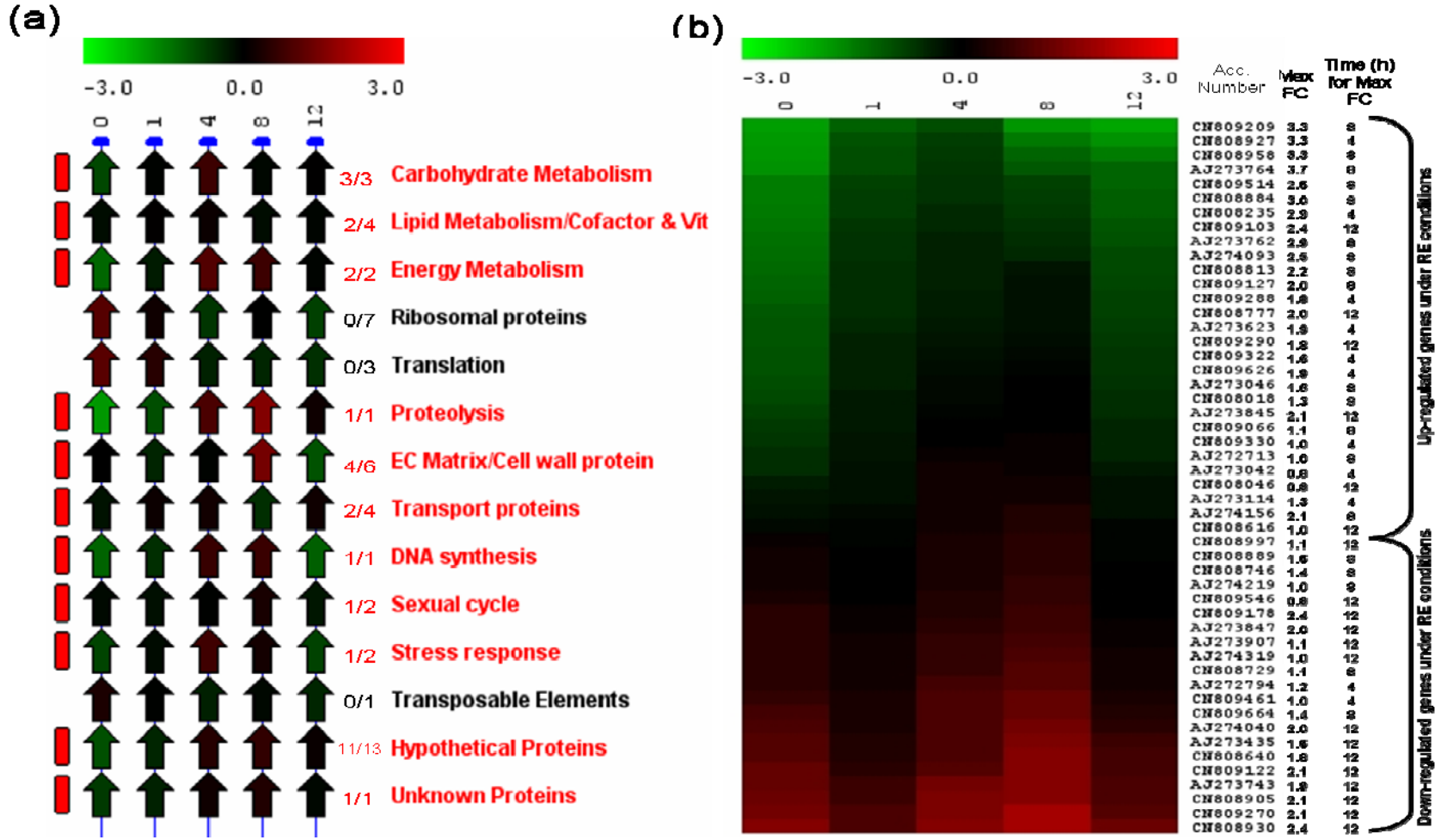

Figure 2-4. Differentially expressed (DE) genes of the entomopathogenic fungus *M. anisopliae* 2575 during growth on RE in a time course (0, 1, 4, 8 and 12 hours) **(a)** linear expression map (LEM) representing the average of the DE genes organized

according to functional groups. Red mark represents the functional groups which average was up-regulated under Root Exudate (RE) conditions. Numbers represent up-regulated genes/total number of DE genes per functional group **(b)** Pattern of expression of the 50 DE genes at all time points evaluated. GenBank accession numbers, the maximum fold change (FC) and the time (hours) when the maximum FC occurred are included.

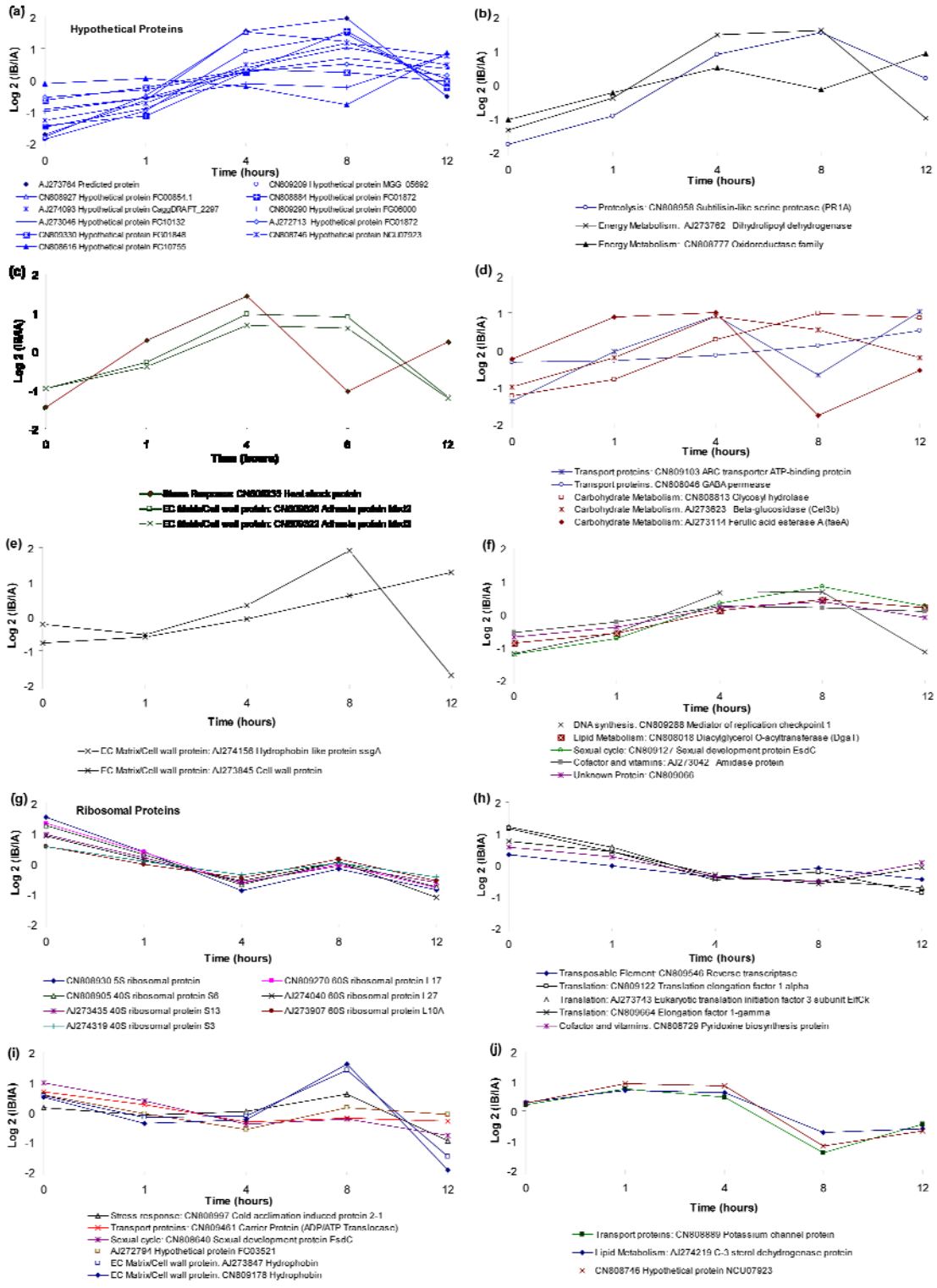

Figure 2-5. Fold change threshold of differentially expressed (DE) genes of the entomopathogenic fungus *M. anisopliae* 2575 while growing on RE on a time course (0,

1, 4, 8 and 12 hours). Figures (a) trough (f) show the threshold of the DE up-regulated genes. Figures (g) through (j) show the threshold of the DE down-regulated genes.

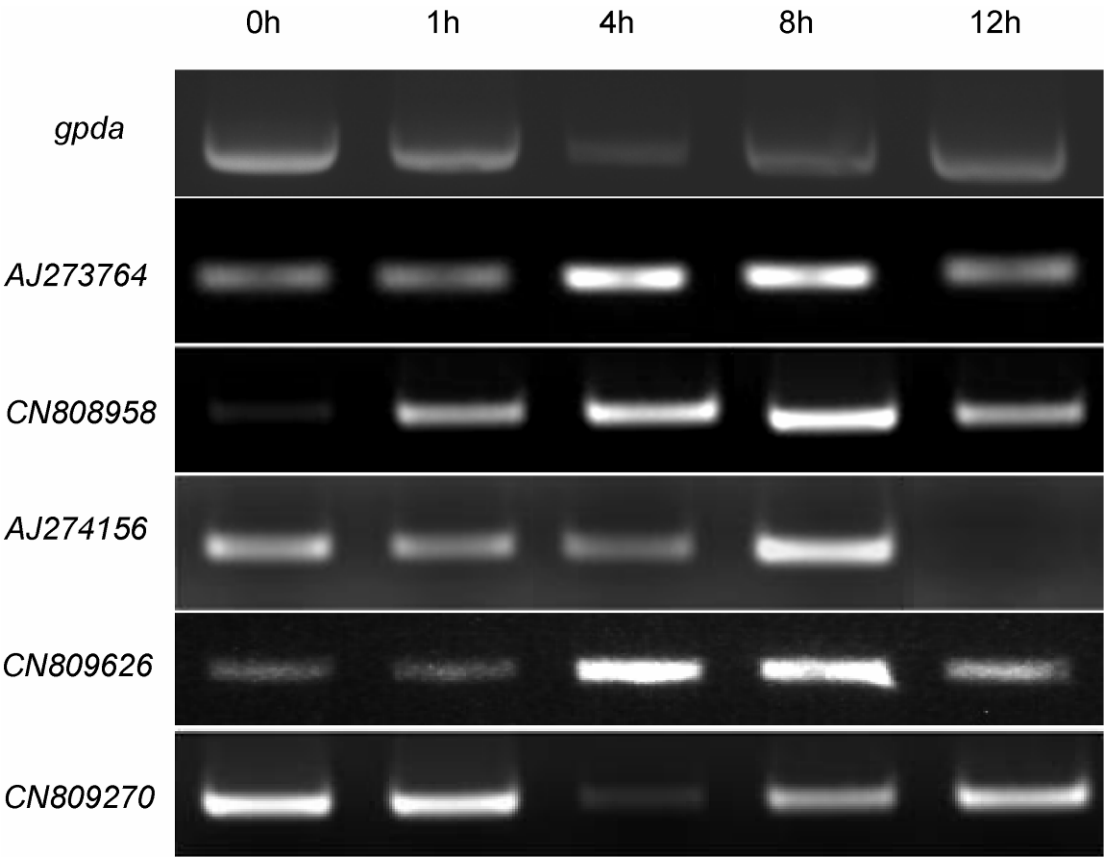

Figure 2-6. Reverse transcriptase PCR (RT-PCR) to validate microarray gene expression of *Metarhizium anisopliae* 2575 growing in root exudates. Five differentially expressed genes were selected: predicted protein (AJ273764), subtilisin-like serine protease PR1A (CN808958), Hydrophobin-like protein (AJ274156), *Metarhizium* adhesin protein Mad2 (CN809626) and a ribosomal protein (CN809270). Glyceraldehyde-3-phosphate dehydrogenase (*gpda*) gene was used as the reference gene.

Table 2-2. Differentially expressed genes of the entomopathogenic fungus *Metrahizium anisopliae* 2575 while growing on root exudates during a time course.

Rank	Accesion No.	EST Library: R=Root C=Cuticle B=Blood	Functional Group	Name	Best Match	E-value	Organism	0 h	1 h	4 h	8 h	12 h	Max. Fold Change	Time (h) to show Max FC	Bayesian Factor
1	AJ273764	C	**Hypothetical Proteins**	Predicted protein	XP_001225688	5.1E-01	*Chaetomium globosum* CBS 148.51	-1.74	-0.65	1.54	1.94	-0.53	**3.7**	8	1.12831E-16
2	AJ273762	C	**Energy Metabolism**	Dihydrolipoyl dehydrogenase	XP_959535	3.0E-47	*Neurospora crassa* OR74A	-1.36	-0.39	1.47	1.59	-1.00	**2.9**	8	6.24732E-13
3	CN809209	R	**Hypothetical Proteins**	Hypothetical protein MGG_05692	XP_360318	6.0E-33	*Magnaporthe grisea* 70-15	-1.88	-0.98	0.89	1.45	-0.23	**3.3**	8	1.25532E-12
4	CN808958	R	**Proteolysis**	Subtilisin-like serine protease PR1A	CAC95049	2.0E-125	*Metarhizium anisopliae* var. *anisopliae*	-1.78	-0.93	0.89	1.54	0.19	**3.3**	8	2.88565E-12
5	CN808927	R	**Hypothetical Proteins**	Hypothetical protein FG00854.1	XP_381030	4.0E-48	*Gibberella zeae* PH-1	-1.81	-0.49	1.52	1.22	0.02	**3.3**	4	9.67705E-12
6	AJ274156	C	**Extracellular Matrix/Cell wall protein**	Hydrophobin-like protein ssgA	P52752	2.0E-41	*Metarhizium anisopliae*	-0.24	-0.54	0.31	1.91	-1.71	**2.1**	8	1.93959E-11
7	CN809178	R	**Extracellular Matrix/Cell wall protein**	Hydrophobin	ABO72628	8.0E-13	*Nomuraea rileyi*	0.49	-0.39	-0.27	1.59	-1.96	**-2.4**	12	2.38741E-11
8	CN808884	R	**Hypothetical Proteins**	Hypothetical protein FG01872	XP_382048	3.0E-33	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	-1.46	-1.14	0.25	1.54	-0.23	**3.0**	8	1.2776E-09
9	CN808235	B	**Stress response**	Heat shock protein	BAE06227	1.5E+00	*Solanum lycopersicum*	-1.45	0.26	1.42	-1.04	0.23	**2.9**	4	1.8846E-08
10	AJ273114	C	**Carbohydrate Metabolism**	Ferulic acid esterase A (faeA)	O42815	3.0E-06	*Aspergillus tubingensis*	-0.25	0.88	1.00	-1.77	-0.56	**1.3**	4	4.69488E-08

Rank	Accesion No.	EST Library: R=Root C=Cuticle B=Blood	Functional Group	Name	Best Match	E-value	Organism	0 h	1 h	4 h	8 h	12 h	Max. Fold Change	Time (h) to show Max FC	Bayesian Factor
11	CN809514	R	**Hypothetical Proteins**	Hypothetical protein NCU01351	XP_961437	6.0E-08	*Neurospora crassa* OR74A	-1.50	-0.91	0.38	1.03	0.47	**2.5**	8	1.0447E-07
12	AJ273847	C	**Extracellular Matrix/Cell wall protein**	Hydrophobin	ABO72628	7.0E-15	*Nomuraea rileyi*	0.55	-0.19	-0.13	1.41	-1.50	**-2.0**	12	1.63555E-07
13	AJ274093	C	**Hypothetical Proteins**	Hypothetical protein CaggDRAFT_2297	ZP_01514615	2.3E-01	*Chloroflexus aggregans* DSM 9485	-1.29	-0.75	0.48	1.16	0.76	**2.5**	8	2.38504E-07
14	CN809103	R	**Transport proteins**	Putative ABC transporter ATP-binding protein	YP_710522	3.3E-02	*Frankia alni* ACN14a	-1.40	-0.05	0.93	-0.68	1.02	**2.4**	12	2.71585E-07
15	CN809626	R	**Extracellular Matrix/Cell wall protein**	Adhesin protein Mad2	ABC65822	5.0E-92	*Metarhizium anisopliae*	-0.97	-0.30	0.95	0.88	-1.18	**1.9**	4	5.17476E-07
16	CN808930	R	**Ribosomal proteins**	5S ribosomal protein	XP_002143231	2.0E-100	*Penicillium marneffei* ATCC 18224	1.52	0.37	-0.90	-0.19	-0.89	**-2.4**	12	5.55366E-07
17	CN809288	R	**DNA synthesis**	Mediator of replication checkpoint 1	NP_594486	6.0E-09	*Schizosaccharomyces pombe* (fission yeast)	-1.18	-0.54	0.67	0.69	-1.13	**1.8**	4	8.73739E-07
18	CN808813	R	**Carbohydrate Metabolism**	Glycosyl hydrolase	ABD49724	7.0E-98	*Metarhizium anisopliae*	-1.25	-0.79	0.28	0.98	0.87	**2.2**	8	1.91653E-06
19	CN809322	R	**Extracellular Matrix/Cell wall protein**	Adhesin protein Mad2	ABC65822	2.0E-136	*Metarhizium anisopliae*	-0.98	-0.41	0.65	0.57	-1.22	**1.6**	4	5.54793E-06
20	CN809270	R	**Ribosomal proteins**	60S ribosomal protein L17	XP_382047	6.0E-89	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	1.33	0.37	-0.66	-0.04	-0.81	**-2.1**	12	2.54178E-05
21	CN808746	R	**Hypothetical Proteins**	Hypothetical protein NCU07923	XP_962439	2.0E-107	*Neurospora crassa* OR74A	0.26	0.92	0.83	-1.18	-0.69	**-1.4**	8	3.00899E-05

Rank	Accesion No.	EST Library: R=Root C=Cuticle B=Blood	Functional Group	Name	Best Match	E-value	Organism	0 h	1 h	4 h	8 h	12 h	Max. Fold Change	Time (h) to show Max FC	Bayesian Factor
22	AJ273845	C	**Extracellular Matrix/Cell wall protein**	Cell wall protein	BAD01559	5.0E-17	*Aspergillus kawachii*	-0.77	-0.60	-0.09	0.60	1.28	**2.1**	12	3.02454E-05
23	CN809127	R	**Sexual cycle**	Sexual development protein EsdC	XP_001262830	2.0E-28	*Neosartorya fischeri* NRRL 181	-1.21	-0.72	0.34	0.83	0.25	**2.0**	8	3.55554E-05
24	CN808905	R	**Ribosomal proteins**	40S ribosomal protein S6	XP_001557655	1.0E-108	*Botryotinia fuckeliana* B05.10	1.27	0.28	-0.71	0.03	-0.78	**-2.1**	12	5.13101E-05
25	CN809122	R	**Translation**	Translation elongation factor 1 alpha	AAR16425	7.0E-146	*Metarhizium anisopliae*	1.17	0.43	-0.48	-0.22	-0.89	**-2.1**	12	0.000105748
26	CN808889	R	**Transport proteins**	Potassium channel protein	ABB90281	4.0E-102	*Gibberella zeae* (anamorph: *Fusarium graminearum*)	0.20	0.74	0.46	-1.43	-0.46	**-1.6**	8	0.000124761
27	AJ274040	C	**Ribosomal proteins**	60S ribosomal protein L27	XP_388032	7.0E-65	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	0.91	0.12	-0.59	0.04	-1.12	**-2.0**	12	0.000146029
28	AJ273743	C	**Translation**	Eukaryotic translation initiation factor 3 subunit EifCk	XP_754692	3.0E-27	*Aspergillus fumigatus* Af293	1.21	0.57	-0.41	-0.51	-0.72	**-1.9**	12	0.000146893
29	CN808777	R	**Energy Metabolism**	Oxidoreductase-short chain family	XP_002152942	3.0E-94	*Penicillium marneffei* ATCC 18224	-1.05	-0.25	0.49	-0.15	0.90	**2.0**	12	0.000225113
30	CN809290	R	**Hypothetical Proteins**	Hypothetical protein FG06000	XP_386176	2.0E-67	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	-1.00	-0.56	-0.11	-0.24	0.79	**1.8**	12	0.000511965
31	AJ273623	C	**Carbohydrate Metabolism**	Beta-glucosidase (Cel3b)	AAP57755	3.0E-74	*Hypocrea jecorina* (*Trichoderma reesei*)	-1.00	-0.21	0.90	0.54	-0.22	**1.9**	4	0.000523778

Rank	Accesion No.	EST Library: R=Root C=Cuticle B=Blood	Functional Group	Name	Best Match	E-value	Organism	0 h	1 h	4 h	8 h	12 h	Max. Fold Change	Time (h) to show Max FC	Bayesian Factor
32	AJ273046	C	**Hypothetical Proteins**	Hypothetical protein FG10132	XP_390308	3.0E-29	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	-0.93	-0.57	0.25	0.69	0.36	**1.6**	8	0.000591119
33	CN808640	B	**Sexual cycle**	Sexual development protein EsdC	XP_001262830	2.0E-28	*Neosartorya fischeri* NRRL 181	0.98	0.36	-0.40	-0.24	-0.79	**-1.8**	12	0.000599879
34	AJ273435	C	**Ribosomal proteins**	40S ribosomal protein S13	XP_387224	1.0E-75	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	0.97	0.18	-0.64	-0.08	-0.64	**-1.6**	12	0.000695675
35	AJ274219	C	**Lipid Metabolism**	C-3 sterol dehydrogenase protein	EDP55243	3.0E-15	*Aspergillus fumigatus* A1163	0.27	0.69	0.62	-0.73	-0.62	**-1.0**	8	0.000985425
36	CN808018	B	**Lipid Metabolism**	Diacylglycerol O-acyltransferase (DgaT)	XP_001267763	3.0E-59	*Aspergillus clavatus* NRRL 1	-0.86	-0.56	0.10	0.46	0.20	**1.3**	8	0.001269504
37	CN808997	R	**Stress response**	Cold acclimation induced protein 2-1	AAY16797	1.0E-05	*Triticum aestivum* (bread wheat)	0.15	-0.11	0.01	0.58	-0.97	**-1.1**	12	0.001514512
38	CN808616	B	**Hypothetical Proteins**	Hypothetical protein FG10755	XP_390931	1.0E-45	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	-0.12	0.05	-0.21	-0.78	0.85	**1.0**	12	0.001526625
39	CN809664	R	**Translation**	Elongation factor 1-gamma	XP_961215	1.0E-77	*Neurospora crassa* OR74A	0.77	0.43	-0.31	-0.59	-0.07	**-1.4**	8	0.001716702
40	AJ273907	C	**Ribosomal proteins**	60S ribosomal protein L10A	AAT74578	1.0E-75	*Chaetomium globosum*	0.55	-0.03	-0.49	0.13	-0.59	**-1.1**	12	0.002696105
41	CN809066	R	**Unknown Proteins**	**Unknown**				-0.68	-0.40	0.20	0.40	-0.09	**1.1**	8	0.002786846
42	CN808729	R	**Cofactor and vitamins**	Pyridoxine biosynthesis protein	XP_001274241	4.0E-87	*Aspergillus clavatus* NRRL 1	0.56	0.26	-0.35	-0.52	0.06	**-1.1**	8	0.003213571

Rank	Accesion No.	EST Library: R=Root C=Cuticle B=Blood	Functional Group	Name	Best Match	E-value	Organism	0 h	1 h	4 h	8 h	12 h	Max. Fold Change	Time (h) to show Max FC	Bayesian Factor
43	AJ272713	C	**Hypothetical Proteins**	Hypothetical protein FG01872	XP_382048	5.0E-44	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	-0.56	-0.30	0.25	0.49	0.15	**1.0**	8	0.003561711
44	AJ274319	C	**Ribosomal proteins**	40S ribosomal protein S3	XP_001555389	7.0E-101	*Botryotinia fuckeliana* B05.10	0.56	0.07	-0.40	0.01	-0.47	**-1.0**	12	0.003683537
45	CN809461	R	**Transport proteins**	Carrier Protein (ADP/ATP Translocase)	XP_386197	2.0E-117	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	0.66	0.24	-0.33	-0.22	-0.31	**-1.0**	4	0.003702196
46	AJ272794	C	**Hypothetical Proteins**	Hypothetical protein FG03521	XP_383697	1.0E-41	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	0.56	-0.06	-0.59	0.15	-0.09	**-1.2**	4	0.004178447
47	CN809330	R	**Hypothetical Proteins**	Hypothetical protein FG01848	XP_382024	3.0E-24	*Gibberella zeae* PH-1 (anamorph: *Fusarium graminearum*)	-0.64	-0.24	0.34	0.24	-0.04	**1.0**	4	0.004453847
48	CN808046	B	**Transport proteins**	GABA (gamma-amino-n-butyrate) permease	XP_746335	1.0E-58	*Aspergillus fumigatus* Af293	-0.33	-0.30	-0.16	0.12	0.51	**0.8**	12	0.005205401
49	CN809546	R	**Transposable Elements**	Reverse transcriptase	ABC24970	1.0E-33	*Monascus pilosus*	0.34	-0.03	-0.38	-0.10	-0.45	**-0.8**	12	0.005796278
50	AJ273042	C	**Cofactor and vitamins**	Amidase protein	ABU75302	5.0E-100	*Ustilaginoidea virens*	-0.55	-0.23	0.24	0.20	0.08	**0.8**	4	0.006030906

Chapter 3: Increased pathogenicity against the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) by *Metarhizium anisopliae* expressing the scorpion toxin (*AaIT*) gene

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3.1 ABSTRACT

Coffee berry borer (CBB) is the Worlds most devastating coffee pest causing an estimated US$500 million worth of losses annually through damage and control costs. *Beauveria bassiana* and *Metarhizium anisopliae* have been employed to control this pest but their low virulence (slow kill and large inoculums) is an important factor constraining their use. *M. anisopliae* (AaIT-Ma549) has been modified to express the scorpion toxin (AaIT) in insect hemolymph and this greatly increased pathogenicity against *Manduca sexta* and *Aedes aegypti*. Here, we demonstrate that AaIT-Ma549 was also dramatically more virulent against CBB, and we provide a much more comprehensive analysis of infection processes and post-mortality development than in the previous research. We evaluated several spore concentrations (10^1 through 10^7 spores/ml) of both the wild type and recombinant strain. At concentrations of 10^1, 10^2 and 10^3 spores/ml, the recombinant strain significantly increased mortality of CBB by 32.2%, 56.6% and 24.6%, respectively. The medial lethal concentration (LC_{50}) was reduced 15.7-fold and the

average survival time (AST) was reduced by 20.1% to 2.98 ± 0.1 days with 10^7 spores/ml. This is the first occasion that an entomopathogenic fungus has been found to kill CBB in less than 3 days. However, AaIT-Ma549 produces significantly fewer spores on cadavers than the parental strain.

Keywords: Coffee; Coffee berry borer; biocontrol; *Metarhizium anisopliae*; AaIT; scorpion neurotoxin, *Androctonus australis* insect toxin.

3.2 INTRODUCTION

Coffee is grown on more than 11 million hectares in over 50 countries in the tropics. Varieties of *Coffea arabica* and *Coffea robusta* are the most important export crops in many countries, especially in South America and East Africa. In 2007, the International Coffee Organization (ICO) reported a world production of 117 millions of exported bags (60-kilo bags) (ICO - International Coffee Organization 2008). Coffee's annual estimated retail value of over US$70 billion makes it the second largest traded commodity in the world after crude oil. Approximately 20 million families throughout the world depend on coffee for their subsistence (Mitchell and Wells 2002).

Coffee plants are subject to attack by many pests and diseases that often cause great damage. Around 900 species of insects feed on coffee plants (leaves, steam, root and/or berries) (Le Pelley 1968, Bustillo et al. 2002). However, the most devastating of these is the coffee berry borer (CBB) *Hypothenemus hampei* Ferrarri (Coleoptera: Curculionidae). CBB's damage to coffee berries regularly causes economic losses ranging from 5% to 25%, and in cases of severe infestation 50% of the crop can be lost (Cardenas and Posada 2001, Wegbe et al. 2003, Durham 2004). CBB costs an estimated US$500 million annually through damage and control methods (Durham 2004).

Because most of the CBB's life cycle is inside the berry, it is particularly difficult to control. The adult females are vulnerable to predators and control agents only while outside the berry. An insecticide (endosulfan) that was an important control agent is being phased out due to its high toxicity to humans and the insect's evolved resistance to the chemical (Brun et al. 1989, Damon 2000). Additionally, the use of chemical pesticides to reduce insect pests raised environmental concerns (Lacey et al. 2001, Phipps

and Park 2002) particularly as coffee is often grown in areas of exceptional biodiversity such as Colombia (Botero and Baker 2001).

Problems with chemical insecticides have stimulated efforts to employ biological control agents, including entomopathogenic fungi (Butt et al. 2001). However, the full potential of biocontrol agents has never been approached. In fact, the employment of all biopesticides represents only about 1.5% of the total crop protection market (Lacey et al. 2001). To date, most research conducted on CBB control has focused on the hyphomycete genera *Metarhizium* and *Beauveria* because their asexual spores (conidia) are relatively easy and cheap to mass produce for field applications. Unfortunately, conventional development of entomopathogenic fungi has been hindered by the perception, often realized, that they kill slowly and are intolerant to common environmental conditions.

Advancements in molecular analysis have stimulated the use of genetic fungal transformation to increase the virulence of entomopathogenic fungi. Wang and St; Leger (2007a) transformed *M. anisopliae* strain 549 to express the 70 aa *Androctonus australis* insect toxin (AaIT) from the Buthid scorpion *Androctonus australis*. The engineered Ma549 strain (named AaIT-Ma549) was tested against both *Manduca sexta* and *Aedes aegypti* and showed higher mortality than the wild type even at an 8-fold lower spore concentration.

The objective of this work is to test if AaIT-Ma549 has an increased virulence against the coffee berry borer as compared with the wild type strain (Ma549). Compared to our previous study (Wang and St Leger 2007a), this paper reports a much more comprehensive analysis of infection processes and post-mortality development e.g. daily

mortality distribution, average survival time (AST), medial lethal concentration (LC_{50}), duration of pathogenic and saprogenic phases and spore production per infected beetle. These parameters are important in evaluating the effectiveness of biocontrol agents since field applications will rely not only on mortality and speed of kill, but also on the ability of the fungus to produce spores and recycle in beetle population.

3.3 MATERIALS AND METHODS

3.3.1 Coffee berry borer

Two-month-old CBB adults were obtained from a mass rearing unit that is held at USDA IBL, Beltsville, MD. CBB adults were taken out from the artificial diet, disinfected for five minutes in a bleach suspension (0.5%), rinsed three times with sterile distilled water and placed in sterile Petri dishes until use.

3.3.2 Fungal strains

Original cultures of the wild type strain *M. anisopliae* 549 (from the ARSEF collection) and the modified strain (AaIT-Ma549) were maintained on Sabouraud dextrose agar (SDA) (Fischer Scientific, Pittsburg, PA).

3.3.3 Germination of fungal spores

Prior to each bioassay, we assessed the percentage of spores capable of germinating. For this, 10 µl spore suspensions (10^5 spores/ml) were incubated at 27ºC on 2.5% Noble agar. After 24 hrs, 3 samples of the agar were placed individually on slides and 100 spores on each sample were counted using light microscopy.

3.3.4 CBB inoculation and incubation

Spores of either Ma549 or AaIT-Ma549 were placed in 10 ml of 0.01% Triton X-100 solution (Sigma Chemical Co., St. Louis, MO), filtered through a cheese cloth and vortexed for 2 minutes. The concentration of spores in each stock suspension was determined with a hemocytometer before preparing a range of inoculums containing 10^1 through 10^7 spores/ml.

Disinfected CBB adults were immersed for two minutes in 10 ml of an inoculum or control (0.01% Triton X-100 solution with no spores). Beetles were then transferred to sterile glass containers containing humid filter paper. The containers were kept in a chamber at 25 ± 2ºC for 21 days. Observations were made daily and the number of dead beetles as well as stages of fungal development on the cadavers (mycelial initiation and sporulation) were recorded.

3.3.5 Experimental Design

The bioassay was set up as a completely randomized design and was performed at two different times with a one month interlude. Four concentrations of spores (10^1, 10^3, 10^5 and 10^7 spores/ml) plus a control containing 0.01% Triton X-100 solution were evaluated first. Seven concentrations of spores (10^1 through 10^7spores/ml) plus a control were evaluated the second time. Each treatment combination (fungal strain by spore concentration) was replicated 40 times (individual beetles in separate vials).

Mortality data was expressed as percentage of mortality (number of dead beetles / total number of beetles per treatment) after 21 days of evaluation of the bioassay and the data was analyzed using the SAS GLIMMIX procedure (SAS Institute Inc. 2006).

Statistical significance among fungal strains and spore concentrations were calculated using the Tukey multiple mean comparison test.

Daily mortality data were analyzed using the survival/reliability (Kaplan-Meier) method of censored data of the JMP 7 software (SAS Institute Inc. 2006) where individuals that survive were censored with a value of one. After inoculating CBB adults with fungal spores, the time of death for each individual beetle was registered. Percentage of individuals that died each day was calculated.

The AST of each treatment combination (fungal strain and concentration) was estimated using the Kaplan-Meier method of censored data of the JMP 7 software. A censored value of one was assigned to live beetles. Statistical differences were calculated between fungal strains at each spore concentration using the Chi square (Wilcoxon) test ($P>0.05$).

The LC_{50} (the number of spores required to kill 50% of CBB populations) was obtained at each treatment combination and was calculated with the PROC Probit procedure of the SAS 9.1.3 software.

The pathogenic and saprogenic phases of the fungus on the insect were followed. The pathogenic phase covers the number of days between infection with spores and the death of the insect. The saprogenic phase was sub-divided into: (1) time from death until mycelium is first observed on the insect cadaver; (2) time from the initial appearance of mycelium until the total coverage of the cadaver; (3) time from total mycelial coverage until conidiophores appear, and (4) time from the first observation of conidiophores until spores are released.

In order to evaluate the production of spores per beetle, five vials containing dead beetles were randomly selected from each treatment combination. Insects were vortexed separately for two minutes in two ml of 0.01% triton X-100. Released spores were counted using a hemocytometer.

Data collected from germination of fungal spores, saprogenic and pathogenic cycle and from spore production on insect cadavers were analyzed using the SAS MIXED procedure and significance among treatments was calculated using the Tukey multiple mean comparison test procedure.

3.4 RESULTS

Transformation of the AaIT construct into Ma 549 did not affect the germination rate of the fungus after 24 h and we did not find any morphological differences among germ tubes of both wild type and engineered strains.

CBB mortality was >96% for both strains at concentrations $>10^4$ spores/ml with no significant differences between the strains. However, at lower spore concentrations, AaIT-Ma549 significantly increased mortality by 32.2% (10^1 spores/ml), 56.6 % (10^2 spores/ml) and 24.6% (10^3spores/ml) (Figure 3-1) ($P<0.0003$).

Figure 2a-g shows that at all spore concentrations daily mortalities of CBB populations treated with AaIT-Ma549 are higher, with mortality being initiated from one to three days earlier than in the CBB population treated with the wild type strain.

Three days post-inoculation with 10^7 spores/ml, 75% of the CBB population infected with AaIT-Ma549 were dead as compared to 51.3% of the population infected with the wild type fungus (Figure 3-2a). With 10^6 spores/ml, 57.5% of CBB infected

with AaIT-Ma549 were dead by day 4 as compared to 27.5% of the population infected with the wild type strain (Figure 3-2b). AaIT-Ma549 (10^5 spores/ml) killed 5% of the CBB population by day 3, 12.5% by day 4 and 23.8% by day 5 as compared to 1.3%, 10% and 13.8% mortality achieved by the wild type (Figure 3-2c). The mortality of beetles infected with 10^4 AaIT-Ma549 spores/ml commenced 3 days earlier than beetles infected with the wild type strain, and 35% of insects infected with AaIT-Ma549 where dead by day 6 as compared to 10% of insects infected with the wild type (Figure 3-2d). At 10^3 spores/ml, AaIT-Ma549 had killed 10% of the CBB by day six, 22% by day seven and killed an additional 22.5% on day eight. In contrast, the wild-type strain had killed just 2.5%, 5% and 11.3% of the CBB by days six, seven and eight, respectively (Figure 3-2e).

Significant differences in the AST were observed between the wild type and AaIT-Ma549 at concentrations between 10^3 and 10^7 spores/ml ($P \leq 0.003$) (Table 3-1). With 10^7 wild-type spores/ml, 50% mortality was reached in 3.73±0.1 days as compared to 2.98±0.1 days with AaIT-Ma549. Thus, AaIT-Ma549 reduced the AST by 20.1% at the highest spore concentration we evaluated. The percent decrease in AST achieved by AaIT-Ma549 is not inversely proportional to spore concentration. The largest decrease in AST (25.1%) by AaIT-Ma549 was obtained with 10^3 spores/ml with AST being significantly reduced from 11.18±0.4 days to 8.37±0.3 days. At concentrations of 10 and 100 spores/ml AaIT-Ma549 produced no significant reduction in AST. The smallest percent reduction of AST (4.6%) was observed with 10 spores/ml (Figure 3-3).

Results for the probit analyses for LC_{50} are shown in Figure 3-4. The number of spores required to kill 50% of the broca is reduced from $5.3x10^2$ wild type spores/ml to

3.4 x10^1 AaIT-Ma549 spores/ml. Thus, it takes 15.7-fold fewer conidia of AaIT-Ma549 to provide the same level of control. Ninety-five percent interval ranges are from 8 to 91 AaIT-Ma549 spores/ml and from 1.7 x10^2 to 1.7 x10^3 wild type spores/ml (Table 3-2). The LC_{90} is reduced from 4.9 x10^3 wild type spores/ml to 8.6 x10^2 AaIT-Ma549 spores/ml, a 5.7-fold reduction (Table 3-2).

Table 3-3 shows the average duration (in days) ± standard error (SE) of the pathogenic and saprogenic phases of CBB infected with wild type or AaIT-Ma549 at each spore concentration. The longest time to complete both phases was 15.7 ± 0.4 days demonstrated by beetles treated with the wild type at a concentration of 10^3 spores/ml. Interestingly, CBB infected with 10 wild type spores/ml demonstrated the longest pathogenic phase (10 days), but subsequently had the shortest time for completing the saprogenic phase (5.3 days).

Figure 3-5 shows that the saprogenic and pathogenic phases of the CBB treated with 10^7 spores/ml lasted 9.2 ± 0.1 days using AaIT-Ma549 and 9.9 ± 0.2 days using the wild-type. The pathogenic phase of AaIT-Ma549 treated beetles was 1.2 days shorter than those infected with the wild type but the time course of the saprogenic phase of AaIT-Ma549 did not change (doted lines, Figure 3-5).

Production of spores per beetle was assessed with CBB treated with 10^7 spores/ml. The average spore production by wild type Ma549 on cadavers was significantly higher (2 x10^6 spores/ml) than that of AaIT-Ma549 (1.2 x10^6 spores/ml) ($P < 0.0001$).

3.5 DISCUSSION

Although the speed of kill is an important parameter when selecting effective biocontrol agents, it is not the only one that needs to be assessed to evaluate the potential utility of fungal pathogens. Comprehensive studies that include other variables (e.g. daily mortality distribution, AST, LC_{50}, duration of the pathogenic and saprogenic phases and spore production per infected beetle) are necessary under lab conditions and field applications in order to fully comprehend the effects of naturally occurring and genetically modified fungal strains on insect pests. These variables also facilitate the evaluation of environmental and economical implications of releasing fungal biocontrol agents into the field.

Previous lab and field evaluations of entomopathogenic fungi to control CBB infestations include the use of *B. bassiana* (Haraprasad et al. 2001, Posada et al. 2002, Posada and Vega 2005) and *M. anisopliae* (Bernal et al. 1994, De la Rosa et al. 1995, Bustillo et al. 1999). Laboratory bioassays of several strains of *M. anisopliae* achieved mortalities of CBB between 32.5% and 95% with10^7 spores/ml (Bernal et al. 1994). Here, we report that even wild type Ma549 achieved mortalities >96% at concentrations $>10^4$ spores/ml. However, AaIT-Ma549 significantly increased mortality with low concentrations of spores (10^1 through 10^3 spores/ml) (Figure 3-1). These results highlight the importance of evaluating different concentrations of spores when comparing the virulence of fungal strains.

De la Rosa et al. (1995) reported LC_{50} values for *M. anisopliae* against CBB that ranged from $4.2x10^6$ spores/ml (strain Ma4) to $1.3x10^7$ spores/ml (strain Ma10). The 15.7 fold reduction in the LC_{50} achieved by AaIT-Ma549 as compared with the wild type

strain should reduce costs of production. To achieve 80% mortality in field applications requires $5x10^{11}$ spores/ha (10^8 spores/tree and 5000 tree/ha on average) (De la Rosa et al. 2000). Based on our lab study, to reach the same level of mortality, AaIT-Ma549 would require $4.3x10^8$ spores/ha (95% up interval).

As well as reducing inoculum loads, expressing AaIT also reduced time to kill. Thus, while both Ma-549 and AaIT-Ma549 at $>10^4$ spores/ml achieved >96% mortalities by 21 days, daily mortality distributions indicate that the modified strain kills CBB earlier (Figure 3-2 a-g). Rapid kill could obviously avoid important economical losses on agricultural crops. Bernal et al (1994) reported that the AST of CBB treated with several strains of *M. anisopliae* at 10^7 spores/ml ranged from 3.4 days (isolate Ma9101) to 5.7days (isolate Ma9107). Samuels et al (2002) reported an AST of 3.4 days when CBB adults were infected with *B. bassiana* (isolate LPP1) at 10^7 spores/ml. The AST of CBB treated with of 10^7 wild type spores/ml is within the range reported by Bernal et al (1994) (3.73 days). The engineered strain reached the AST in 2.97±0.18 days; the first report of an entomopathogenic fungus killing broca in less than 3 days.

The duration of the pathogenic and saprogenic phases of CBB infected with *B. bassiana* at 1 $x10^7$ spores/ml was reported by Posada and Vega (2005). Comparing 50 strains, they found that the shortest time to complete both pathogenic and saprogenic phases was 5.6±1.4 days (strain togo-Gbadi Gaodo-5453) and the longest time was 15.1±1.2 days (strain I.coast-Ayenoua-5482). *M. anisopliae* is less virulent to CBB than *B. bassiana* (Bernal et al. 1994, De la Rosa et al. 2000). Here, we report that the duration of the pathogenic and saprogenic phases of CBB infected with 10^7 spores/ml AaIT-Ma549 is 9.2±0.1 days. Thirty of the *B. bassiana* strains evaluated by Posada and Vega

(2005) took longer than this, suggesting that the *AaIT* gene could potentially also improve the pathogenic performance of *B. bassiana* strains.

Even though the modified strain is more virulent, the wild type strain produces significantly more spores on cadavers. Rapid kill by fungal strains could reduce their ability to exploit host tissues for spore production (St. Leger et al. 1992b). A quantification of spore production on dipteran and lepidopteran hosts was not performed by Wang and St. Leger (2007) so the relation of our data to other pathogen–host systems is unclear. The fact that the modified strain produced significantly lower amounts of spores on beetle cadavers is potentially an important environmental feature that could reduce rates of dispersal.

AaIT has already provided the most promising recombinant baculoviruses (Zlotkin et al. 2000), with improved performance against lepidopteran larvae in several field trials (Sun et al. 2002). However, many insects not susceptible to baculoviruses are targeted by *M. anisopliae*. These studies are providing an opportunity to diversify the deployment of this useful, very well studied toxin, which like *M. anisopliae* has already passed many regulatory hurdles. Social acceptance of transgenic organisms involves issues that will also have to be addressed for many technologies under consideration. It presumably would depend on the properties of the fungus we develop, and may require mitigating technologies to block the pathogens spread and gene flow to other microbes. However, if transgene expression should allow a fungus to rapidly deplete pest populations then the technology would likely seem more acceptable to people whose livelihood is threatened. In the end, it will be their choice.

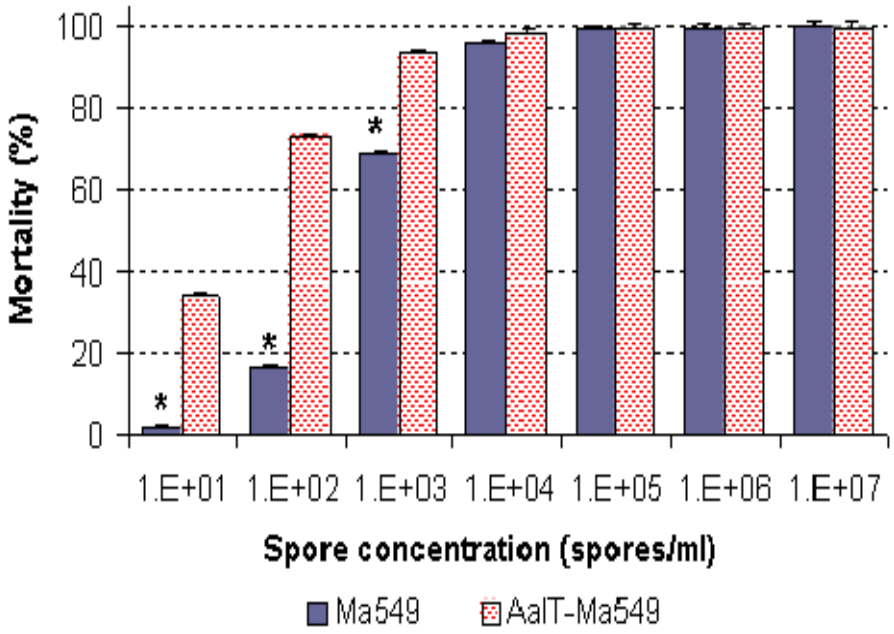

Figure 3-1. Mortality of coffee berry borer adults 21 days after challenged with *Metarhizium anisopliae* Ma549 wild type and the modified isolate AaIT-Ma549 at seven spore concentrations. Control group was treated with no spores. Significant differences ($P < 0.0003$) are denoted with * (Tukey multiple mean comparison test).

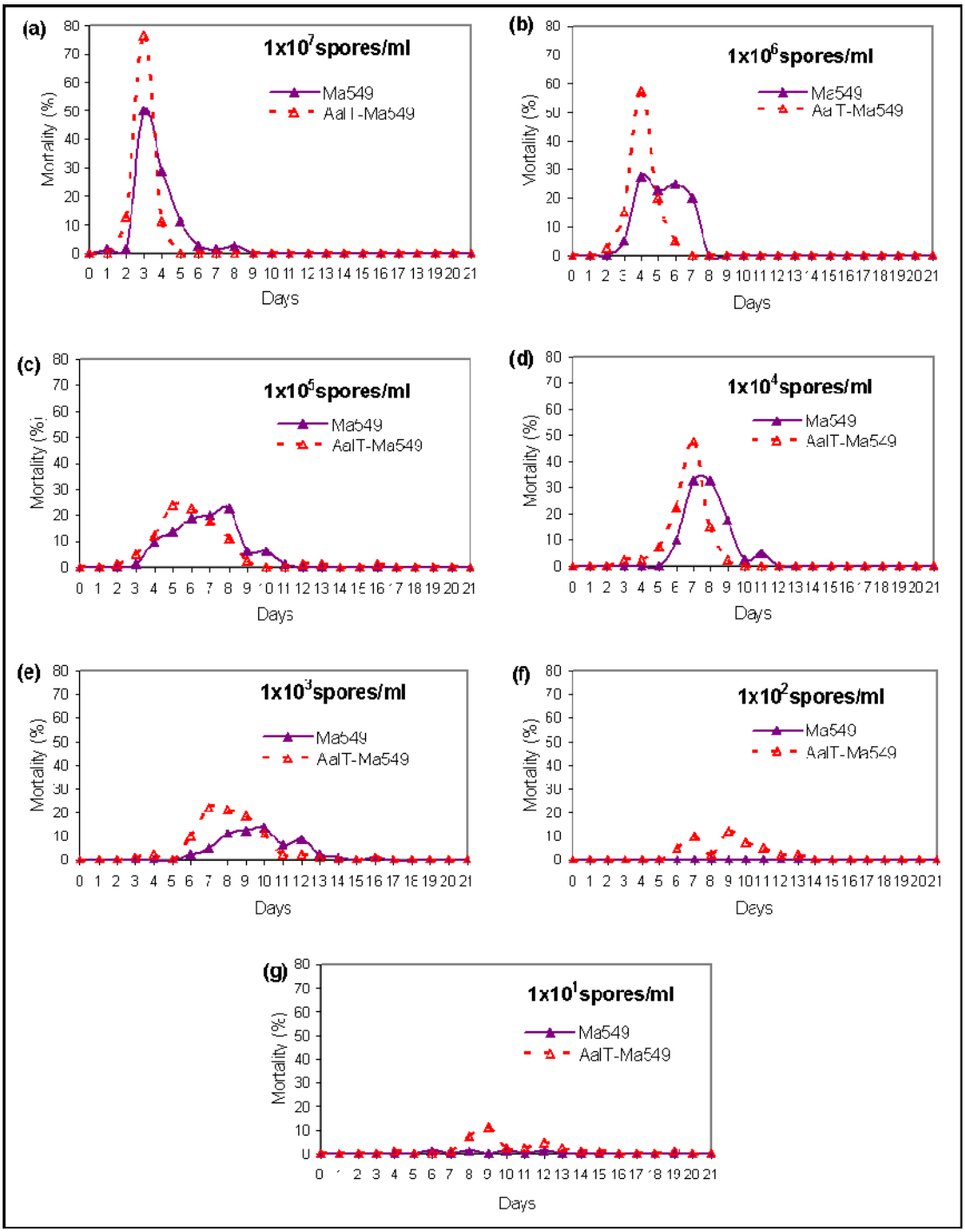

Figure 3-2. Daily mortality distribution of coffee berry borer challenged with Ma549 wild type and AaIT-Ma549 at seven spore concentrations. Control presented no mortality due to fungal spores, hence is not included.

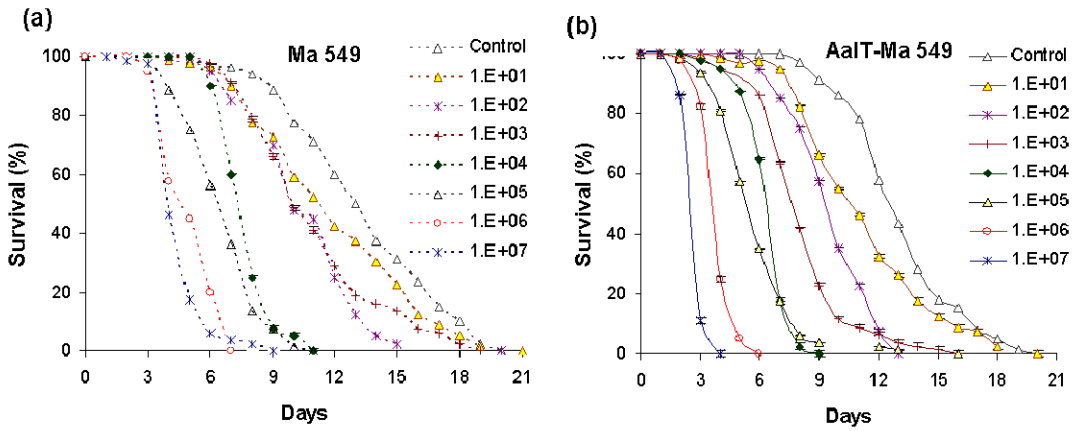

Figure 3-3. Average survival time (AST) for Ma549 and AaIT-Ma549 on coffee berry borer adults at seven spore concentrations. (a) Ma549, (b) AaIT-Ma549. Controls are CBB adults treated with 0.01% Triton X-100 containing no spores.

Table 3-1. Average Survival time (AST) of Ma549 and AaIT-Ma549 at several spore concentrations.

Spore Concentration	N	Ma 549			AaIT-Ma549			Chi Square (Wilcoxon Test)	DF	Prob>Chi Square	% reduction
		Mean (days)	±	SE	Mean (days)	±	SE				
Control	80	13.53	±	0.4	13.29	±	0.3	0.30	1	0.583	
10^1	80	12.05	±	0.4	11.50	±	0.4	3.28	1	0.070	4.6
10^2	40	10.83	±	0.4	9.78	±	0.3	3.28	1	0.070	9.7
10^3	80	11.18	±	0.4	8.37	±	0.3	37.09	1	<0.001	25.1
10^4	40	7.88	±	0.2	6.65	±	0.2	18.20	1	<0.001	15.6
10^5	80	6.78	±	0.2	6.08	±	0.2	8.78	1	0.003	10.3
10^6	40	5.18	±	0.2	4.10	±	0.1	14.26	1	<0.001	20.8
10^7	80	3.73	±	0.1	2.98	±	0.1	28.68	1	<0.001	20.1

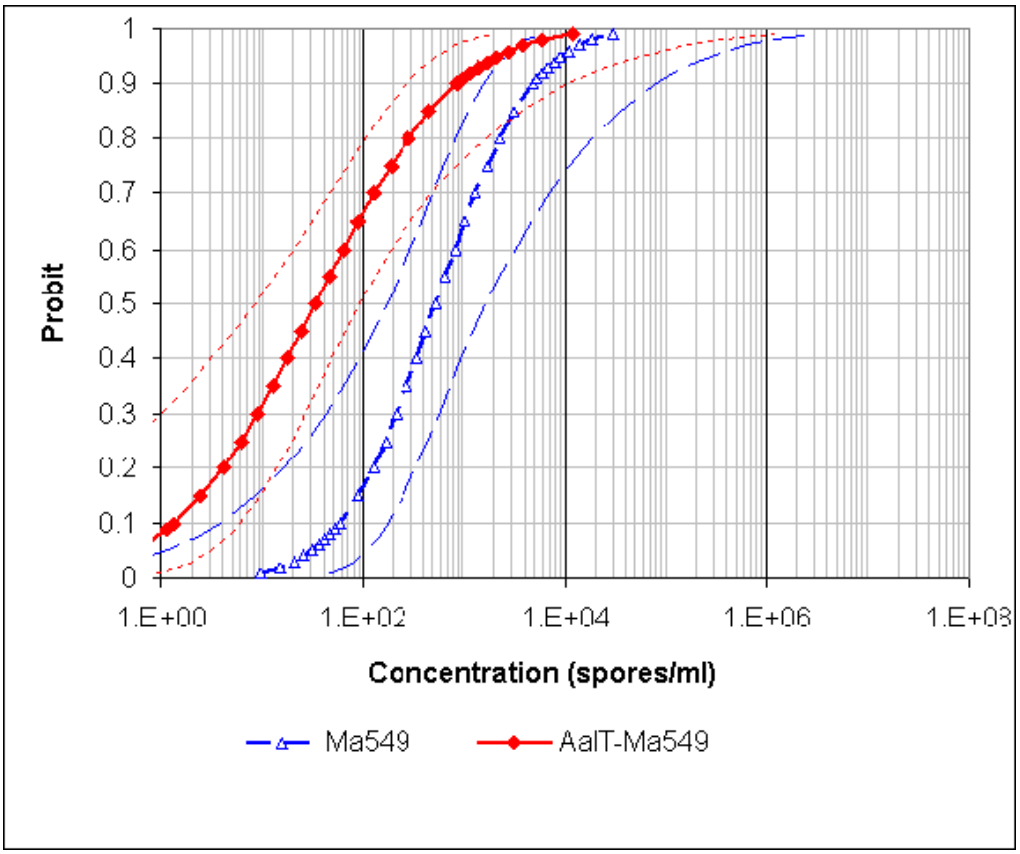

Figure 3-4. Mortality response of coffee berry borer to different spore concentrations of Ma549 wild type and AaIT-Ma549. Doted lines represent 95% coefficient intervals.

Table 3-2. Probit analysis of Ma549 and AaIT-Ma549.

Prob	Ma 549 Chi Sq=19.09; P value=<0.0001			AaIT-Ma549 Chi Sq=24.02; P value=<0.0001			FOLD CHANGE	Spore Reduction
	Dose	95% Down	95% Up	Dose	95% Down	95% Up		
0.01	9.5E+00	1.0E-01	4.6E+01	9.5E-02	2.8E-04	8.9E-01	99.9	9.4E+00
0.02	1.5E+01	2.7E-01	6.5E+01	1.9E-01	1.0E-03	1.5E+00	80.4	1.5E+01
0.03	2.0E+01	4.9E-01	8.0E+01	2.9E-01	2.2E-03	2.0E+00	70.1	2.0E+01
0.04	2.6E+01	7.7E-01	9.5E+01	4.1E-01	4.1E-03	2.5E+00	63.2	2.5E+01
0.05	3.1E+01	1.1E+00	1.1E+02	5.3E-01	6.6E-03	3.0E+00	58.1	3.0E+01
0.06	3.6E+01	1.5E+00	1.2E+02	6.7E-01	1.0E-02	3.6E+00	54.1	3.5E+01
0.07	4.1E+01	2.0E+00	1.4E+02	8.1E-01	1.4E-02	4.2E+00	50.8	4.0E+01
0.08	4.7E+01	2.5E+00	1.5E+02	9.7E-01	2.0E-02	4.7E+00	48.0	4.6E+01
0.09	5.2E+01	3.1E+00	1.6E+02	1.1E+00	2.6E-02	5.3E+00	45.6	5.1E+01
0.1	5.8E+01	3.8E+00	1.8E+02	1.3E+00	3.5E-02	6.0E+00	43.5	5.6E+01
0.15	8.8E+01	8.6E+00	2.5E+02	2.5E+00	1.1E-01	9.5E+00	35.8	8.6E+01
0.2	1.2E+02	1.6E+01	3.4E+02	4.0E+00	2.5E-01	1.4E+01	30.7	1.2E+02
0.25	1.7E+02	2.7E+01	4.4E+02	6.2E+00	5.3E-01	1.9E+01	26.9	1.6E+02
0.3	2.1E+02	4.3E+01	5.7E+02	9.0E+00	1.0E+00	2.7E+01	23.9	2.1E+02
0.35	2.7E+02	6.4E+01	7.4E+02	1.3E+01	1.8E+00	3.6E+01	21.4	2.6E+02
0.4	3.4E+02	9.1E+01	9.6E+02	1.8E+01	3.1E+00	4.8E+01	19.2	3.3E+02
0.45	4.3E+02	1.3E+02	1.3E+03	2.5E+01	5.2E+00	6.6E+01	17.4	4.0E+02
0.5	**5.3E+02**	**1.7E+02**	**1.7E+03**	**3.4E+01**	**8.5E+00**	**9.1E+01**	**15.7**	**5.0E+02**
0.55	6.6E+02	2.3E+02	2.3E+03	4.6E+01	1.3E+01	1.3E+02	14.2	6.1E+02
0.6	8.2E+02	3.0E+02	3.2E+03	6.4E+01	2.1E+01	1.9E+02	12.9	7.6E+02
0.65	1.0E+03	3.8E+02	4.7E+03	8.9E+01	3.1E+01	2.9E+02	11.6	9.5E+02
0.7	1.3E+03	5.0E+02	7.0E+03	1.3E+02	4.7E+01	4.8E+02	10.4	1.2E+03
0.75	1.7E+03	6.4E+02	1.1E+04	1.9E+02	6.9E+01	8.5E+02	9.2	1.5E+03
0.8	2.3E+03	8.3E+02	1.9E+04	2.8E+02	1.0E+02	1.7E+03	8.1	2.0E+03
0.85	3.2E+03	1.1E+03	3.5E+04	4.6E+02	1.6E+02	3.7E+03	6.9	2.7E+03
0.9	4.9E+03	1.6E+03	8.0E+04	8.6E+02	2.7E+02	1.1E+04	5.7	4.0E+03
0.91	5.4E+03	1.7E+03	9.8E+04	1.0E+03	3.0E+02	1.4E+04	5.4	4.4E+03
0.92	6.1E+03	1.9E+03	1.2E+05	1.2E+03	3.4E+02	1.9E+04	5.2	4.9E+03
0.93	6.9E+03	2.0E+03	1.5E+05	1.4E+03	3.9E+02	2.6E+04	4.9	5.4E+03
0.94	7.9E+03	2.3E+03	2.0E+05	1.7E+03	4.6E+02	3.6E+04	4.6	6.1E+03
0.95	9.2E+03	2.5E+03	2.8E+05	2.2E+03	5.5E+02	5.5E+04	4.3	7.0E+03
0.96	1.1E+04	2.9E+03	4.0E+05	2.8E+03	6.7E+02	8.8E+04	3.9	8.2E+03
0.97	1.4E+04	3.4E+03	6.2E+05	3.9E+03	8.5E+02	1.6E+05	3.5	9.9E+03
0.98	1.9E+04	4.3E+03	1.1E+06	6.1E+03	1.2E+03	3.5E+05	3.1	1.3E+04
0.99	3.0E+04	6.0E+03	2.9E+06	1.2E+04	1.9E+03	1.2E+06	2.5	1.8E+04

Table 3-3. Pathogenic and saprogenic phases of the CBB. The statistics were performed estimating the average and standard error for the data that were completed in all the development stages of the fungus through its pathogenic and saprogenic phases (Mean in days).

Spore concentration (spores/ml)	Fungal isolate	N	Pathogenic phase		Saprogenic phase								Total Saprogenic phase (Death - Spore release)		Total Saprogenic and Pthogenic phases (Inoculation - Spore release)	
			Inoculation -Death		Death – Mycelium initiation		Mycelium initiation – Total mycelium coverage		Total mycelium coverage - Spores formation		Spore formation - Spores release					
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
10^1	Ma549	3	10.0	1.0	1.5	0.0	1.5	0.5	1.0	0.0	1.3	0.3	5.3	0.8	15.3	0.3
	AaIT-Ma549	22	9.1	0.5	1.4	0.2	2.1	0.3	1.0	0.1	1.8	0.2	6.2	0.4	15.3	0.6
10^3	Ma549	45	9.5	0.4	1.4	0.2	1.9	0.2	1.0	0.0	1.7	0.1	6.1	0.3	15.7	0.4
	AaIT-Ma549	68	8.1	0.3	1.3	0.1	1.9	0.2	0.9	0.1	1.8	0.1	5.9	0.3	14.0	0.4
10^4	Ma549	40	7.9	0.2	1.3	0.1	2.1	0.2	1.0	0.0	1.8	0.1	6.1	0.2	14.0	0.2
	AaIT-Ma549	39	6.5	0.2	1.1	0.1	2.0	0.2	1.0	0.1	1.7	0.1	5.7	0.2	12.4	0.2
10^5	Ma549	78	6.7	0.2	1.2	0.1	1.7	0.2	0.9	0.0	1.8	0.1	5.6	0.2	12.4	0.2
	AaIT-Ma549	77	5.9	0.3	1.2	0.1	1.7	0.2	0.9	0.0	2.0	0.0	5.8	0.2	11.7	0.3
10^6	Ma549	40	5.1	0.2	1.0	0.1	1.9	0.2	1.0	0.1	1.8	0.1	5.7	0.2	10.8	0.2
	AaIT-Ma549	40	4.0	0.1	1.0	0.1	1.9	0.1	1.0	0.1	2.0	0.0	5.8	0.1	9.9	0.1
10^7	Ma549	78	3.7	0.2	1.1	0.1	2.1	0.2	1.0	0.0	2.0	0.1	6.2	0.2	9.9	0.2
	AaIT-Ma549	75	2.9	0.1	1.0	0.1	2.2	0.1	1.0	0.1	2.1	0.1	6.2	0.1	9.2	0.1

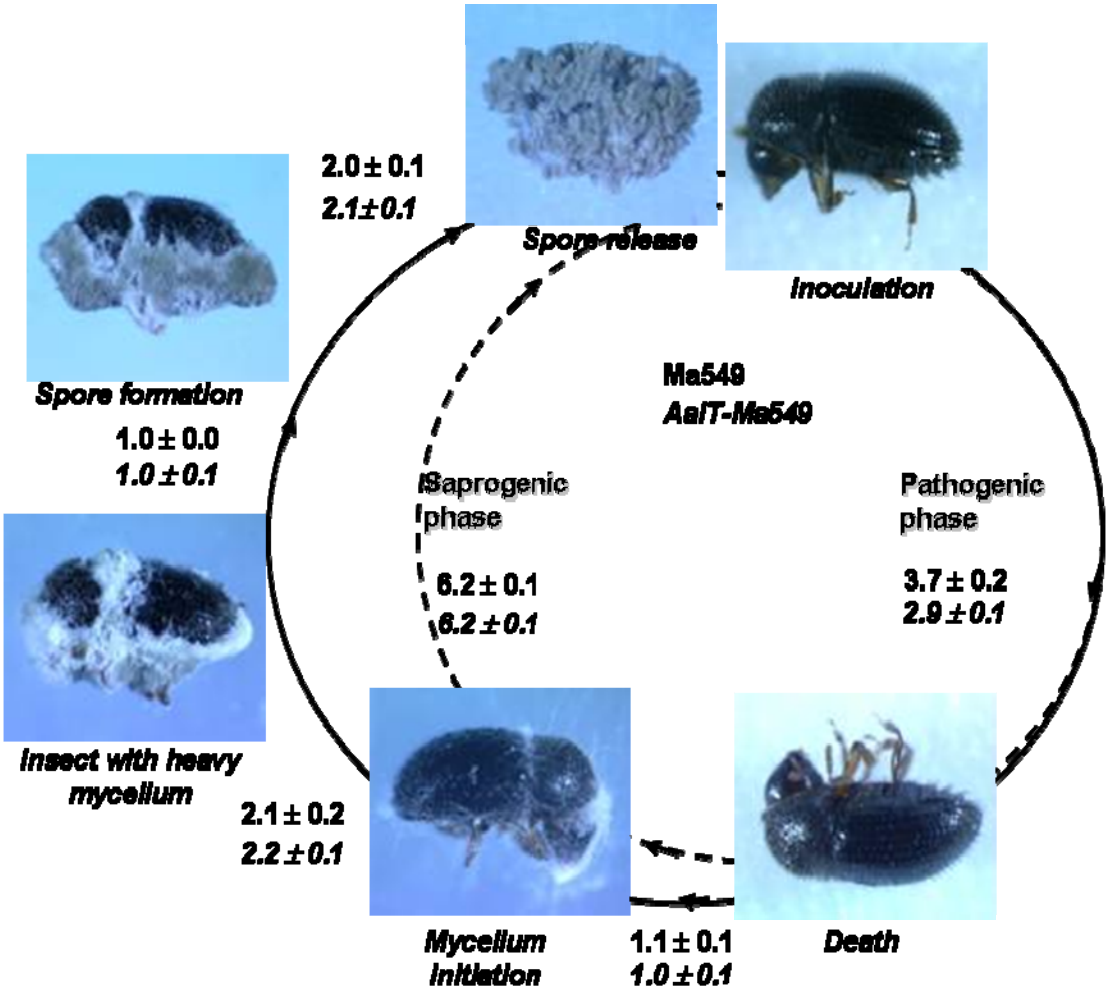

Figure 3-5. Duration (in days) of pathogenic and saprogenic phases of Ma549 and AaIT-Ma549 (italic font) infecting coffee berry borer at a concentration of 10^7 spores/ml.

Chapter 4: Single and double transformations of *Beauveria bassiana* with *M. anisopliae* esterase (*Mest1*) or/and scorpion toxin (*AaIT*) genes increases pathogenicity against the Colorado potato beetle *Leptinotarsa decemlineata*

4.1 ABSTRACT

Despite some successes, the use of entomopathogenic fungi as alternatives to chemical insecticides has not occurred on a very large scale due to their slow speed of kill and a requirement for large inoculums. Using the fungus as a delivery vehicle for foreign toxins represents a powerful approach for improving their pathogenicity. We inserted the *M. anisopliae AaIT* gene into three strains of *Beauveria bassiana* with high (ARSEF 252), medium (ARSEF 8998) and low (ARSEF 9184) mortality against the Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) (Coleoptera: Chrysomelidae) second instar larvae. A second gene from *M. anisopliae* (*esterase – Mest1*) that accelerates the pathogens developmental processes was also used to generate single (AaIT or Mest1) and double transformants (AaIT-Mest1) of each *B. bassiana* strain. The pathogenicity of wild type and single and double recombinant strains was evaluated against second instar CPB at concentrations of $1x10^4$ and $5x10^4$spores/mm^2 of potato leaves. Mortality rates were strain- and dose- dependant and increased from 16.1 to 36.7% in single transformants (AaIT or Mest1) and from 7.1 to 33.5% in double transformants (AaIT-Mest1). The average survival time (AST) was reduced by up to 33% (from 5.4±0.6 to 3.6±0.3 days) with 252AaIT-Mest1 ($5x10^4$ spores/mm^2) and the medial lethal concentration (LC_{50}) was

reduced by up to 5.9-fold if the strain 9184AaIT-Mest1 was used. Singly, both toxins demonstrated increased killing power against second instar CPB. However, combining *AaIT* and *Mest1* genes did not produce synergistic effects.

4.2 INTRODUCTION

The use of fungi as biological control agents of insect pests is hindered by environmental and biotic processes (e.g., sensitivity to solar radiation, extreme temperatures and desiccation, microbial antagonism and host immune response) that can either inhibit or delay outbreaks of fungal disease in insect populations (Lacey et al. 2001). Molecular manipulation of entomopathogenic fungi focuses on improving parameters that increase their virulence (speed of kill), restrict or widen host range, reduce inoculum loads and/or alter saprophytic competence. This could theoretically lead to designing the ideal biocontrol agent for a particular insect pest and one that may be regionally specific (St. Leger 2007).

The selection of genes that offer the greatest immediate potential for improving the efficacy of fungi for pest control is an issue of prime importance in fungal transformation. The most effective toxins produced by generalist strains of the entomopathogenic fungus *Metarhizium anisopliae* are the destruxins (Samuels et al. 2002, Soledade et al. 2002). Unfortunately, for the purposes of genetic engineering, destruxins are secondary metabolites and encoded by genes that are too large at 20 Kb for convenient molecular manipulations. Some fungal genes that have been selected to create transgenic strains of *M. anisopliae* with improved virulence against insect pests include cuticle-degrading enzymes such as subtilisin protease (*Pr1A*) (St. Leger et al. 1996, Hu and St. Leger 2002a) and chitinases (*Chit1*) (Screen et al. 2001). *Metarhizium* esterase gene (*Mest1*) is another candidate to improve fungal virulence. *Mest1* activity is restricted to the lipid droplets of conidia and conidial germ tube and contributes to the rapid mobilization of stored lipids which accelerates fungal germination and growth into the insect. *Mest1* may therefore

help the fungus to establish the infection before the insect has time to produce a significant immune response (Wang and St. Leger, unpublished).

The insertion of non-fungal genes, such as those encoding arthropod toxins, has also been explored in an attempt to increase virulence of entomopathogens. Recombinant baculovirus containing the 70 amino acid *Androctonus australis* insect-specific toxin (AaIT) from the Buthid scorpion *Androctonus australis* has already provided promising control of lepidopteran larvae in the field (Sun et al. 2002). At nanomolar levels, the AaIT modifies the sodium channel and binds to the insect neuronal membrane causing immediate and sustained contraction of all insect's body muscles (Zlotkin et al. 1999, Zlotkin et al. 2000, Ji et al. 2002, Karbat et al. 2004).

M. anisopliae strain 549 was transformed to express AaIT (Wang and St Leger 2007a). The expression of this gene was driven by the promoter region of the *M. anisopliae* collagen-like protein (*Mcl1*) gene. This protein allows fungal hyphal bodies to evade detection by hemocytes during the infection process (Wang and Leger 2006). The *Mcl1* promoter not just produced rapid and high-level of expression of the toxin in *Manduca sexta* blood but also restricted its expression to insect's hemolymph making the AaIT even more specific for use in insect control. The transformant strain (named AaIT-549) was able to kill insect pests faster than the wild type strain (up to 38% faster in mosquitoes) (Wang and St Leger 2007a) and reduced inoculum loads that cause 50% mortality (LC_{50}) by 22-fold against larvae of tobacco hornworm *M. sexta* (Lepidoptera:Sphingidae), by 9-fold against adults of yellow fever mosquitoes *Aedes aegypti* (Diptera:Culicidae) (Wang and St Leger 2007a) and by 15.7-fold against the coffee berry borer (CBB) *Hypothenemus hampei* (Coleoptera: Curculionidae) (Pava-

Ripoll et al. 2008).

However, *M. anisopliae* has not been found in nature attacking CBB or the Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Coleoptera: Chrisomelidae). For this reason, biocontrol of these pests has relied on the use of the entomopathogenic fungus *Beauveria bassiana*. Genetic engineering of *B. bassiana* strains, has already improved pathogenicity of this fungus against selected agricultural pests. Thus, overexpression of chitinase in *B. bassiana* increased virulence against the aphid, *Myzus persicae* (Hemiptera: Aphididae) (Fan et al. 2007). Additionally, the insertion of *M. anisopliae Pr1A* and *Mest1* genes has been successful in increasing the virulence of *B. bassiana* strains against coleopteran pests including the CBB (Rodriguez and Gongora 2005).

Nonetheless, optimal pathogenicity may require manipulation of not just a single gene but several genes encoding enzymes and toxins that may act synergistically or additively. However, gene interactions may be antagonistic causing reduced efficacy of the fungal pathogen. Lu et al. (2008) cotransformed *B. bassiana* with the *AaIT* gene and the *M. anisopliae Pr1A* gene which led to degradation of the AaIT toxin by the protease gene suggesting that protein interactions need to be evaluated in more detail.

In this study, we inserted either the insect-specific scorpion toxin gene *(AaIT)* or the *M. anisopliae* esterase gene (*Mest1*) into three strains of *B. bassiana*: ARSEF 252, ARSEF 8998 and ARSEF 9184 that showed high, medium and low mortality against the Colorado potato beetle, respectively. In order to evaluate any additive or synergistic effects between genes, we constructed double transformants of the three *B. bassiana* strains containing both *AaIT* and *Mest1* genes. The effectiveness of wild type, single and

double transformants of *B. bassiana* was tested against second instar larvae of CPB.

4.3 MATERIALS AND METHODS

4.3.1 Fungal strains

Three strains of *B. bassiana* were selected for genetic transformation and screening in this study: (1) Strain ARSEF 252 (isolated from *L. decemlineata* in a greenhouse in Maine, USA) is widely used for biocontrol applications against several insect pests including CPB (Campbell et al. 1985, Wraight et al. 1998, Castrillo et al. 2008). (2) Strain ARSEF 8998 was a natural infection found in adult CPB while we were running our experiments at the greenhouse, University of Maryland, College Park. The strain was isolated and registered in the ARSEF collection. (3) Strain ARSEF 9184 was originally found in a Geometrid lepidopteran in Colombia. Single spore isolates of each strain were grown on Sabouraud dextrose agar (SDA) (Fischer Scientific, Pittsburg, PA) for 3 weeks at 27°C before collection of the spores for genetic transformation.

4.3.2 Vector construction

The pMcl1prAaIT plasmid was provided by Wang and St Leger (2007a). This plasmid contains the gene encoding the 70 amino acid AaIT placed upstream of the *M. anisopliae Mcl1* promoter to target expression of the toxin into the insect hemolymph (Wang and Leger 2006). The Mcl1-AaIT insert was digested with *BamHI* and inserted into restricted Ti master vector pFBarGFP at the *BglII* site. The Ti master vector was described by Fang et al. (2009b) and contains the herbicide phosphinothricin (glufosinate ammonium, ppt), resistance gene *bar* and the enhanced green fluorescent protein gene *egfp*.

The *M. anisopliae* esterase gene (*Mest1*) was amplified by PCR using the primers Mest1F (5′-CGGGATCCCGACCACCAACATAACATCCATCA-3′, a *BamHI* site is underlined) and Mest1R (5′-TCCCCCGGGGGAGGCAGCTGGGGTCGCTCCGA-3′, *SmaI* site is underlined). The digested PCR product was inserted into the corresponding sites of a pBarGPE1 plasmid so that the *Mest1* was under the control of a constitutive *Aspergillus nidulans gpdA* promoter. The Mest1 cassette was then released by cleavage with *BglII,* and inserted into the *BglII* restriction site of the Ti master vector pFBarGFP.

The resulting plasmids, pMcl1AaIT-FBarGFP and pgpdaMest1-FBarGFP, were introduced into *A. tumefaciens* AGL-1 as described by Lazo et al. (1991).

4.3.3 *Agrobacterium tumefaciens* mediated transformation of *B. bassiana* strains

A. tumefaciens-genetic transformation of *B. bassiana* strains was conducted based on the protocol designed by Fang et al. (2009b). Fungal spores were placed in sterile 0.01% Tween 20 (Sigma Chemical Co., St. Louis, MO), filtered through glass wool and vortexed for 2 min. The concentration of spores of each strain was determined with a hemocytometer and adjusted to 10^2 and 10^6 spores/ml. *A. tumefaciens* containing Ti plasmid pMcl1AaIT-FBarGFP or pgpdaMest1-FBarGFP was grown at 29°C overnight on LB supplemented with kanamycin (50 µg/ml) and carbencilline (50 µg/ml) and diluted to an OD_{660} nm value of 0.15 with the induction medium (IM) (Covert et al. 2001) supplemented with Acetosyringone (AS) (200 µM) (Sigma-Aldrich Co., St. Louis, MO) and MES (40 mM) (Sigma-Aldrich Co., St. Louis, MO). Acetosyringone is a phenolic inducer of the virulence genes present on the Ti plasmid. The *A. tumefaciens* culture was then grown at 29°C while shaking at 250 rpm for ~4 hours (until reaching an OD_{660} value between 0.5–0.8). Bacterial and spore suspensions were mixed in equal amounts and 200

µl of the mixture was spread on a black filter paper that was previously placed on IM plates supplemented with AS (200 µM) and MES (40 mM). Two days after incubation at 27°C, the filter paper was transferred to an M-100 plate containing 300 µg/ml cefotaxime to inhibit growth of *A. tumefaciens* and 10 µg/ml of the herbicide ppt (Fisher Scientific International, Inc., Pittsburgh, PA) to select transformants. After 2 days of growth, the black filter paper was overlaid with M-100 agar containing 5 µg/mL of ppt and incubated again at 27°C. Putative transformants were visible 5-6 days after incubation and were transferred to fresh M-100 media containing 10 µg/ml of ppt. Single transformants with the *AaIT* gene were constructed first and double transformation with the *Mest1* gene was then performed as described above but using a final concentration of glufosinate ammonium of 30 µg/ml instead of 10 µg/ml. Transformants were further confirmed with a fluorescent microscope set to a wavelength of 490 nm to observe green fluorescent protein (GFP) expression. Twenty colonies of each of the transformant strains expressing GFP were randomly selected, then placed on SDA and incubated at 27°C for 2-3 weeks. Morphological characteristics of the colonies were observed on SDA and those colonies in which gene insertion did not affect normal growth and conidiation were selected. Single transformants containing either the *AaIT* or the *Mest1* gene and double transformant containing both genes (*AaIT-Mest1*) were constructed for each *B. bassiana* strain.

4.3.4 Expression verification

The expression of the *AaIT* gene in single (-AaIT) and double transformants (AaIT-Mest1) was confirmed through reverse transcriptase PCR (RT-PCR) and western blot analysis. Spores of each *B. bassiana* transformant were grown for 30 h on 100 ml of

Sabouraud dextrose broth (SDB). Mycelia was collected through vacuum filtration, washed three times with sterile distilled water and transferred to *Manduca sexta* blood for 24 h as described previously (Wang et al. 2005). After that time, fungal mycelia were separated from *M. sexta*'s blood through vacuum filtration and total RNA was immediately extracted using the Qiagene Rneasy Mini kit (Valencia, CA, USA) according to manufacturer's instructions. RT-PCR was performed using the primers designed from the synthetic *AaIT* gene (Wang and St Leger 2007a). *M. sexta* blood was stored at -20°C for further western blot analysis. This experiment was repeated twice.

Western blot analyses were performed using a sheep anti - *A. australis* hector venom (MicroPharm Co., UK) as primary antibody and Donkey Anti-Goat IgG, AP (Promega BioSciences, CA, USA) as secondary antibody. Detection of AaIT was performed on 16.5% Tris/Tricine gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Wang and St. Leger (2007).

To test the activity of AaIT we used the insect muscle contraction assay (Zlotkin et al. 2000) where the fourth instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) were injected with 50 µl of *M. sexta* blood supernatant from 12 hours-old cultures of *B. bassiana* wt and transformant.

Insertion of the *Mest1* gene was performed through PCR amplification using Mest1 primers described before. To test the activity of the *Mest1* gene we evaluated the clearing zones of single and double transformant strains when growing on SDA + 0.4% tributyrin (Sigma Chemical Co., St. Louis, MO) (Fan and Mattey 1999, Singh et al. 2006).

4.3.5 Bioassay

We performed a screening bioassay in order to determine the pathogenicity of *B. bassiana* wild type and single and double transformant strains. Additional bioassays to calculate the median lethal concentration (LC_{50}) (the number of spores required to kill 50% of CPB larvae populations) were performed among selected strains.

4.3.5.1 Fungal spore preparation

Cultures of *B. bassiana* (wild type and transformant strains) were maintained on SDA for 2-3 weeks at 27°C prior to the each bioassay. Spores of each fungal strain were collected and placed in 5 ml of 0.01% Tween 20, filtered through glass wool and vortexed for 2 minutes. The concentration of spores was determined with a hemocytometer. For the screening bioassay, inoculum suspensions of $1x10^9$ and $5x10^9$ spores/ml were prepared. For the LC_{50} bioassay, inoculums of $2.5x10^6$, $5x10^7$, $1x10^8$, and $2x10^8$ spores/ml were prepared. Before running each bioassay, we assessed the percentage of germination of fungal spores as described previously (Pava-Ripoll et al. 2008).

4.3.5.2 Colony of Colorado potato beetle

Adults of CPB from a laboratory colony that is imidacloprid-resistant (Eastman strain from Maine, USA) were kindly provided by Dr. Galen Dively, Department of Entomology, University of Maryland, College Park. Beetles were maintained on fresh potato leaves (*Solanum tuberosum*) in cages placed in the greenhouse at the University of Maryland, College Park, MD at 25°C, 60-70% RH and 14:10 h L/D photoperiod.

CPB egg masses were collected daily, surface sterilized with 5% bleach solution for two minutes, rinsed three times with sterile distilled water, placed on sterile plates and stored at 12°C for a max of 8 days (to synchronize larval stages). Eggs where then transferred to a controlled chamber (Percival Scientific, Inc., Perry, IA, USA) at 25±1.0 °C, 60-70% RH and 14:10 h L/D photoperiod for hatching and larval rearing. Groups of 25-30 newly emerged larvae were transferred to new Petri dishes and feed with fresh surface-sterilized potato leaves until they reached the second instar larval stage and were used in bioassays.

4.3.5.3 Inoculation of fungal spores

Greenhouse-grown potato leaves were collected, surface sterilized (as described before) and dried on sterile tissue paper. Discs of known area (1000 mm^2) were cut and placed on 35 mm diameter Petri dishes (Ignoffo et al. 1983). For screening bioassays, 100 µL of either $1x10^9$ or $5x10^9$ spores/ml suspension were spread on the whole surface area of the potato leaf disc and let dry for five minutes before adding CPB larvae. For the LC_{50} bioassay, 100 µL of $2x10^7$, $1x10^8$, $5x10^8$, and $2.5x10^9$ spores/ml were added to the potato discs. Four replicates containing four larvae each were performed per treatment combination (fungal strain and spore concentration). Petri dishes containing CPB second instar larvae feeding on leaf discs with a known concentration of fungal spores were placed in the controlled chamber (same conditions as before). After 24 h of incubation, individual larvae from each plate were transferred to individual Petri dishes containing fresh potato leaves and put on plastic containers that were kept in the controlled chamber for a period 12 days. Observations were made daily and fresh potato leaves were added as required. The number of dead CPB larvae and the stage of fungal development on the

cadavers (mycelial initiation and sporulation) were recorded. Verification of GFP fluorescence by transformant strains on infected cadavers was performed under the fluorescent microscope.

4.3.6 Experimental Design

Each bioassay was set up as a completely randomized design. The screening bioassay was repeated at two different times with a two month interlude. Two concentrations of spores ($1x10^4$ and $5x10^4$ spores/mm^2) plus a control containing 0.01% Tween 20 solution, and twelve fungal strains: three wild type (Bb252wt, Bb8998wt and Bb9184wt), six single transformants with either the *AaIT* gene (Bb252-AaIT, Bb8998-AaIT and Bb9184-AaIT) or the *Mest1* gene (Bb252-Mest1, Bb8998-Mest1 and Bb9184-Mest1) and three double transformants (Bb252AaIT-Mest1, Bb8998AaIT-Mest1 and Bb9184AaIT-Mest1) were evaluated in the screening bioassay. The LC_{50} bioassay was also repeated two times with a month interlude and four concentrations of spores ($2x10^3$, $1x10^4$, $5x10^4$, and $2.5x10^5$ spores/mm^2) plus a control (0.01% Tween 20) and four fungal strains: two wild type (Bb252wt and Bb9184wt) and two double transformants (Bb252AaIT-Mest1 and Bb9184AaIT-Mest1) were evaluated.

Mortality data was expressed as percentage of mortality (number of dead larvae due to fungal infection / total number of larvae per treatment) 12 days post-inoculation and the data was analyzed using the SAS GLIMMIX procedure (SAS Institute Inc. 2006). Statistical significance between fungal strains and spore concentrations were calculated using the Tukey multiple mean comparison test.

The average survival time (AST) for the CPB for each treatment combination was estimated using the Kaplan-Meier method of censored data of the JMP 7 software. A censored value of one was assigned to live larvae. Statistical differences were calculated between fungal strains at each spore concentration using the Chi square (Wilcoxon) test ($P<0.05$).

The LC_{50}, expressed as spores/mm^2, was obtained at each treatment combination in the LC_{50} bioassay and was calculated with the PROC Probit procedure of the SAS 9.2 software.

4.4 RESULTS

4.4.1 *Beauveria bassiana* transformation and characterization

Agrobacterium tumefaciens-mediated transformation of three strains of *B. bassiana* (ARSEF 252, 8998 and 9184) followed the protocol described by Fang et al. (2009b). Single transformations with the scorpion toxin gene (*AaIT*) were performed using the pMcl1AaIT-FBarGFP plasmid and single transformations with the *M. anisopliae* esterase gene (*Mest1*) were performed using the pgpdaMest1-FBarGFP plasmid. Glufosinate ammonium-resistant colonies expressing GFP were chosen from the selection plates. With either plasmid, transformation efficiencies were dependant on fungal strains, and averaged 75±2.8%, 88±2.8% and 67±2.1% for strains 252, 8998 and 9184, respectively.

Twenty glufosinate ammonium-resistant colonies expressing GFP were randomly selected from single transformants with either the scorpion toxin (Bb252-AaIT, Bb8998-AaIT and Bb9184-AaIT) or *Mest1* gene (Bb252-Mest1, Bb8998-Mest1 and Bb9184-

Mest1) and placed on SDA at 27°C for three weeks to check morphological characteristics and conidiation. The insertion of the plasmid did not affect these characteristics in most (70±10%) of the colonies (14±2 out of 20 colonies, on average), indicating that disruption of physiologically important genes had not occurred.

To proceed with double transformation, four single transformant colonies expressing the *AaIT* gene were further selected from each strain (Bb252-AaIT, Bb8998-AaIT and Bb9184-AaIT). The insertion of the pgpdaMest1-FBarGFP plasmid was also performed using the *A. tumefaciens*-mediated transformation protocol with a higher concentration of glufosinate ammonium (30 µg/mL) on the selection media. Putative high concentration-glufosinate ammonium-resistant colonies were present on plates 7-8 days later (about 2 days delayed compared to single transformant colonies). The expression of GFP was also confirmed under a fluorescent microscope but a visual increase in green fluorescence could not select for putative double transformant colonies. The average transformation efficiencies of double transformants were 44.5±3.3% for strain 252-AaIT and 40±2.2% for both 8998-AaIT and 9184-AaIT strains.

Morphological characteristics and conidiation of putative double transformants were analyzed in ten randomly selected glufosinate ammonium-resistant colonies as described before. The insertion of the *Mest1* gene in strains containing the *AaIT* gene did not affect growth and conidition in 55%, 65% and 62.5% of selected colonies of strains 252AaIT-Mest1, 8998AaIT-Mest1 and 9184AaIT-Mest1, respectively.

The expression of the *AaIT* gene by the *Metarhizium Mcl1* promoter was confirmed through RT-PCR analyses after mycelia of single and double transformants were grown in *M. sexta* blood or SDB (Figure 4-1a). The secretion of the AaIT toxin was

confirmed by Western blot analysis (Figure 4-1b). The activity of the AaIT toxin was tested by performing the insect muscle contraction assay on fourth instar larvae of the greater wax moth, *Galleria mellonella*. The injection of culture supernatants from single and double transformants grown on *M. sexta* blood triggered body contraction in wax moth larvae (Figure 4-2).

To verify the integration of the *M. anisopliae* esterase gene into the fungal genome of *B. bassiana*, putative single and double transformants containing the *Mest1* gene were amplified by PCR using specific primers described previously. Colonies with positive bands were selected and the activity of the *Mest1* gene was confirmed by detection of clearing zones on SDA + 0.4% tributyrin (Figure 4-3).

4.4.2 Virulence of *B. bassiana* wild type and transformant strains on Colorado potato beetle (CPB) second instar larva

Germination rates of wild type and single and double transformant strains of *B. bassiana* were between 82 and 93% in all bioassays performed and we did not find any morphological differences in germ tubes of wild type or engineered strains.

Mortality rates of CPB larvae were strain- and dose- dependent (Figure 4-4). Comparing wild type (wt) strains, 252wt presented the highest mortality rate (55±8.5% and 91±1.8% for $1x10^4$ and $5x10^4$ spores/mm^2, respectively) (Figure 4-4a), strain 8998wt presented a medium mortality rate (32±7.2% and 78±8.2% for $1x10^4$ and $5x10^4$ spores/mm^2, respectively) (Figure 4-4b) and strain 9184wt presented the lowest mortality rate (20±6.6% and 63±9.2% for $1x10^4$ and $5x10^4$ spores/mm^2, respectively) (Figure 4-4c).

Mortality rates of CPB larvae caused by all *B. bassiana* single and double

transformants were higher at the lowest concentration of spores evaluated: at $1x10^4$ spores/mm^2, mortality rates increased from 19% (strain 252-AaIT) to 37% (strain 9184-AaIT) when compared with wild type strains whereas at $5x10^4$ spores/mm^2, mortality rates increased from 5% (strain 252-AaIT) to 24% (strain 9184-AaIT) (Figure 4-4 a-c).

Overall, single and double transformants presented higher mortality rates as compared with the wild type but there was not a significant increase in the mortality rates of double transformants when compared with single transformants. The mortality rates of single and double transformants of strain 8998, the strain 252 double transformant and the 9184 single transformant expressing the scorpion toxin gene were significantly higher as compared to their wild type strains (Figure 4-4a-c) ($P<0.05$).

Figure 4-5 shows the average survival time (AST) of all strains evaluated at $1x10^4$ spores/mm^2 and $5x10^4$ spores/mm^2 (Figure 4-5 a-c). Chi-square tests performed across groups of strains showed significant differences between the wild type and single and double transformants at both concentrations of spores ($P<0.001$).

Table 4-1 shows the time (in days) that it took each wild type and transformant strain to kill 50% of the CPB second instar larvae population. Reductions in AST are also strain-dependant and neither single or double transformants nor spore concentrations produced the same pattern of reduction in each strain. With $1x10^4$ spores/mm^2, the reduction in AST by single transformant strains ranged from 12.5% (strain 252-AaIT) to 31.3% (strain 8998-AaIT) whereas reduction in AST of double transformants ranged from 19.1% (strain 9184-AaIT) to 32.3% (strain 252AaIT-Mest1). With $5x10^4$ spores/mm^2, the reduction in AST by single transformant strains ranged from 12.8% (strain 9184-Mest1) to25.6% (strain 252-Mest1) whereas reduction in AST of double transformants ranged

from 15.9% (strain 9184AaIT-Mest1) to 33% (strain 252AaIT-Mest1).

Results for the probit analyses for LC_{50} of wild type and double transformant strains are shown in Figures 4-6a (strain 252) and 4-6b (strain 9184). For strain 252, the number of spores required to kill 50% of the CPB second instar larvae population was reduced from $3x10^4$ wild type spores/mm^2 to 9.9 $x10^3$ 252AaIT-Mest1 spores/mm^2. Thus, it takes 3-fold fewer conidia of 252AaIT-Mest1 to provide the same level of control. The LC_{90} is reduced from $2.3x10^6$ wild type spores/mm^2 to 3.5 $x10^5$ 252AaIT-Mest1 spores/mm^2, a 6.8-fold reduction (Table 4-2).

For strain 9184, the number of spores required to kill 50% of the CPB second instar larvae population was reduced from $5.2x10^4$ wild type spores/mm^2 to $8.7x10^3$ 9184AaIT-Mest1 spores/mm^2. Thus, it takes 5.9-fold fewer conidia of 9184AaIT-Mest1 to provide the same level of control. The LC_{90} is reduced from $8.5x10^6$ wild type spores/mm^2 to 3.3 $x10^5$ 252AaIT-Mest1 spores/mm^2, a 25.9-fold reduction (Table 4-3).

4.5 Discussion

4.5.1 *Beauveria bassiana* transformation and characterization

There is much interest in the application of recombinant DNA technologies for the production of hypervirulent entomopathogenic fungal strains, particularly if they can also overcome adverse environmental conditions (Lacey et al. 2001). Here, we demonstrated that *A. tumefaciens*-mediated transformation is an efficient means of inserting the *M. anisopliae* esterase gene, *Mest1* or the insect-specific arthropod toxin gene, *AaIT* into the genome of the entomopathogenic fungus *B. bassiana.* The resulting transgenic fungal strains demonstrated increased virulence against second instar larvae of

the CPB. The *A. tumefaciens* transformation protocol can also be used for co-transformation of *AaIT* and *Mest1* genes. *B. bassiana* double transformants were successfully selected using a three-fold higher concentration of the herbicide glufosinate ammonium in the selection media.

As previously reported by Jin et al. (2008), the use of GFP as a selectable and visible marker was an important tool for the selection of our single transformants, however expression of GFP did not visually increase in double transformants. The expression of GFP in engineered *B. bassiana* strains facilitated the visualization of the infection process of the fungus in all stages of the life cycle of the CPB (Figure 4-7). Thus, the insertion of fluorescent markers is a useful tool not just for monitoring the release of transformant strains into the field (Hu and St. Leger 2002a, O'Brien 2008) but also for monitoring all stages of the fungal infection process and for the diagnosis of disease before the fungus substantially fills the insect's body (Figures 4-2 and 4-7).

The *Mcl1* promoter was sufficient to drive rapid and hemolymph-specific expression of the *AaIT* gene in *M. anisopliae* (Wang and St Leger 2007a). Interestingly, although in *B. bassiana* the *Mcl1* promoter expressed low levels of AaIT toxin in SDB it expressed much higher levels in hemolymph indicating that some regulatory signals causing differential expression of the Mcl1 promoter are conserved in *M. anisopliae* and *B. bassiana* (Figure 4-1a).

4.5.2 Virulence of *B. bassiana* wild type and single and double transformant strains on Colorado potato beetle (CPB) second instar larva

The CPB is an insect pest of commercial importance to solanaceous crops in North America, Europe and Asia. Both adult and larvae feed on foliage causing complete defoliation of the crops if left uncontrolled (Wraight and Ramos 2002). The ability of CPB to develop resistance to chemical insecticides has motivated the use of biopesticides such as *B. bassiana* (Ignoffo et al. 1983, Hajek et al. 1987, Storch and Dill 1987, Furlong and Groden 2003), *Bacillus thuringiensis* (Ignoffo et al. 1982, Ferro et al. 1997, Costa et al. 2001) or a synergistic combination of the two (Costa et al. 2001, Wraight and Ramos 2005).

Although *B. bassiana* has been registered as an important natural enemy of all stages of the CPB, the virulence of fungal strains in lab and field have been very variable and their efficacy depends upon the fungal strain utilized for control and the developmental stage of the insect at the time of application. Miranpuri & Kachaturians (1995) reported mortalities of immature and adult stages of CPB infected by fifteen strains of *B. bassiana* ranging from 36% (strain USRR2533) to 97% (strain GK2016) after treatment with 10^8 spores/ml. Here, we report that the mortality of CPB second instar larvae infected by wild type strains of *B. bassiana* ARSEF 252, 8998 and 9184 was 91±1.8%, 78±8.2% and 63±9.2%, respectively when using a concentration of $5x10^4$ spores/mm^2 potato leaves.

The insertion of single gene (*AaIT* or *Mest1*) and double genes (*AaIT-Mest1*) in each of the three *B. bassiana* strains increased mortalities of second instar CPB as compared to infection with wild type strains. However, levels of statistical significance

varied depending on the combination of fungal strain and gene inserted (Figure 4-4). For example, at $1x10^4$ spores/mm^2 mortality rates significantly increased by 30.8% in the double transformant, 252AaIT-Mest1, by 36.7% in the single transformant 9184AaIT and by 30.7% in both single and double transformants of strain 8998 (Figure 4-4). These results highlight the importance of evaluating several fungal strains when evaluating the impact on virulence of different transgenes.

Concentrations of *B. bassiana* wild type spores required to kill 50% of our beetle population were $3x10^4$ spores/mm^2 for strain ARSEF 252 and $5.2x10^4$ spores/mm^2 for strain ARSEF 9184. Fold reductions of 3 and 5.9 were observed using double transformant strains 252Mest1-AaIT and 9184Mest1-AaIT, respectively (Tables 4-2 and 4-3). The greatest fold reduction in LC_{50} values was observed by the double transformant of the lowest virulence strain of *B. bassiana* (ARSEF 9184). This result suggests that entomopathogenic fungal strains with low virulence can benefit more from molecular manipulation than strains that are already highly pathogenic.

As well as reducing inoculum loads, the insertion of single genes (*AaIT* or *Mest1)* and double genes (*AaIT* and *Mest1*) into three *B. bassiana* strains also reduced the time to kill second instar CPB. Miranpuri & Kachaturians (1995) reported that the AST of immature stages of CPB treated with 10^8 spores/ml *B. bassiana* (strain GK2016) ranged from 1.2 to 4.5 days. Ignoffo et al. (1983) reported an AST from 2 to 4.5 days when CPB first instar larvae were infected with 10^5 and 10^2 *B. bassiana* conidia/$mm^2$of potato leaves, respectively. Here we report that at $5x10^4$ spores/mm^2 the lowest AST (3.2±0.3 days) was observed by the engineered strain 8998-AaIT whereas the highest AST (4.3±0.5 days) was observed by engineered strains 252-AaIT and 9184-Mest1. At 10^4

spores/mm^2 the lowest AST (4.4±0.5 days) was observed by the engineered strains 252AaIT-Mest1 and 8998-AaIT and the highest AST (5.6±0.6 days) was observed by engineered strain 9184-AaIT. Thus, the insertion of single or double genes in *B. bassiana* strains with high, medium and low mortality against CPB second instar larvae did not produce a simple pattern of increased virulence.

The propensity of CPB to evolve resistance to chemical insecticides has increased the economic impact of this beetle worldwide (Mota-Sanchez et al. 2006, Alyokhin et al. 2007) as well as increased the need for additional management tools (Gokce et al. 2006). However, CPB has also acquired resistance to biological insecticides and CPB accumulates chemicals from host plants (such as alkaloids) that are known to affect bacterial and fungal pathogens (Krischik 1991). Steroidal glycoalkaloids such as tomatin and solanine reduce the virulence of *B. bassiana* to CPB because conidial and hyphal growth is inhibited by the consumption of potato foliage (Hare and Andreadis 1983, da Costa et al. 2002). Additionally, the innate CPB biota can protect the beetle against fungal infections as enteric bacteria (particularly *Pseudomonas* strains B3G and 2V1D) isolated from CPB fourth instar larvae were found to completely inhibit the growth of *B. bassiana* (ARSEF 6721) in *in vitro* bioassays (Blackburn et al. 2008).

Despite these factors that can increase resistance of CPB to *B. bassiana* and the variation in virulence of fungal strains to different stages of CPB, we have shown that the efficacy of three strains of *B. bassiana* can be increased through the insertion of fungal (*Mest1*) or arthropod toxin (*AaIT*) genes into their genomes. Singly, both toxins produced increased mortality against second instar CPB. However, a combination of both *AaIT* and *Mest1* genes did not produce synergistic effects. The combination of other

genes of either fungal or arthropod origin is worth attempting in order to achieve more virulent strains for the control of populations of the Colorado potato beetle that are rapidly evolving to acquire resistant to both chemical and biological pesticides.

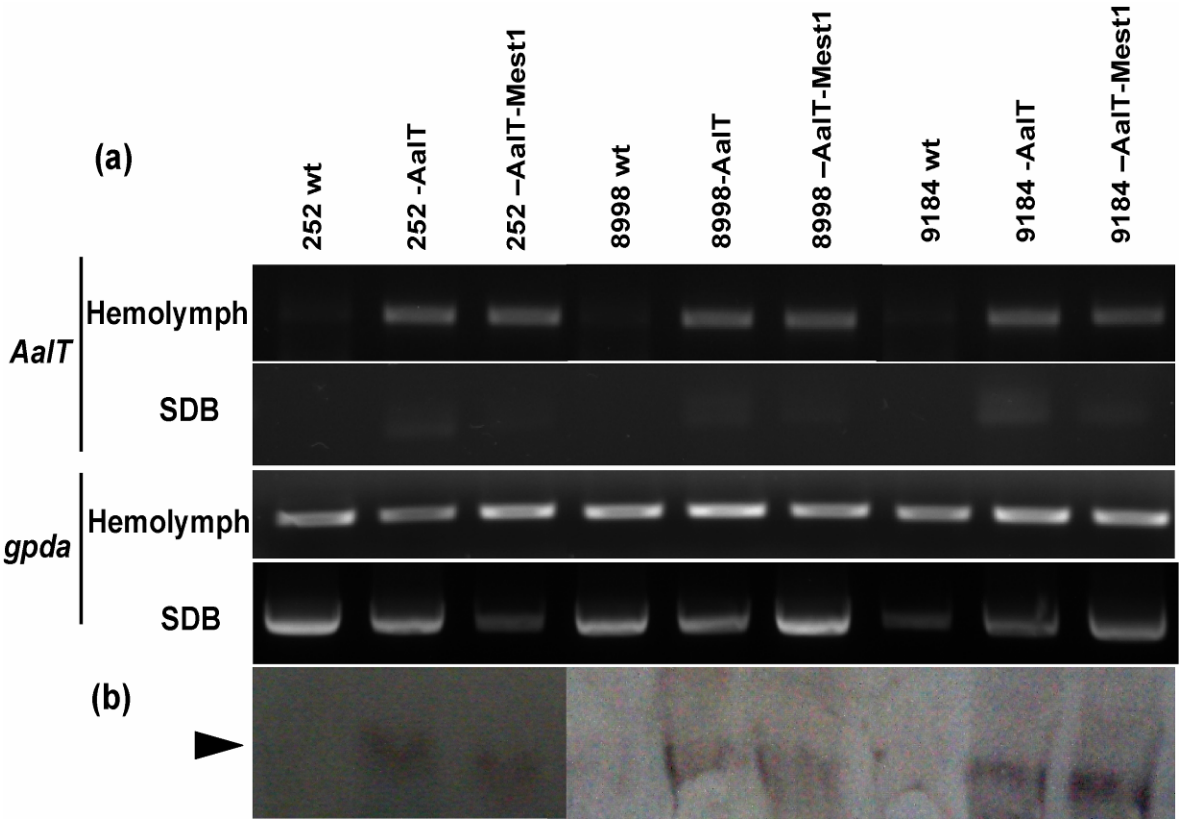

Figure 4-1. Expression of the *AaIT* gene driven by *Metarhizium anisopliae*'s *Mcl1* promoter in three *Beauveria bassiana* strains (ARSEF 252, 8998 and 9184). **(a)** RT-PCR analysis of single transformants with the *AaIT* gene and double transformants containing both *AaIT* and *Mest1* genes. The expression of AaIT in *M. sexta* haemolymph is higher than in Sabouraud dextrose broth (SDB). **(b)** Western blot analysis of *M. sexta* hemolymph used to culture wild type and single and double transformants of three *B. bassiana* strains.

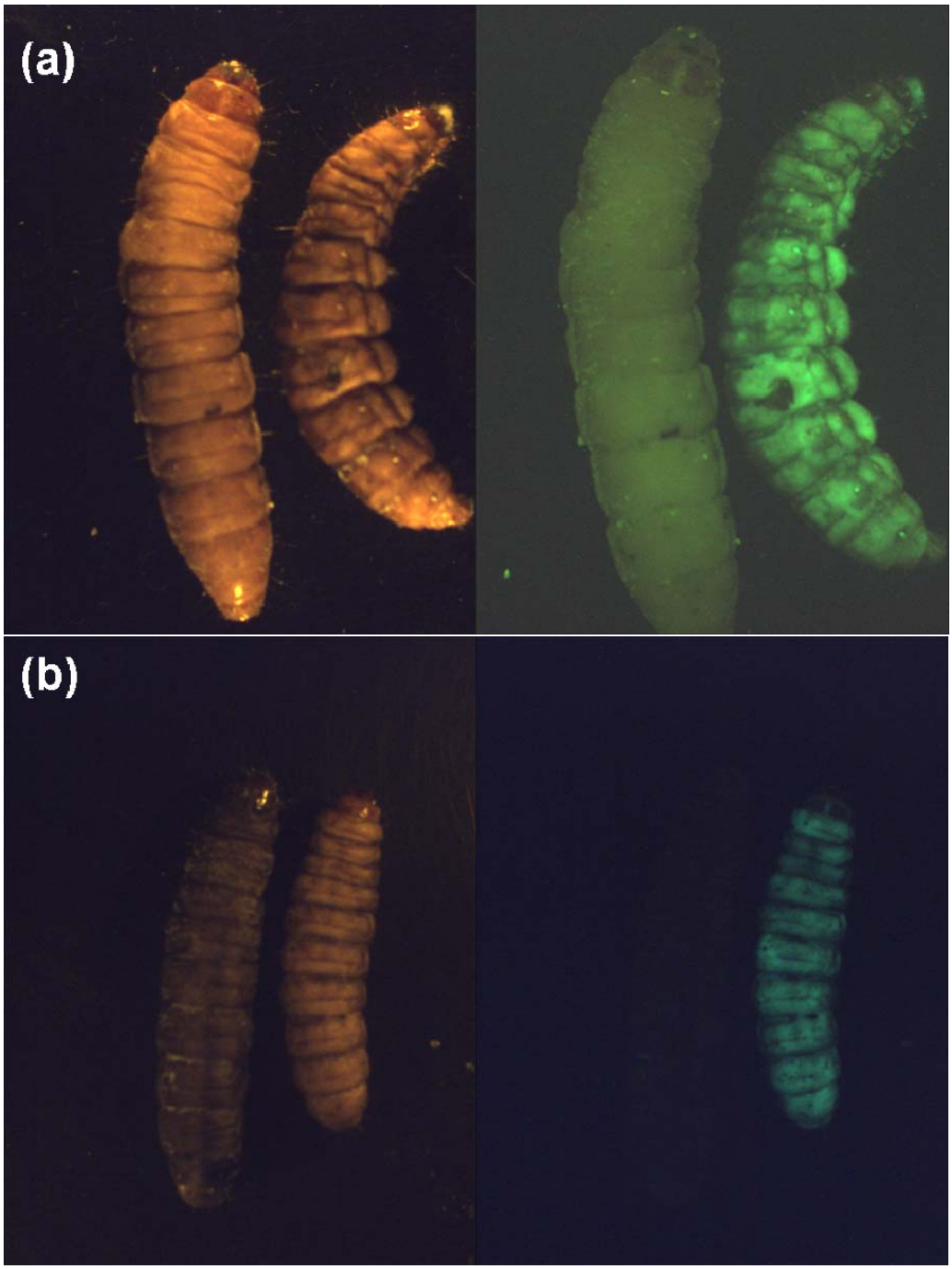

Figure 4-2. AaIT paralysis assay on *Galleria mellonella* fourth instar larvae. **(a)** *Beauveria bassiana* ARSEF 8998 wild type and single transformant 8998-AaIT expressing the green fluorensce protein (GFP). **(b)** *B. bassiana* ARSEF 9184 wild type and double transformant 9184AaIT-Mest1 expressing GFP.

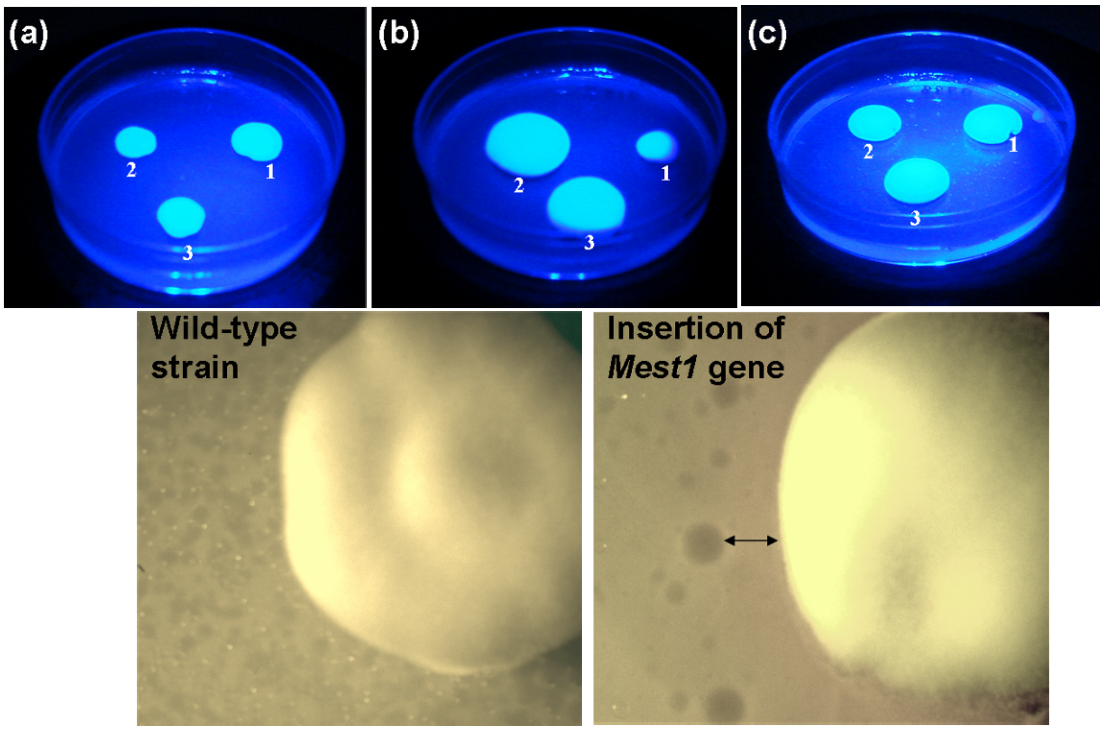

Figure 4-3. Colonies of *Beauveria bassiana* growing on Sabouraud dextrose agar plus 0.4% tributyrin. **(a)** *B. bassiana* ARSEF 252. **(b)** *B. bassiana* ARSEF 8998. **(c)** *B. bassiana* ARSEF 9184. Wild type (1), single transformants containing the *Metarhizium anisopliae*'s esterase gene (*Mest1*) (2) and double transformants containing *AaIT* and *Mest1* genes (3) In figures at the bottom a clear zone is produced by the activity of *Mest1* gene in transformant strains as compared with wild-type strains.

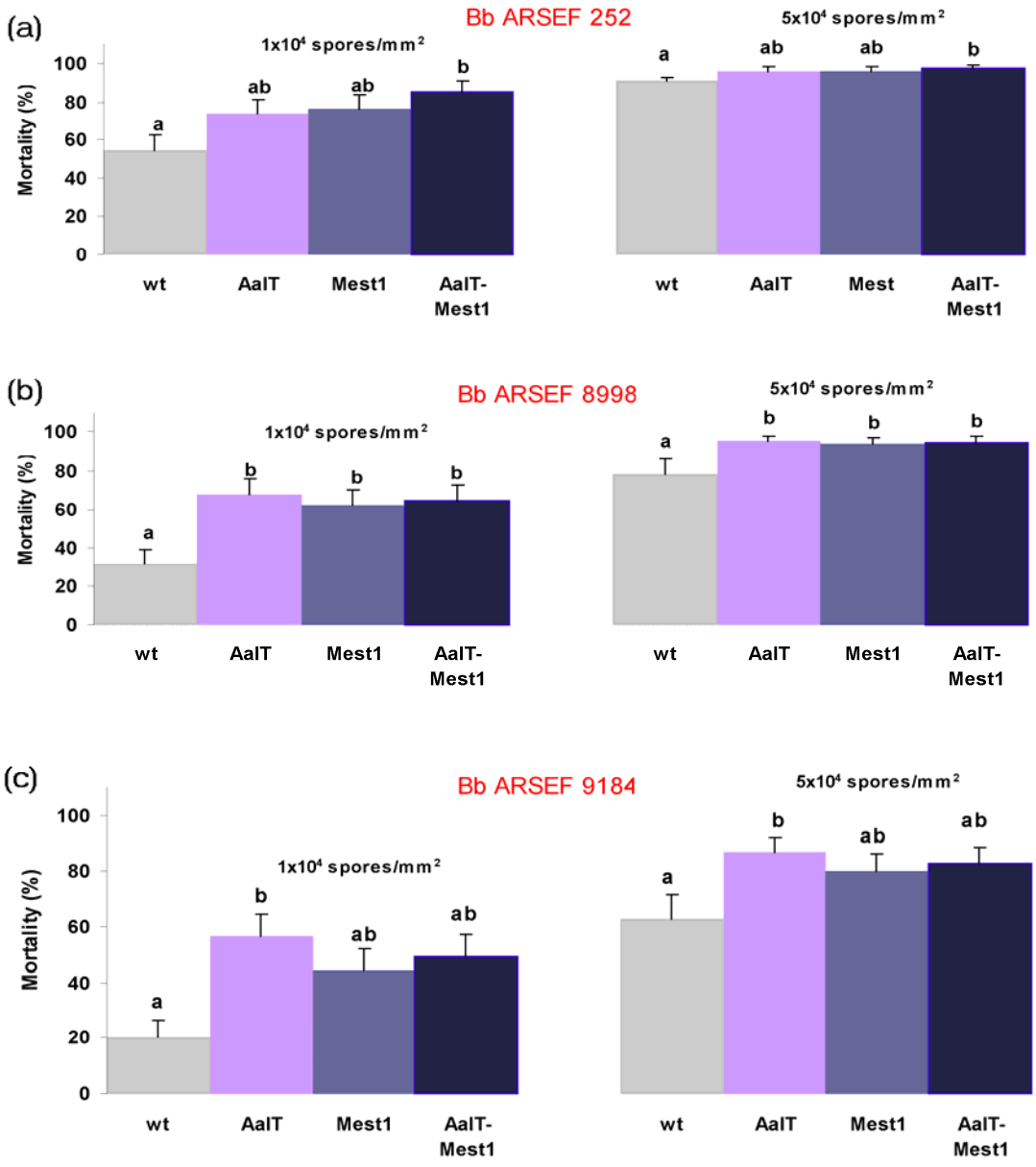

Figure 4-4. Mortality of Colorado potato beetle (CPB) second instar larvae after being challenged with $1x10^4$ and $5x10^4$ spores/mm^2 of *Beauveria bassiana* wild type, single transformants with either the *AaIT* or the *Mest1* gene and double transformant containing both *AaIT* and *Mest1* genes. **(a)** *B. bassiana* ARSEF 252. **(b)** *B. bassiana* ARSEF 8998. **(c)** *B. bassiana* ARSEF 9184. Treatments with the same letter are not significantly different ($P<0.005$) (Tukey multiple mean comparison test).

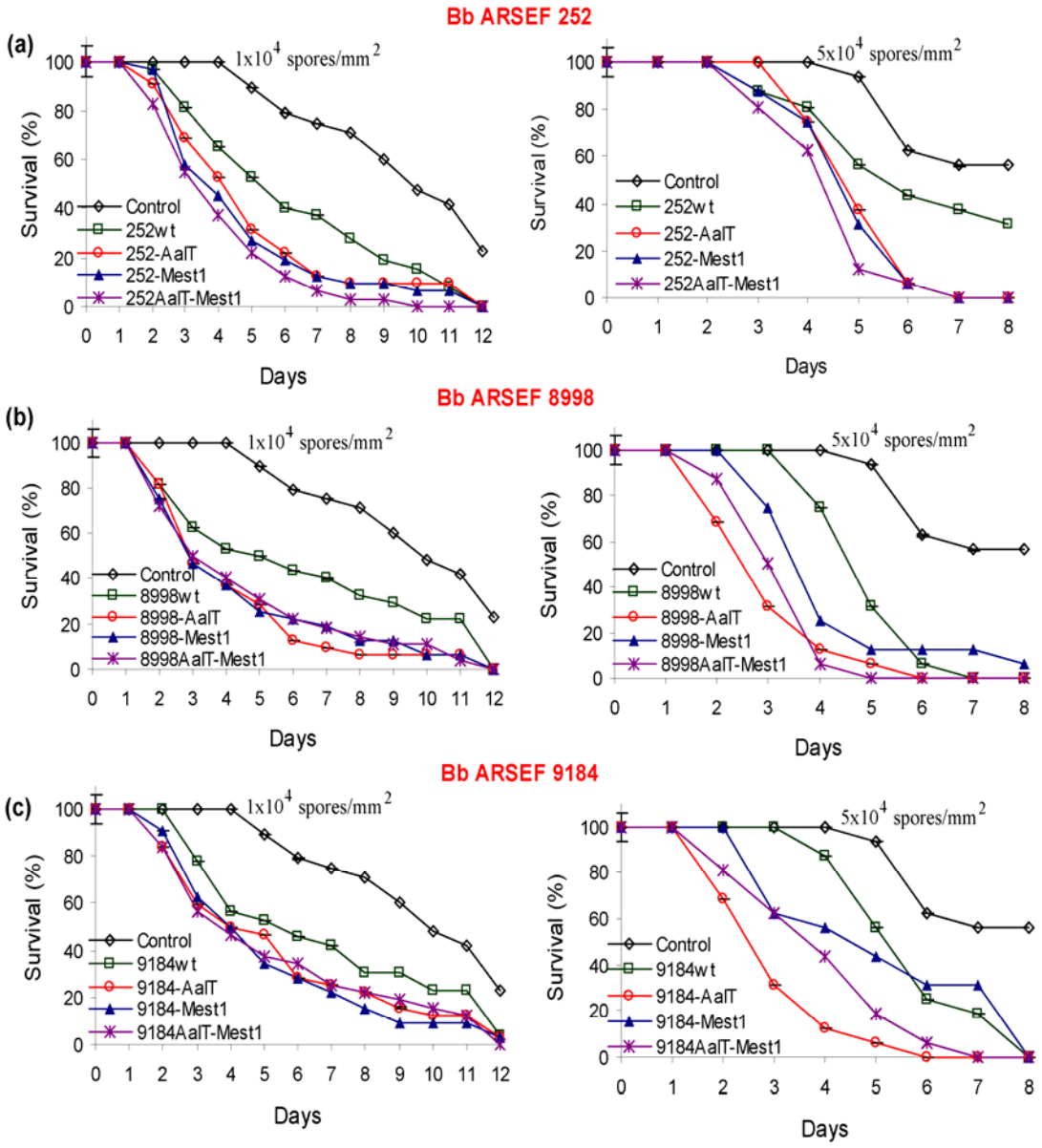

Figure 4-5. Average survival time (AST) of Colorado potato beetle (CPB) second instar larvae after being challenged with $1x10^4$ and $5x10^4$ spores/mm^2 of *Beauveria bassiana* wild type, single transformants with either the *AaIT* or the *Mest1* gene and double transformant containing both *AaIT* and *Mest1* genes. **(a)** *B. bassiana* ARSEF 252. **(b)** *B. bassiana* ARSEF 8998. **(c)** *B. bassiana* ARSEF 9184. Controls are CPB larvae treated with 0.01% Tween 20 containing no spores.

Table 4-1. Average Survival time (AST) of Colorado potato beetle (CPB) second instar larvae after being challenged with wild type, single transformants with either *AaIT* or *Mest1* and double transformants (*AaIT-Mest1*) of three strains of *Beauveria bassiana* (ARSEF 252, 8998 and 9184) at two concentrations of spores ($1x10^4$ spores/mm^2 and $5x10^4$ spores/mm^2). Mean in days ± standard error (SE). The percentage of reduction on transformant strains is calculated based on their wild type strains.

Fungal Strain	$1x10^4$ spores/mm^2											
	Bb ARSEF 252					Bb ARSEF 8998				Bb ARSEF 9184		
	N	Mean	±	SE	% Reduction	Mean	±	SE	% Reduction	Mean	±	SE	% Reduction
Control	48	9.6	±	0.4		9.6	±	0.4		9.6	±	0.4	
wild type	32	6.5	±	0.5		6.4	±	0.7		6.8	±	0.6	
AaIT	32	5.3	±	0.5	**12.5**	4.4	±	0.5	**31.3**	5.6	±	0.6	**17.6**
Mest1	32	4.8	±	0.5	**26.2**	4.6	±	0.5	**28.1**	5.3	±	0.5	**22.1**
AaIT-Mest1	32	4.4	±	0.4	**32.3**	4.7	±	0.5	**26.6**	5.5	±	0.6	**19.1**
	$5x10^4$ spores/mm^2											
Fungal Strain	Bb ARSEF 252					Bb ARSEF 8998				Bb ARSEF 9184		
	N	Mean	±	SE	% Reduction	Mean	±	SE	% Reduction	Mean	±	SE	% Reduction
Control	16	5.6	±	0.2		5.6	±	0.2		5.6	±	0.2	
wild type	16	5.4	±	0.6		4.1	±	0.2		4.9	±	0.3	
AaIT	16	4.3	±	0.3	**20.9**	3.2	±	0.3	**22.7**	4.1	±	0.4	**15.4**
Mest1	16	4.0	±	0.3	**25.6**	3.4	±	0.4	**17.6**	4.3	±	0.5	**12.8**
AaIT-Mest1	16	3.6	±	0.3	**33.0**	3.4	±	0.3	**17.6**	4.1	±	0.3	**15.9**

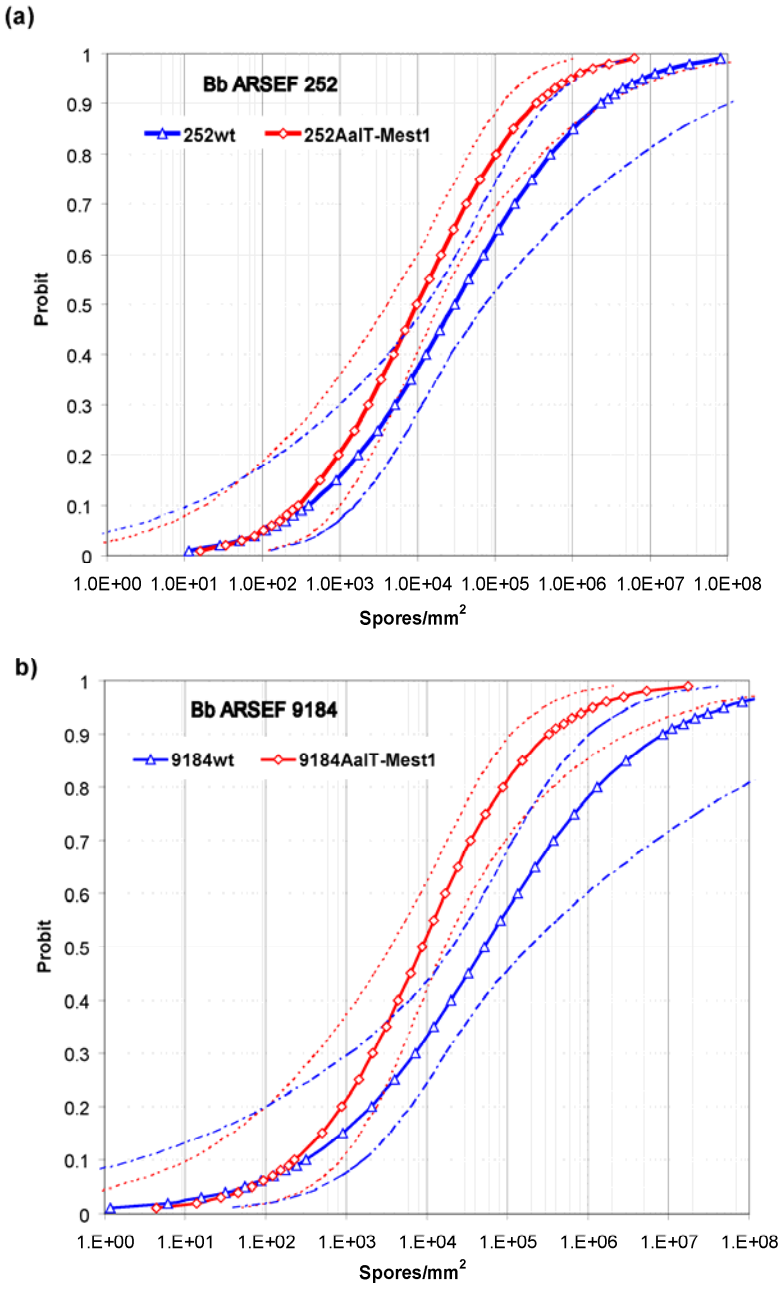

Figure 4-6. Median lethal concentration (LC_{50}) of *Beauveria bassiana* wild type and double transformants containing both *AaIT* and *Mest1* genes on Colorado potato beetle (CPB) second instar larvae. **(a)** *B. bassiana* ARSEF 252. **(b)** *B. bassiana* ARSEF 9184. Doted lines represent 95% coefficient intervals.

Table 4-2. Probit analysis of *Beauveria bassiana* ARSEF 252 wild type and double transformant containing both *AaIT* and *Mest1* genes on Colorado potato beetle (CPB) second instar larvae.

Probit	Bb ARSEF 252wt Chi Sq=19.26; P value=<0.0001			Bb ARSEF 252AaIT-Mest1 Chi Sq=25.55; P value=<0.0001			FOLD CHANGE	Spore Reduction
	Dose	95% Down	95% Up	Dose	95% Down	95% Up		
0.01	11.2	0.0	130.0	15.9	0.2	121.3	0.7	-5
0.02	28.4	0.1	249.4	33.8	0.6	210.8	0.8	-5
0.03	51.0	0.3	377.7	54.5	1.3	299.8	0.9	-3
0.04	79.3	0.7	516.7	78.1	2.3	391.0	1.0	1
0.05	113.6	1.4	667.3	104.7	3.7	485.6	1.1	9
0.06	154.3	2.3	830.3	134.4	5.5	584.2	1.1	20
0.07	201.7	3.8	1006.0	167.3	7.9	687.2	1.2	34
0.08	256.4	5.8	1196.0	203.4	10.8	795.2	1.3	53
0.09	319.0	8.5	1401.0	243.1	14.4	908.3	1.3	76
0.10	390.0	12.2	1621.0	286.4	18.7	1027.0	1.4	104
0.15	896.1	52.7	2989.0	564.4	55.2	1715.0	1.6	332
0.20	1736.0	166.8	4930.0	967.8	130.0	2594.0	1.8	768
0.25	3061.0	440.5	7700.0	1537.0	268.7	3728.0	2.0	1524
0.30	5095.0	1031.0	11749.0	2329.0	511.7	5205.0	2.2	2766
0.35	8169.0	2198.0	17913.0	3423.0	919.7	7168.0	2.4	4746
0.40	12785.0	4326.0	27869.0	4932.0	1583.0	9840.0	2.6	7853
0.45	19720.0	7885.0	45146.0	7023.0	2631.0	13608.0	2.8	12697
0.50	**3.0E+04**	**1.3E+04**	**7.7E+04**	**9.9E+03**	**4.2E+03**	**1.9E+04**	**3.0**	**20266**
0.55	4.6E+04	2.1E+04	1.4E+05	1.4E+04	6.6E+03	2.8E+04	3.3	3.2E+04
0.60	7.1E+04	3.2E+04	2.7E+05	2.0E+04	1.0E+04	4.2E+04	3.6	5.1E+04
0.65	1.1E+05	4.8E+04	5.6E+05	2.9E+04	1.5E+04	6.7E+04	3.9	8.3E+04
0.70	1.8E+05	7.1E+04	1.2E+06	4.2E+04	2.2E+04	1.1E+05	4.2	1.4E+05
0.75	3.0E+05	1.1E+05	3.0E+06	6.4E+04	3.2E+04	2.1E+05	4.6	2.3E+05
0.80	5.3E+05	1.6E+05	7.9E+06	1.0E+05	4.8E+04	4.1E+05	5.1	4.2E+05
0.85	1.0E+06	2.7E+05	2.5E+07	1.8E+05	7.4E+04	9.4E+05	5.8	8.4E+05
0.90	2.3E+06	4.9E+05	1.1E+08	3.5E+05	1.3E+05	2.7E+06	6.8	2.0E+06
0.91	2.9E+06	5.7E+05	1.6E+08	4.1E+05	1.4E+05	3.5E+06	7.0	2.5E+06
0.92	3.6E+06	6.6E+05	2.3E+08	4.9E+05	1.6E+05	4.7E+06	7.3	3.1E+06
0.93	4.5E+06	7.9E+05	3.6E+08	5.9E+05	1.9E+05	6.4E+06	7.7	3.9E+06
0.94	5.9E+06	9.5E+05	5.8E+08	7.4E+05	2.2E+05	9.1E+06	8.0	5.2E+06
0.95	8.0E+06	1.2E+06	9.9E+08	9.4E+05	2.7E+05	1.4E+07	8.5	7.1E+06
0.96	1.2E+07	1.5E+06	1.9E+09	1.3E+06	3.4E+05	2.2E+07	9.1	1.0E+07
0.97	1.8E+07	2.1E+06	4.2E+09	1.8E+06	4.4E+05	3.9E+07	9.9	1.6E+07
0.98	3.2E+07	3.2E+06	1.2E+10	2.9E+06	6.3E+05	8.4E+07	11.0	2.9E+07
0.99	8.1E+07	6.1E+06	6.3E+10	6.2E+06	1.1E+06	2.8E+08	13.0	7.5E+07

Table 4-3. Probit analysis of *Beauveria bassiana* ARSEF 9184 w wild type and double transformant containing both *AaIT* and *Mest1* genes on Colorado potato beetle (CPB) second instar larvae.

Probit	Bb ARSEF 9184wt Chi Sq=14.78; P value=<0.0001			Bb ARSEF 9184AaIT-Mest1 Chi Sq=23.049; P value=<0.0001			FOLD CHANGE	Spore Reduction
	Dose	95% Down	95% Up	Dose	95% Down	95% Up		
0.01	1.2	0.0	38.2	4.4	0.0	49.5	0.3	-3
0.02	6.1	0.0	114.5	14.0	0.1	114.3	0.4	-8
0.03	16.1	0.0	219.5	27.8	0.3	187.7	0.6	-12
0.04	32.1	0.0	350.6	45.6	0.8	268.1	0.7	-13
0.05	55.3	0.1	507.0	67.0	1.5	355.0	0.8	-12
0.06	86.6	0.2	688.4	92.2	2.5	447.8	0.9	-6
0.07	126.9	0.4	895.0	121.1	3.9	546.5	1.0	6
0.08	177.5	0.8	1127.0	153.8	5.8	650.9	1.2	24
0.09	239.4	1.5	1386.0	190.2	8.3	761.0	1.3	49
0.10	313.8	2.5	1673.0	230.6	11.4	877.0	1.4	83
0.15	919.4	21.7	3576.0	495.5	40.5	1549.0	1.9	424
0.20	2065.0	106.7	6475.0	881.4	104.2	2396.0	2.3	1184
0.25	4030.0	386.8	10873.0	1418.0	225.7	3463.0	2.8	2612
0.30	7226.0	1147.0	17773.0	2149.0	439.4	4822.0	3.4	5077
0.35	12281.0	2908.0	29378.0	3134.0	796.1	6585.0	3.9	9147
0.40	20173.0	6414.0	50918.0	4461.0	1369.0	8937.0	4.5	15712
0.45	32466.0	12404.0	95264.0	6259.0	2261.0	12194.0	5.2	26207
0.50	**5.2E+04**	**2.1E+04**	**1.9E+05**	**8.7E+03**	**3.6E+03**	**1.7E+04**	**5.9**	**43032**
0.55	8.2E+04	3.4E+04	4.3E+05	1.2E+04	5.6E+03	2.4E+04	6.8	7.0E+04
0.60	1.3E+05	5.2E+04	1.0E+06	1.7E+04	8.4E+03	3.6E+04	7.8	1.2E+05
0.65	2.2E+05	7.9E+04	2.6E+06	2.4E+04	1.2E+04	5.7E+04	9.0	1.9E+05
0.70	3.7E+05	1.2E+05	7.2E+06	3.5E+04	1.8E+04	9.7E+04	10.5	3.4E+05
0.75	6.6E+05	1.8E+05	2.3E+07	5.4E+04	2.7E+04	1.8E+05	12.4	6.1E+05
0.80	1.3E+06	2.9E+05	8.6E+07	8.6E+04	4.0E+04	3.8E+05	15.0	1.2E+06
0.85	2.9E+06	5.2E+05	4.3E+08	1.5E+05	6.3E+04	9.4E+05	19.0	2.8E+06
0.90	8.5E+06	1.1E+06	3.8E+09	3.3E+05	1.1E+05	3.3E+06	25.9	8.2E+06
0.91	1.1E+07	1.3E+06	6.5E+09	4.0E+05	1.3E+05	4.5E+06	28.0	1.1E+07
0.92	1.5E+07	1.6E+06	1.2E+10	4.9E+05	1.6E+05	6.4E+06	30.5	1.5E+07
0.93	2.1E+07	2.0E+06	2.4E+10	6.3E+05	1.9E+05	9.5E+06	33.6	2.0E+07
0.94	3.1E+07	2.6E+06	5.1E+10	8.2E+05	2.3E+05	1.5E+07	37.5	3.0E+07
0.95	4.8E+07	3.5E+06	1.3E+11	1.1E+06	2.9E+05	2.5E+07	42.7	4.7E+07
0.96	8.3E+07	5.1E+06	3.8E+11	1.7E+06	3.8E+05	4.8E+07	49.9	8.2E+07
0.97	1.7E+08	8.1E+06	1.6E+12	2.7E+06	5.5E+05	1.1E+08	61.0	1.6E+08
0.98	4.4E+08	1.5E+07	1.1E+13	5.4E+06	9.0E+05	3.5E+08	80.6	4.3E+08
0.99	2.2E+09	4.6E+07	3.1E+14	1.7E+07	2.1E+06	2.4E+09	129.1	2.2E+09

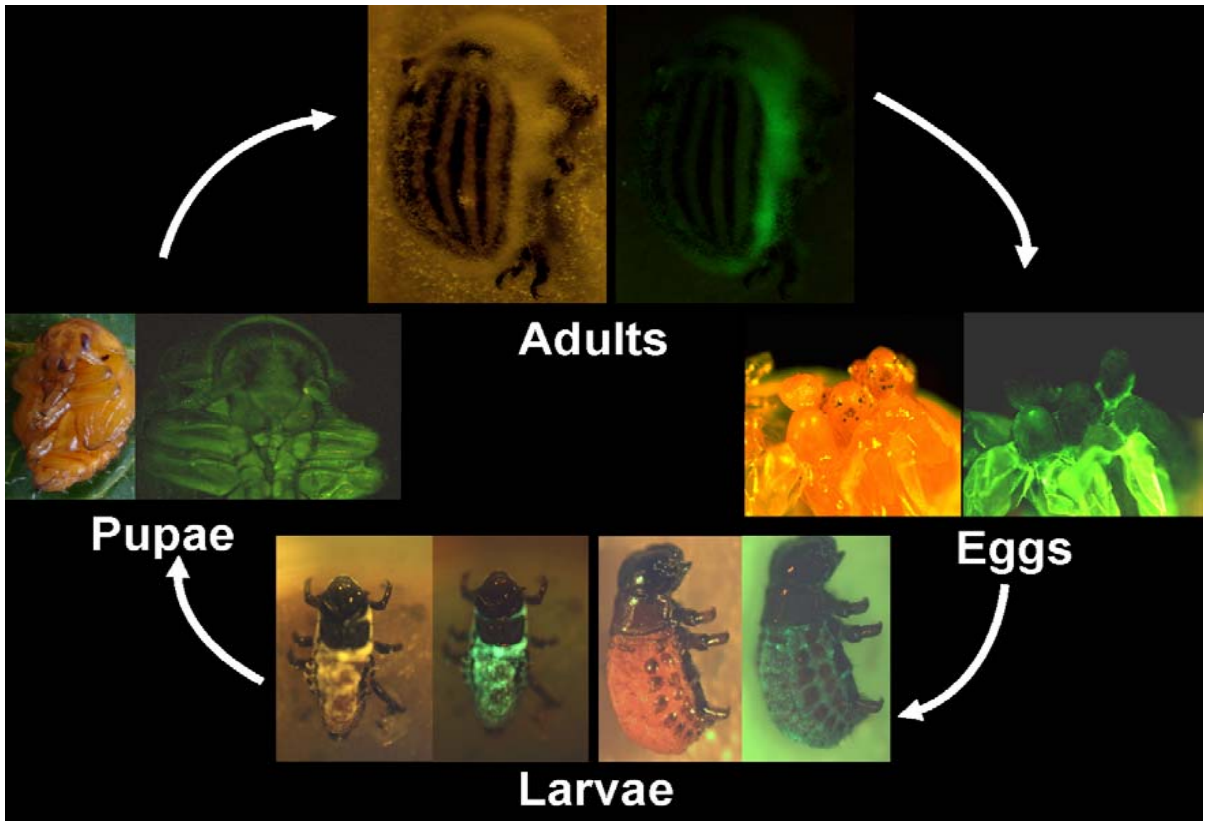

Figure 4-7. Life cycle of Colorado potato beetle (CPB) *Leptinotarsa decemlineata* infected with *Beauveria bassiana* ARSEF 8998 containing both *AaIT* and *Mest1* genes and the green fluorescence protein (GFP). All stages of CPB are susceptible to fungal infection and the expression of GFP evidence the infection process before the fungus is being visible in the insect's body

CONCLUDING REMARKS

The results of this research have emphasized the versatility of the entomopathogenic fungus *Metarhizium*.

M. anisopliae contains biologically distinct subtypes with generalist or specialist host ranges. This kind of pathogen diversity is a rich source of inference that can be used to develop models of the evolution of pathogens and pathogenicity and provide a novel perspective on the evolution and strategies of highly specialized fungi. First, using *Metarhizium* orthologous subtilisin coding sequences (Pr1H and Pr1A) I established the divergence times for individual *Metarhizium* species, subspecies and strains. Strains can be selected representing evolutionary distances ranging from <1 to 8 MY and the Pleistocene epoch (2 MYA - 10,000 YA) shows extensive radiation of *M. anisopliae* subtypes. Climate change during the past 10 million years (late Cenozoic era) was critical for the evolution of many species and the diversification of modern animals, flowering plants, trees, and associated insects. The evolution of large grazing animals in the Cenozoic also contributed to the spread of grasses that eventually covered 31% of the planet's land. This may have favored the diversification of *M. anisopliae*, which has been found to be most abundant in undisturbed pasture soils, 2-6 cm deep, where it can reach 10^6 propagules/g.

Ancestral reconstruction suggests that specialist *M. anisopliae* var. *acridium* strains adapted to orthopteran hosts evolved independently in both Australia and South America. The *M. robertsii* clade evolved in the America's from a soil dwelling ancestor with orthopteran and coleopteran hosts and dispersed worldwide as broad host range

strains. Some more recently evolved *M. anisopliae* strains still have a localized geographical distribution, particularly in Asia and the America's, but do not have localized hosts since they are found on soil and a variety of insect orders such as Blattodea, Lepidoptera, Hemiptera and Orthoptera. Using *Metarhizium*'s Pr1H and Pr1A coding sequences I could demonstrate that most amino acid sites are highly conserved and adaptive evolution affects only a few sites of the Pr1A coding sequence. Adaptive evolution is at least partly responsible for evolutionary innovations and species divergence of this entomopathogenic fungus and combined with knowledge of estimated dates of fungal divergence can help us to understand genome scale evolution and adaptations of *Metarhizium* to specific hosts and different environments.

Secondly, knowing that *Metarhizium* diversified greatly in a time of grass land expansion (the late Cenozoic era) and has adapted to soil environments produced this question: What are *Metarhizium* adaptations to grassland soils? To answer this question I first evaluated the germination of spores from eleven strains of entomopathogenic fungi (genera *Beauveria* and *Metarhizium*) and two soil saprophytic fungi (the non-rhizospheric fungus *Aspergillus niger* and the best studied rhizosphere competent fungus *Trichoderma harzianum*) at several concentrations of bean root exudates (RE) (1, 2.5, 5, 10 and 20 mg/ml). My results showed that at concentrations of RE <1 mg/ml *M. anisopliae* strain ARSEF 2575 showed higher germination rates than *T. harzianum*.

Knowledge of the ability of *M. anisopliae* strain ARSEF 2575 to germinate under low concentrations of RE encouraged further research on the genetic and physiological factors controlling rhizosphere competence. While a set of functionally related genes could be commonly expressed as this fungus adapts to different environments (insect

cuticle, insect blood and root exudates), there might also exist additional subset of genes specific to each environment. Using microarray analyses, I identified the subset of genes that *M. anisopliae* expressed after 1, 4, 8 and 12 hours of growth in 5 mg/ml of RE. Results showed that fifty genes (2.9%) were differentially expressed (DE) at all time points and they were organized into functionally related groups. I also demonstrated that the up-regulation of genes under RE conditions occurs at earlier time points (mainly 4 and 8 hours post-inoculation into RE) and that these up-regulated genes have fold change (FC) increases between 0.8 and 3.7.

The expression of the subtilisin-like serine protease (Pr1A) gene (CN808958) increased through time under RE conditions having the maximum expression ratio (1.5) after 8 hours; this is a 3.3 FC increase. This fact documents the rapid adaptation of this fungus to new environments and the ability of this fungus to use some of the same set of genes under different conditions even though they express secreted products (insect host or RE). However, the rapid expression of specific genes is also an interesting finding. The gene *Mad2* (for *Metarhizium* adhesin-like protein 2) is responsible for the attachment of the fungus to plant surfaces and is crucial for effective persistence and colonization of roots. I found this gene (CN809626) to be actively expressed in RE and reached a maximum FC value of 1.9 after 4 h. Interestingly, a fragment of the EST sequence of the root library (CN809322) originally designated as an unknown protein, presented 98% similarity (E value 2e-136) to the MAD2 transcript (CN809626) and although expressed at lower levels than MAD2 showed an identical pattern of gene expression through time in RE . This suggests that this transcript is also actively involved in the attachment of the cells to plant surfaces. I also found a large number of hypothetical and orphans proteins

(41.4% of DE genes) that were also up-regulated indicating that many previously uncharacterized genes may have functions related to saprophytic survival. The rhizosphere competence of *M. anisopliae* and knowledge of the genes involved as it adapts to soil environments, particularly at early time points is important when considering the potential commercial use of biological control agents toward root herbivores and soil-borne plant pathogens.

Finally, I demonstrated that genetic enhancement of entomopathogenic fungi is an excellent option to increase fungal virulence against insect pests when using lower inoculum loads. I bioassayed the insertion of genes of arthropod origin (*AaIT* gene) in *M. anisopliae* against the most economically important insect pest of coffee plantations world wide, the coffee berry borer (CBB) *Hypothenemus hampei* (Coleoptera: Curcolionidae). The genetic modified strain (AaIT-549) increased mortality up to 56.6% and reduced inoculum loads that cause 50% mortality (LC_{50}) by 15.7-fold. The average survival time (AST) was also reduced by 20.1% since time to kill decreased from 3.73±0.1 days using 10^7 wild-type spores/ml to 2.98±0.1 days using the same concentration of AaIT-Ma549 spores.

Giving the results obtained with the AaIT-549 against a coleopteran pest I extrapolated this approach to strains of an unrelated pathogen, *Beauveria bassiana*. I inserted the *AaIT* gene into three strains of *B. bassiana* with high, medium and low mortality against the Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) (Coleoptera: Chrysomelidae) second instar larvae. I also used a second gene from *M. anisopliae* (*esterase – Mest1*) that mobilizes internal nutrients of the pathogen speeding developmental processes and generated single (AaIT or Mest1) and double transformants

(AaIT-Mest1) of each *B. bassiana* strain. The pathogenicity of wild type and single and double recombinant strains was evaluated against CPB second instar larvae at concentrations of $1x10^4$ and $5x10^4$spores/mm^2 of potato leaves.

Mortality rates were strain- and dose- dependant and increased from 16.1 to 36.7% in single transformants (AaIT or Mest1) and from 7.1 to 33.5% in double transformants (AaIT-Mest1). The average survival time (AST) was reduced up to 33% (from 5.4±0.6 to 3.6±0.3 days) with the double recombinant strain 252AaIT-Mest1 at $5x10^4$ spores/mm^2 and the medial lethal concentration (LC_{50}) was reduced up to 5.9-fold with strain 9184AaIT-Mest1. Singly, both toxins demonstrated increased killing power against the CPB second instar larvae. However, synergistic effects derived from the combination of both *AaIT* and *Mest1* genes did not produce an increased magnitude of fungal hypervirulence.

My results showed that the efficacy of transformed fungal strains depends on the genera of entomopathogenic fungus used (i.e. *Beauveria* or *Metarhizium*), the fungal strains selected, the type of gene (s) inserted and the insect pest tested. Insects have tendency to develop resistance to chemical insecticides increasing their economic importance and the search for alternative control methods. However, some pests such as the Colorado potato beetle have also acquired resistance to biological insecticides by taking up toxic ingredients of their host plants (Solanaceaes). Steroidal glycoalkaloids such as tomatin and solanine have been shown to reduce the virulence of *B. bassiana* to CPB as conidial and hyphal growth is inhibited by the consumption of potato foliage. Additionally, CPB contains innate enteric biota that can protect the beetle against fungal infections. These facts may help explain differences in the effectiveness of transformed

fungal strains on the coleopteran insect pests tested in this research and at the same time suggests that the impact of genetic enhancement will vary considerably with the fungal strain and insect pest.

BIBLIOGRAPHY

Alyokhin, A., G. Dively, M. Patterson, C. Castaldo, D. Rogers, M. Mahoney, and J. Wollam. 2007. Resistance and cross-resistance to imidacloprid and thiamethoxam in the Colorado potato beetle *Leptinotarsa decemlineata*. Pest Management Science 63: 32-41.

Angelini, C., L. Cutillo, D. De Canditiis, M. Mutarelli, and M. Pensky. 2008. BATS: A Bayesian user-friendly software for Analyzing Time Series microarray experiments. Bmc Bioinformatics 9.

Attenborough, D. 1984. The Living Planet: A Portrait of the Earth. British Broadcasting Corporation., London, UK.

Aziz, N. H., M. Z. ElFouly, A. A. ElEssawy, and M. A. Khalaf. 1997. Influence of bean seedling root exudates on the rhizosphere colonization by *Trichoderma lignorum* for the control of *Rhizoctonia solani*. Botanical Bulletin of Academia Sinica 38: 33-39.

Bagga, S., G. Hu, S. E. Screen, and R. J. St Leger. 2004. Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene 324: 159-169.

Bais, H. P., S. W. Park, T. L. Weir, R. M. Callaway, and J. M. Vivanco. 2004. How plants communicate using the underground information superhighway. Trends in Plant Science 9: 26-32.

Barley, K. P. 1970. The configuration of the root system in relation to nutrient uptake Advances in Agronomy 22: 159-201.

Berbee, M. L., and J. W. Taylor. 2001. Fungal molecular evolution: Gene trees and geologic time. The Mycota: A comprehensive treatise on fungi as experimental systems for basic and applied research. Systematics and evolution, Part B: 229-245.

Bernal, M. G., A. E. Bustillo, and F. J. Posada. 1994. Virulencia de aislamientos de *Metarhizium anisopliae* y su eficacia en campo sobre *Hypothenemis hampei.* Revista Colombiana de Entomologia 20: 225-228.

Bidochka, M. J., J. E. Kasperski, and G. A. M. Wild. 1998. Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. Canadian Journal of Botany-Revue Canadienne De Botanique 76: 1198-1204.

Bidochka, M. J., A. M. Kamp, T. M. Lavender, J. Dekoning, and J. N. A. De Croos. 2001. Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: Uncovering cryptic species? Applied and Environmental Microbiology 67: 1335-1342.

Bischoff, J. F., S. A. Rehner, and R. A. Humber. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101: 512-530.

Blackburn, M. B., D. E. Gundersen-Rindal, D. C. Weber, P. A. W. Martin, and R. R. Farrar. 2008. Enteric bacteria of field-collected Colorado potato beetle larvae inhibit growth of the entomopathogens *Photorhabdus temperata* and *Beauveria bassiana*. Biological Control 46: 434-441.

Botero, J., and P. S. Baker. 2001. Coffee and biodiversity, a producer-country perspective., pp. 94-103. *In* P. S. Baker [ed.], Coffee Futures: A source book of

some critical issues confronting the coffee industry. CAB International, Wallingford, UK.

Boucias, D. G., and J. C. Pendland. 1987. Detection of protease inhibitors in the hemolymph of resistant *Anticarsia gemmatalis* which are inhibitory to the Entomopathogenic Fungus, *Nomuraea rileyi*. Experientia 43: 336-339.

Brode, P. F., C. R. Erwin, D. S. Rauch, B. L. Barnett, J. M. Armpriester, E. S. F. Wang, and D. N. Rubingh. 1996. Subtilisin BPN' variants: Increased hydrolytic activity on surface-bound substrates via decreased surface activity. Biochemistry 35: 3162-3169.

Bromham, L., and D. Penny. 2003. The modern molecular clock. Nature Reviews Genetics 4: 216-224.

Brun, L. O., C. Marcillaud, V. Gaudichon, and D. M. Suckling. 1989. Endosulfan Resistance in *Hypothenemus hampei* (Coleoptera, Scolytidae) in New Caledonia. Journal of Economic Entomology 82: 1310-1316.

Bryan, P. N. 2000. Protein engineering of subtilisin. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology 1543: 203-222.

Bucking, H., J. Abubaker, M. Govindarajulu, M. Tala, P. E. Pfeffer, G. Nagahashi, P. Lammers, and Y. Shachar-Hill. 2008. Root exudates stimulate the uptake and metabolism of organic carbon in germinating spores of Glomus intraradices. New Phytologist 180: 684-695.

Bustillo, A. E., R. Cardenas, and F. J. Posada. 2002. Natural enemies and competitors of *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) in Colombia. Neotropical Entomology 31: 635-639.

Bustillo, A. E., M. G. Bernal, P. Benavides, and B. Chaves. 1999. Dynamics of *Beauveria bassiana* and *Metarhizium anisopliae* infecting *Hypothenemus hampei* (Coleoptera : Scolytidae) populations emerging from fallen coffee berries. Florida Entomologist 82: 491-498.

Butt, T. M., C. Jackson, and N. Magan. 2001. Introduction fungal biocontrol agents: progress, problems and potential. , pp. 1-8. *In* T. M. Butt, C. Jackson and N. Magan [eds.], Fungi as biocontrol agents. CAB International, Willingford, U.K.

Campbell, R. K., T. E. Anderson, M. Semel, and D. W. Roberts. 1985. Management of the Colorado potato beetle using the entomogenous fungus *Beauveria bassiana*. American Potato Journal 62: 29-37.

Cardenas, R., and F. J. Posada. 2001. Los insectos y otros habitantes de cafetales y platanales. Comite Departamental de Cafeteros del Quindio - Cenicafe, Armenia, Colombia.

Castrillo, L. A., T. A. Ugine, M. J. Filotas, J. P. Sanderson, J. D. Vandenberg, and S. P. Wraight. 2008. Molecular characterization and comparative virulence of *Beauveria bassiana* isolates (Ascomycota : Hypocreales) associated with the greenhouse shore fly, *Scatella tenuicosta* (Diptera : Ephydridae). Biological Control 45: 154-162.

Chul, K. S., B. Y. Goo, L. D. Gyu, and K. Y. Heon. 1996. Antifungal activities of *Metarhizium anisopliae* against *Fusarium oxysporum*, *Botrytis cinerea* and *Alternaria solani*. Korean Journal of Mycology 24: 49-55.

Costa, S. D., M. E. Barbercheck, and G. G. Kennedy. 2001. Mortality of Colorado potato beetle (*Leptinotarsa decemlineata*) after sublethal stress with the CryIIIA

delta-endotoxin of *Bacillus thuringiensis* and subsequent exposure to *Beauveria bassiana*. Journal of Invertebrate Pathology 77: 173-179.

Covert, S. F., P. Kapoor, M. H. Lee, A. Briley, and C. J. Nairn. 2001. *Agrobacterium tumefaciens* mediated transformation of *Fusarium circinatum*. Mycological Research 105: 259-264.

Cunningham, B. C., and J. A. Wells. 1987. Improvement in the alkaline stability of subtilisin using an efficient random mutagenesis and screening procedure. Protein Engineering 1: 319-325.

da Costa, G. L., M. I. M. Sarquis, A. M. L. de Moraes, and V. Bittencourt. 2002. Isolation of *Beauveria bassiana* and *Metarhizium anisopliae* var. *anisopliae* from *Boophilus microplus* tick (Canestrini, 1887), in Rio de Janeiro State, Brazil. Mycopathologia 154: 207-209.

Damon, A. 2000. A review of the biology and control of the coffee berry borer, *Hypothenemus hampei* (Coleoptera : Scolytidae). Bulletin of Entomological Research 90: 453-465.

De la Rosa, W., J. L. Godinez-Aguilar, and R. Alatorre-Rosas. 1995. Biological activity of five strains of *Metarhizium anisopliae*, upon the coffee berry borer *Hypothenemus hampei* (Coleoptera: Scolytidae). Entomophaga 40: 403-412.

De la Rosa, W., R. Alatorre, J. F. Barrera, and C. Toriello. 2000. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycetes) upon the coffee berry borer (Coleoptera : Scolytidae) under field conditions. Journal of Economic Entomology 93: 1409-1414.

Deenihan, A., J. Donlan, J. Breen, and R. Moles. 2009. Mid-term impacts of excluding large grazing animals on a Burren grass/scrubland patch. Biology and Environment: Proceedings of the Royal Irish Academy 109B: 107–113.

Dix, N. J., and J. Webster. 1995. Fungal ecology. Chapman & Hall, London, UK.

Donoghue, P. C. J., and M. J. Benton. 2007. Rocks and clocks: calibrating the Tree of Life using fossils and molecules. Trends in Ecology & Evolution 22: 424-431.

Driver, F., R. J. Milner, and J. W. H. Trueman. 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycological Research 104: 134-150.

Durham, S. 2004. Stopping the coffee berry borer from boring into profits, pp. 10-11, Agricultural Research Magazine.

Eijsink, V. G. H., G. Vriend, J. R. Vanderzee, B. Vandenburg, and G. Venema. 1992. Increasing the thermostability of the neutral proteinase of *Bacillus stearothermophilus* by improvement of internal hydrogen bonding. Biochemical Journal 285: 625-628.

Elad, Y., I. Chet, and J. Katan. 1980. *Trichoderma harzianum* - biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology 70: 119-121.

Fan, X. L., and M. Mattey. 1999. Small enzymes with esterase activities from two thermophilic fungi, *Emericella nidulans* and *Talaromyces emersonii*. Biotechnology Letters 21: 1071-1076.

Fan, Y. H., W. G. Fang, S. J. Guo, X. Q. Pei, Y. J. Zhang, Y. H. Xiao, D. M. Li, K. Jin, M. J. Bidochka, and Y. Pei. 2007. Increased insect virulence in *Beauveria*

bassiana strains overexpressing an engineered chitinase. Applied and Environmental Microbiology 73: 295-302.

Ferro, D. N., A. C. Slocombe, and C. T. Mercier. 1997. Colorado potato beetle (Coleoptera: Chrysomelidae): Residual mortality and artificial weathering of formulated *Bacillus thuringiensis* subsp. *tenebrionis*. Journal of Economic Entomology 90: 574-582.

Freimoser, F. M., S. Screen, S. Bagga, G. Hu, and R. J. St Leger. 2003. Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiology-Sgm 149: 239-247.

Furlong, M. J., and E. Groden. 2003. Starvation induced stress and the susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata*, to infection by *Beauveria bassiana*. Journal of Invertebrate Pathology 83: 127-138.

Gokce, A., M. E. Whalon, H. Cam, Y. Yanar, I. Demirtas, and N. Goren. 2006. Plant extract contact toxicities to various developmental stages of Colorado potato beetles (Coleoptera: Chrysomelidae). Annals of Applied Biology 149: 197-202.

Gron, H., M. Meldal, and K. Breddam. 1992. Extensive comparison of the substrate preferences of 2 subtilisins as determined with peptide-substrates which are based on the principle of intramolecular quenching. Biochemistry 31: 6011-6018.

Hadar, Y., I. Chet, and Y. Henis. 1979. Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. Phytopathology 69: 64-68.

Hajek, A. E., R. S. Soper, D. W. Roberts, T. E. Anderson, K. D. Biever, D. N. Ferro, R. A. Lebrun, and R. H. Storch. 1987. Foliar applications of *Beauveria bassiana* (Balsamo) Vuillemin for control of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) - an overview of pilot test - Results from the Northern United States. Canadian Entomologist 119: 959-974.

Hale, M. G., L. D. Moore, and G. J. Griffin. 1978. Root exudates and exudation, pp. 475. *In* Y. R. Dommergues and S. V. Krupa [eds.], Interactions between non-pathogenic soil microorganisms and plants. Elsevier Scientific Publishing Company, Amsterdam.

Haraprasad, N., S. R. Niranjana, H. S. Prakash, H. S. Shetty, and S. Wahab. 2001. *Beauveria bassiana* - A potential mycopesticide for the efficient control of coffee berry borer, *Hypothenemus hampei* (Ferrari) in India. Biocontrol Science and Technology 11: 251-260.

Hare, J. D., and T. G. Andreadis. 1983. Variation in the susceptibility of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) when reared on different host plants to the fungal pathogen, *Beauveria bassiana* in the field and laboratory. Environmental Entomology 12: 1891-1896.

Harman, G. E. 2006. Overview of mechanisms and uses of *Trichoderma* spp. Phytopathology 96: 190-194.

Harman, G. E., and M. Shoresh. 2007. The mechanisms and applications of symbiotic opportunistic plant symbionts, pp. 374. *In* M. Vurro and J. Gressel [eds.], Novel

Biotechnologies for Biocontrol Agent Enhancement and Management Springer Netherlands.

Harman, G. E., I. Chet, and R. Baker. 1980. *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium spp* or *Rhizoctonia solani*. Phytopathology 70: 1167-1172.

Hay, W. W., E. Soeding, R. M. DeConto, and C. N. Wold. 2002. The late Cenozoic uplift - climate change paradox. International Journal of Earth Sciences 91: 746-774.

Hu, G., and R. J. St. Leger. 2002a. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that It Is rhizosphere competent. Applied Environmental Microbiology 68: 6383-6387.

Hu, G., and R. J. St. Leger. 2002b. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that It Is rhizosphere competent. Appl. Environ. Microbiol. %R 10.1128/AEM.68.12.6383-6387.2002 68: 6383-6387.

ICO - International Coffee Organization. 2008. Statistics. http://www.ico.org/trade_statistcs.asp/.

Ignoffo, C. M., C. Garcia, and M. Kroha. 1982. Susceptibility of the Colorado potato beetle *Leptinotarsa decemlineat*a to *Bacillus thuringiensis*. Journal of Invertebrate Pathology 39: 244-246.

Ignoffo, C. M., C. Garcia, M. Kroha, A. Samsinakova, and S. Kalalova. 1983. A leaf surface-treatment bioassay for determining the activity of conidia of *Beauveria bassiana* against *Leptinotarsa decemlineata*. Journal of Invertebrate Pathology 41: 385-386.

Jarzembowski, E. A. 1995. Early Cretaceous insect faunas and palaeoenvironment. Cretaceous Research 16: 681-693.

Ji, S. J., F. Liu, E. Q. Li, and Y. X. Zhu. 2002. Recombinant scorpion insect toxin AaIT kills specifically insect cells but not human cells. Cell Research 12: 143-150.

Jin, K., Y. J. Zhang, Z. B. Luo, Y. H. Xiao, Y. H. Fan, D. Wu, and Y. Pei. 2008. An improved method for *Beauveria bassiana* transformation using phosphinothricin acetlytransferase and green fluorescent protein fusion gene as a selectable and visible marker. Biotechnology Letters 30: 1379-1383.

Karbat, I., F. Frolow, O. Froy, N. Gilles, L. Cohen, M. Turkov, D. Gordon, and M. Gurevitz. 2004. Molecular basis of the high insecticidal potency of scorpion alpha-toxins. Journal of Biological Chemistry 279: 31679-31686.

Kasuga, T., T. J. White, and J. W. Taylor. 2002. Estimation of nucleotide substitution rates in Eurotiomycete fungi. Molecular Biology and Evolution 19: 2318-2324.

Krischik, V. A. 1991. Specific or generalized plant defense: Reciprocal interactions between herbivores and pathogens. *In* P. Barbosa, V. A. Krischik and C. G. Jones [eds.], Microbial mediation of plant-herbivore interactions. John Wiley & Sons, New York.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics 5: 150-163.

Lacey, L. A., R. Frutos, H. K. Kaya, and P. Vail. 2001. Insect pathogens as biological control agents: Do they have a future? Biological Control 21: 230-248.

Lazo, G. R., P. A. Stein, and R. A. Ludwig. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Bio-Technology 9: 963-967.

Le Pelley, R. H. 1968. Pests of Coffee. Longmans Green & Co., Ltd., London.

Maddison, W. P., and D. R. Maddison. 2009. Mesquite: a modular system for evolutionary analysis. Version 2.6. . http://mesquiteproject.org.

McPhalen, C. A., and M. N. G. James. 1988. Structural comparison of two serine proteinase-protein inhibitor complexes - Eglin-C-subtilisin Carlsberg and Ci-2-subtilisin novo. Biochemistry 27: 6582-6598.

Milner, R. J. 1992. Selection and characterization of strains of *Metarhizium anisopliae* for control of soil insects in Australia, pp. 200-207, Biological control of locusts and grasshoppers. CAB International; International Institute of Tropical Agriculture.

Miranpuri, G. S., and G. G. Khachatourians. 1995. Comparative virulence of different isolates of *Beauveria bassiana* and *Verticillium lecani* against Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Journal of Insect Science 8: 160-166.

Mitchell, K., and P. Wells. 2002. Spilling the beans on the coffee trade. The fairtrade foundation, http://www.fairtrade-advocacy.org/documents/Spilling_Beans_Trade.pdf.

Mota-Sanchez, D., R. M. Hollingworth, E. J. Grafius, and D. D. Moyer. 2006. Resistance and cross-resistance to neonicotinoid insecticides and spinosad in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). Pest Management Science 62: 30-37.

Muller, A., W. Hinrichs, W. M. Wolf, and W. Saenger. 1994. Crystal structure of calcium-free Proteinase K at 1.5 Angstrom resolution. Journal of Biological Chemistry 269: 23108-23111.

Narhi, L. O., Y. Stabinsky, M. Levitt, L. Miller, R. Sachdev, S. Finley, S. Park, C. Kolvenbach, T. Arakawa, and M. Zukowski. 1991. Enhanced stability of subtilisin by three point mutations. Biotechnology and Applied Biochemistry 13: 12-24.

Nikoh, N., and T. Fukatsu. 2000. Interkingdom host jumping underground: Phylogenetic analysis of entomoparasitic fungi of the genus *Cordyceps*. Molecular Biology and Evolution 17: 629-638.

O'Brien, T. R. 2008. *Metarhizium anisopliae*'s persistence as a saprophyte, genetic basis of adaptation and role as a plant symbiont. Ph.D. Thesis, Entomology. University of Maryland, College Park.

Pantoliano, M. W., M. Whitlow, J. F. Wood, S. W. Dodd, K. D. Hardman, M. L. Rollence, and P. N. Bryan. 1989. Large increases in general stability for subtilisin Bpn' through incremental changes in the free energy of unfolding. Biochemistry 28: 7205-7213.

Pava-Ripoll, M., F. J. Posada, B. Momen, C. Wang, and R. S. Leger. 2008. Increased pathogenicity against coffee berry borer, Hypothenemus hampei (Coleoptera : Curculionidae) by Metarhizium anisopliae expressing the scorpion toxin (AaIT) gene. Journal of Invertebrate Pathology 99: 220-226.

Phipps, R. H., and J. R. Park. 2002. Environmental benefits of genetically modified crops: Global and European perspectives on their ability to reduce pesticide use. Journal of Animal and Feed Sciences 11: 1-18.

Pingel, R. L., and L. C. Lewis. 1996. The fungus *Beauveria bassiana* (Balsamo) Vuillemin in a corn ecosystem: Its effect on the insect predator *Coleomegilla maculata* De Geer. Biological Control 6: 137-141.

Pond, S. L. K., S. D. W. Frost, and S. V. Muse. 2005. HyPhy: hypothesis testing using phylogenies. Bioinformatics 21: 676-679.

Posada, D., and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.

Posada, F. J., and F. E. Vega. 2005. A new method to evaluate the biocontrol potential of single spore isolates of fungal entomopathogens. Journal of Insect Science 5.

Posada, F. J., V. E. Osorio, and S. E. Velasquez. 2002. Evaluación de la patogenicidad de *Beauveria bassiana* sobre la broca del café empleando el método de aspersión foliar. Revista Colombiana de Entomología 28: 139-144.

Prior, C. 1992. Discovery and characterization of fungal pathogens for locust and grasshopper control, pp. 159-180, Biological Control of locusts and grasshoppers. CAB International, UK.

Quesada-Moraga, E., J. A. Navas-Cortés, E. A. A. Maranhao, A. Ortiz-Urquiza, and C. Santiago-Álvarez. 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. Mycological Research 111: 947-966.

Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews 62: 597-+.

Redecker, D., R. Kodner, and L. E. Graham. 2000. Glomalean fungi from the Ordovician. Science 289: 1920-1921.

Remy, W., T. N. Taylor, H. Hass, and H. Kerp. 1994. Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proceedings of the National Academy of Sciences of the United States of America 91: 11841-11843.

Roddam, L. F., and A. C. Rath. 1997. Isolation and characterization of *Metarhizium anisopliae* and *Beauveria bassiana* from subantarctic Macquarie Island. Journal of Invertebrate Pathology 69: 285-288.

Rodriguez, M. L., and C. E. Gongora. 2005. Transformation of *Beauveria bassiana* Bb9205 with *pr1A*, *pr1J*, and *ste1* genes of *Metarhizium anisopliae* and evaluation of the pathogenicity on the coffee berry borer. Revista Colombiana de Entomologia 31: 51-58.

Rovira, A. D. 1965. Plant root exudates and their influence upon soil microorganisms, pp. 571. *In* K. F. Baker, W. C. Snyder, R. R. Baker and a. others [eds.], Ecology of soil-borne plant pathogens, prelude to biological control. University of California Press, Berkeley, Los Angeles, California.

Samuels, R. I., R. C. Pereira, and C. A. T. Gava. 2002. Infection of the coffee berry borer *Hypothenemus hampei* (Coleoptera : Scolytidae) by Brazilian isolates of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*

(Deuteromycotina : Hyphomycetes). Biocontrol Science and Technology 12: 631-635.

SAS Institute Inc. 2006. SAS/Procedures guide version SAS® 9.1.3 computer program, version By SAS Institute Inc., Cary, NC, USA.

Screen, S. E., G. Hu, and R. J. St Leger. 2001. Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. Journal of Invertebrate Pathology 78: 260-266.

Siezen, R. J., and J. A. M. Leunissen. 1997. Subtilases: The superfamily of subtilisin-like serine proteases. Protein Science 6: 501-523.

Singh, R., N. Gupta, V. K. Goswami, and R. Gupta. 2006. A simple activity staining protocol for lipases and esterases. Applied Microbiology and Biotechnology 70: 679-682.

Soledade, M., C. Pedras, L. I. Zaharia, and D. E. Ward. 2002. The destruxins: synthesis, biosynthesis, biotransformation, and biological activity. Phytochemistry 59: 579-596.

Spatafora, J. W., G. H. Sung, J. M. Sung, N. L. Hywel-Jones, and J. F. White. 2007. Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. Molecular Ecology 16: 1701-1711.

St. Leger, R. J. 2008. Studies on adaptations of *Metarhizium anisopliae* to life in the soil. Journal of Invertebrate Pathology 98: 271-276.

St. Leger, R. J., A. K. Charnley, and R. M. Cooper. 1986. Cuticle degrading enzymes of entomopathogenic fungi: Mechanisms of interaction between pathogen enzymes and insect cuticle. Journal of Invertebrate Pathology 47: 295-302.

St. Leger, R. J., D. C. Frank, D. W. Roberts, and R. C. Staples. 1992a. Molecular cloning and regulatory analysis of the cuticle-degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. European Journal of Biochemistry 204: 991-1001.

St. Leger, R. J., L. Joshi, M. J. Bidochka, and D. W. Roberts. 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proceedings of the National Academy of Sciences of the United States of America 93: 6349-6354.

St. Leger, R. J., B. May, L. L. Allee, D. C. Frank, R. C. Staples, and D. W. Roberts. 1992b. Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae*. Journal of Invertebrate Pathology 60: 89-101.

St. Leger, R. J. S. 2007. *Metarhizium anisopliae* as a model for studying bioinsecticidal host pathogen interactions, pp. 179-204, Novel Biotechnologies for Biocontrol Agent Enhancement and Management. Springer.

Stidham, T. A., and J. A. Stidham. 2000. A New Miocene band-winged grasshopper (Orthoptera: Acrididae) from Nevada. Annals of the Entomological Society of America 93: 405-407.

Storch, R. H., and J. F. Dill. 1987. *Beauveria bassiana* for control of Colorado potato beetle Coleoptera: Chrysomelidae in Maine USA. Maine Agricultural Experiment Station Technical Bulletin (Orono): 1-8.

Sun, X. L., X. W. Chen, Z. X. Zhang, H. L. Wang, J. J. A. Bianchi, H. Y. Peng, J. M. Vlak, and Z. H. Hu. 2002. Bollworm responses to release of genetically modified *Helicoverpa armigera* nucleopolyhedroviruses in cotton. Journal of Invertebrate Pathology 81: 63-69.

Sung, G. H., G. O. Poinar, and J. W. Spatafora. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. Molecular Phylogenetics and Evolution 49: 495-502.

Sung, G. H., J. M. Sung, N. L. Hywel-Jones, and J. W. Spatafora. 2007a. A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. Molecular Phylogenetics and Evolution 44: 1204-1223.

Sung, G. H., N. L. Hywel-Jones, J. M. Sung, J. J. Luangsa-Ard, B. Shrestha, and J. W. Spatafora. 2007b. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Studies in Mycology: 5-59.

Tajima, F. 1993. Simple methods for testing the molecular evolutionary clock hypothesis. Genetics 135: 599-607.

Volkl, P., P. Markiewicz, K. O. Stetter, and J. H. Miller. 1994. The sequence of a subtilisin-type protease (Aerolysin) from the hyperthermophilic Archaeum *Pyrobaculum aerophilum* reveals sites important to thermostability. Protein Science 3: 1329-1340.

Wagner, B. L., and L. C. Lewis. 2000. Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. Applied and Environmental Microbiology 66: 3468-3473.

Walker, T. S., H. P. Bais, E. Grotewold, and J. M. Vivanco. 2003. Root exudation and rhizosphere biology. Plant Physiology 132: 44-51.

Wang, C., and R. J. S. Leger. 2006. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proceedings of the National Academy of Sciences of the United States of America 103: 6647-6652.

Wang, C. S., and R. J. St Leger. 2007a. A scorpion neurotoxin increases the potency of a fungal insecticide. Nature Biotechnology 25: 1455-1456.

Wang, C. S., and R. J. St Leger. 2007b. The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryotic Cell 6: 808-816.

Wang, C. S., G. Hu, and R. J. St Leger. 2005. Differential gene expression by *Metarhizium anisopliae* growing in root exudate and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. Fungal Genetics and Biology 42: 704-718.

Wang, S. B., A. Leclerque, M. Pava-Ripoll, W. G. Fang, and R. J. St Leger. 2009a. Comparative genomics using microarrays reveals divergence and loss of virulence associated genes in host-specific strains of the insect pathogen *Metarhizium anisopliae*. Eukaryotic Cell 8: 888-898.

Wang, S. B., A. Leclerque, M. Pava-Ripoll, W. G. Fang, and R. J. St Leger. 2009b. Comparative Genomics Using Microarrays Reveals Divergence and Loss of Virulence-Associated Genes in Host-Specific Strains of the Insect Pathogen Metarhizium anisopliae. Eukaryotic Cell 8: 888-898.

Wegbe, K., C. Cilas, B. Decazy, C. Alauzet, and B. Dufour. 2003. Estimation of production losses caused by the coffee berry borer (Coleoptera : Scolytidae) and calculation of an economic damage threshold in Togolese coffee plots. Journal of Economic Entomology 96: 1473-1478.

Whalley, W. M., and G. S. Taylor. 1973. Influence of pea root exudates on germination of conidia and Chlamydospores of physiologic races of *Fusarium oxysporum* F. Pisi. Annals of Applied Biology 73: 269-276.

Wolfger, H., Y. M. Mamnun, and K. Kuchler. 2001. Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Research in Microbiology 152: 375-389.

Woo, S. L., F. Scala, M. Ruocco, and M. Lorito. 2006. The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. Phytopathology 96: 181-185.

Wraight, S. P., and M. E. Ramos. 2002. Application parameters affecting field efficacy of *Beauveria bassiana* foliar treatments against Colorado potato beetle *Leptinotarsa decemlineata*. Biological Control 23: 164-178.

Wraight, S. P., and M. E. Ramos. 2005. Synergistic interaction between *Beauveria bassiana* and *Bacillus thuringiensis tenebrionis* based biopesticides applied against field populations of Colorado potato beetle larvae. Journal of Invertebrate Pathology 90: 139-150.

Wraight, S. P., R. I. Carruthers, C. A. Bradley, S. T. Jaronski, L. A. Lacey, P. Wood, and S. Galaini-Wraight. 1998. Pathogenicity of the entomopathogenic

fungi *Paecilomyces* spp. and *Beauveria bassiana* against the silverleaf whitefly, *Bemisia argentifolii*. Journal of Invertebrate Pathology 71: 217-226.

Yang, Z. 2003. Adaptive molecular evolution., pp. 229-254. *In* D. J. Balding, M. Bishopa and C. Cannings [eds.], Handbook of Statistical Genetics, Second ed. John Wiley & son Ltda.

Yokoyama, E., M. Arakawa, K. Yamagishi, and A. Hara. 2006. Phylogenetic and structural analyses of the mating-type loci in Clavicipitaceae. Fems Microbiology Letters 264: 182-191.

Zimmermann, G. 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Science and Technology 17: 879-920.

Zlotkin, E., A. L. Devonshire, and J. W. Warmke. 1999. The pharmacological flexibility of the insect voltage gated sodium channel: toxicity of AaIT to knockdown resistant (*kdr*) flies. Insect Biochemistry and Molecular Biology 29: 849-853.

Zlotkin, E., Y. Fishman, and M. Elazar. 2000. AaIT: From neurotoxin to insecticide. Biochimie 82: 869-881.