The rhizosphere-competent entomopathogen *Metarhizium anisopliae* expresses a specific subset of genes in plant root exudate

Monica Pava-Ripoll,[1] Claudia Angelini,[2] Weiguo Fang,[1] Sibao Wang,[1] Francisco J. Posada[1] and Raymond St Leger[1]

[1]Department of Entomology, University of Maryland, College Park, MD 20742, USA

[2]Istituto per le Applicazioni del Calcolo, 'Mauro Picone', CNR-Napoli, Italy

Correspondence
Monica Pava-Ripoll
monicapavaripoll@hotmail.com

Metarhizium anisopliae and *Beauveria bassiana* are ubiquitous insect pathogens and possible plant symbionts, as some strains are endophytic or colonize the rhizosphere. We evaluated 11 strains of *M. anisopliae* and *B. bassiana*, and two soil saprophytes (the non-rhizospheric *Aspergillus niger* and the rhizosphere-competent *Trichoderma harzianum*) for their ability to germinate in bean root exudates (REs). Our results showed that some generalist strains of *M. anisopliae* were as good at germinating in RE as *T. harzianum*, although germination rates of the specialized acridid pathogen *Metarhizium acridum* and the *B. bassiana* strains were significantly lower. At RE concentrations of <1 mg ml^{-1}, *M. anisopliae* strain ARSEF 2575 showed higher germination rates than *T. harzianum*. Microarrays showed that strain 2575 upregulated 29 genes over a 12 h period in RE. A similar number of genes (21) were downregulated. Upregulated genes were involved in carbohydrate metabolism, lipid metabolism, cofactors and vitamins, energy metabolism, proteolysis, extracellular matrix/cell wall proteins, transport proteins, DNA synthesis, the sexual cycle and stress response. However, 41.3 % of the upregulated genes were hypothetical or orphan sequences, indicating that many previously uncharacterized genes have functions related to saprophytic survival. Genes upregulated in response to RE included the subtilisin Pr1A, which is also involved in pathogenicity to insects. However, the upregulated *Mad2* adhesin specifically mediates adhesion to plant surfaces, demonstrating that *M. anisopliae* has genes for rhizosphere competence that are induced by RE.

INTRODUCTION

The entomopathogen *Metarhizium anisopliae* is a soil fungus with worldwide distribution (Zimmermann, 2007). Some strains of *M. anisopliae* are rhizosphere-competent (Hu & St. Leger, 2002; St. Leger, 2008), which may explain the persistence of *M. anisopliae* in soils for long periods of time in the absence of an insect host (Bidochka *et al.*, 2001). In grasslands, populations of *M. anisopliae* can reach up to 10^6 propagules g^{-1} at 2–6 cm deep (Milner, 1992).

M. anisopliae is presumably subject to different selective pressures during insect colonization and soil survivability (Prior, 1992; St. Leger, 2008; Wang *et al.*, 2005), but

overall, the genetic groups of *M. anisopliae* and their geographical distribution seem shaped by their adaptability to specific soils and habitat types rather than for their pathogenicity to insects (Bidochka *et al.*, 1998; Quesada-Moraga *et al.*, 2007). It is likely that the distribution of different genotypes of *M. anisopliae* will depend 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). In addition insects, plants and microbial populations will interact at the soil–root interface to affect *Metarhizium* populations (St. Leger, 2008). Similarly, complex interactions occur with the best-known rhizosphere-competent fungus *Trichoderma harzianum*. This fungus is also used as a biocontrol agent because it can parasitize plant fungal pathogens and establishes a symbiotic relationship with the roots of some plants, increasing plant growth and productivity (Harman, 2006). Complex interactions between multiple strains of *Trichoderma*, the roots of plants and other fungal pathogens suggest an established molecular cross-talk between them (Woo *et al.*, 2006). However, the genetic and physiological factors controlling

rhizosphere competence in fungi, including *Trichoderma*, are little understood (St. Leger, 2007). In fact, for many economically important pathogens, the least studied aspect of their biology involves the extended periods for which they survive in soil in the absence of a suitable host (Kerry, 2000). Thus, while a lot of research has been performed on the entomopathogenicity of *M. anisopliae*, its saprophytic lifestyle has received little consideration. Presumably, the same functionally related genes could be adaptive for multiple different environments [insect cuticle, insect blood and root exudates (REs)], but there might also exist different subsets of genes that are adaptive to each environment (Wang *et al.*, 2005).

In this study, we performed a time-course evaluation of gene expression by *M. anisopliae* growing on plant REs as the first step in elucidating pathways that are used by this fungus for soil survivability and adaptability. We need this knowledge to be able to predict and control outbreaks of plant or animal disease. In addition, lack of knowledge of the fate of fungal genotypes at the population and ecosystem levels creates an inherent uncertainty about the efficacy, survivability and environmental risk posed by any introduced or engineered fungi. 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.

METHODS

Bean REs. In order to simulate rhizospheric conditions in the laboratory, *M. anisopliae* was grown on bean REs from black pea seeds (*Vigna unguiculata* subsp. *unguiculata*). To obtain the RE, bean seeds were disinfected by soaking them for 2 min in 95 % ethanol, rinsed in sterile distilled water and transferred to 30 % hydrogen peroxide (Fisher Scientific) for 1 min. The disinfected seeds were rinsed three times in sterile distilled water and placed on wet, sterile paper for initial germination. Seedlings were then transferred to containers with sterile distillated water and kept there connected to an aquarium air pump until roots were formed (about 1 week). The water in which the plants were growing was collected in 50 ml sterile tubes and freeze-dried. The dried RE was dissolved in sterile water to produce a stock solution of 40 mg ml^{-1}, and filter-sterilized before storage at $-20\ ^\circ$C.

Fungal strains and spore germination in REs. We evaluated 11 entomopathogens from the genera *Beauveria* and *Metarhizium*, and two soil saprophytes, *Aspergillus niger* ATCC 10574 (non-rhizospheric) and *T. harzianum* strain T22, a model rhizosphere-competent strain (Harman, 2006), at five concentrations of bean RE (1, 2.5, 5, 10 and 20 mg ml^{-1}). Information about the geographical area and insect host (if entomopathogenic) of each fungal strain is shown in Supplementary Table S1.

Each fungal strain was grown on Sabouraud dextrose agar (SDA) (Fisher Scientific) for 2 weeks at 27 $^\circ$C. A final concentration of 10^4 spores ml^{-1} in 0.01 % Tween 20 was prepared, and 100 µl was added to sterile tubes containing 900 µl of the selected concentration of RE. Fungal spores were also added to 0.1 % yeast extract (Fisher Scientific) and sterile distilled water as positive and negative controls,

respectively. Three replicates were performed for each treatment combination (fungal strains and RE concentrations).

The percentage spore germination of each fungus was determined after 24 h incubation at 27 $^\circ$C with shaking (250 r.p.m.). In order to detect differences between *T. harzianum* and *M. anisopliae*, we also evaluated their germination at concentrations lower than 1 mg ml^{-1} (0.01, 0.05, 0.1 and 0.5 mg ml^{-1}).

Data were analysed using one-way analysis of variance (ANOVA) (SAS Institute Inc, 2006). A *P* value <0.05 determined the significance level.

Culture conditions and total RNA extraction. For microarray analyses, *M. anisopliae* mycelium inocula from Sabouraud dextrose broth (SDB) cultures (Wang *et al.*, 2005) were suspended in 20 ml sterile distilled water. Five millilitres of the mycelial suspension was transferred to four 50 ml flasks, each containing sufficient RE to give a final concentration of 5 mg RE ml^{-1}. This relatively high concentration was not limiting to growth during the course of the experiment, thus avoiding changes in gene expression due to nutritional deprivation alone. Samples were incubated at 27 $^\circ$C at 220 r.p.m., and mycelium was collected from individual flasks after 1, 4, 8 and 12 h of incubation. Total RNA was immediately extracted from the samples using the Qiagen RNeasy Mini kit according to the manufacturer's instructions, and treated with Qiagen DNase I. Two biological replicates were performed.

cDNA microarray. For microarray analysis, we used cDNAs isolated from three libraries that were previously constructed using mycelia of *M. anisopliae* (ARSEF 2575), grown on insect cuticle (Freimoser *et al.*, 2003), or grown in insect blood or plant REs (Wang *et al.*, 2005). PCR-amplified expressed sequence tags (ESTs) were spotted on γ-aminopropyl II-coated glass slides (Corning, Inc.), following the standard protocol developed at the University of Maryland Biotechnology Institute (http://www.umbi.umd.edu/carb/core-facilities/microarray-services/facility.php). A total set of 1748 unigenes (870 from cuticle, 276 from haemolymph and 602 from the root library) were printed in triplicate per slide. Blanks using water and saline sodium citrate (SSC) buffer were also included 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).

Microarray design and RNA hybridization. To analyse expression profiles in RE, we applied a loop design protocol, where every time point was compared with the previous time point. The contrast between adjacent time points provides 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 h) was amplified and directly labelled with Cy3 (green) and Cy5 (red) cyanine dyes using the Ovation Aminoallyl system (Nugen). Five slides were hybridized per replicate (time 0 vs 1 h, 1 h vs 4 h, 4 h vs 8 h, 8 h vs 12 h and 12 h vs time 0). Competitive hybridization of the second biological replicate was performed using a reverse dye-assignment to eliminate bias from dye incorporation.

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 an Axon 4200 microarray scanner (Molecular Devices). To obtain differential signal expression, the TIFF images generated (two image files per hybridized slide) were analysed using the Spotfinder program (http://www.tm4.org/spotfinder.html). Data were then normalized using the LOWESS (LOcally WEighted Scatterplot Smoothing) technique with MIDAS software (http://www.tm4.org/midas.html). MIDAS output files included standard deviation regularization, low-intensity filters and in-slide replicate analysis.

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). To account for the 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 of 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.

Validation of differentially expressed genes by RT-PCR. Total RNA extracted for microarray analysis was also used for RT-PCR using the VersocDNA kit (Thermo Fisher Scientific) and oligo-dT primers according to the manufacturer's protocol. The cDNA product was subsequently amplified by PCR using gene-specific primers. As a control, RT-PCRs were also performed using the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (*gpda*) gene.

RESULTS

Fungal strains and spore germination on bean REs

All fungal strains showed some germination in RE at 1 mg ml^{-1}. *T. harzianum* and the broad-host-range *M. anisopliae* strain 2105 showed the highest germination rates at the five concentrations of RE (ranging from 1 to 20 mg ml^{-1}) (Fig. 1a). These germination rates ranged from 96.8 to 99.3 %, and did not differ significantly from each other. *M. anisopliae* strains 2575, 549 and 1080 with a broad host range had germination rates ranging from 76.6 to 93.9 % at 1 mg ml^{-1} of RE, germination rates ranging from 91.6 to 95.2 % at 2.5 mg RE ml^{-1}, and germination rates ranging from 87 to 93 % at 5 mg ml^{-1}. These germination rates were not significantly different from those of the model rhizospheric fungus *T. harzianum* at the same concentrations of RE. However, strain 2575 showed a significantly lower germination rate as compared with *T. harzianum* at concentrations greater than 5 mg RE ml^{-1} (Fig. 1a).

The specialist *M. acridum* strain 324, specific to locusts and related grasshoppers (Orthoptera: Acrididae), is not rhizosphere-competent (O'Brien, 2008). We also tested other strains of *Metarhizium* with unknown host ranges, including *M. anisopliae* strains 4600 and 4620, which were isolated from the Tasmanian soldier fly (Diptera: Stratiomyidae), and *M. anisopliae* strain 2974, recently reclassified in the Agriculture Research Service Entomopathogenic Fungal Collection (ARSEF) collection as *Metarhizium brunneum*, originally isolated from a mosquito (Diptera: Culicidae) (Supplementary Table S1). These strains have very low rhizosphere competence as determined by microcosm studies (O'Brien, 2008). *M. acridum* 324 showed its highest germination rate (47.3 %) at 1 mg RE ml^{-1} and its lowest germination rate (24.7 %) at 20 mg RE ml^{-1}. Germination rates of *M. acridum* 324 were significantly different from

those of both *T. harzianum* and *A. niger* at each concentration of RE (Fig. 1a). The lowest germination frequencies with 1 mg RE ml^{-1} were shown by *M. anisopliae* strains 4620 (22.5 %), 2974 (12.5 %) and 4600 (6.5 %), and *A. niger* (19.3 %). However, germination rates of the *M. anisopliae* strains increased at higher concentrations of RE (except at 20 mg ml^{-1}, when strains 4620 and 2974 showed slightly reduced germination rates), while the germination rate of *A. niger* declined with increasing concentrations of RE (germination rates of *A. niger* were 19.3, 14, 13, 2 and 0 % at concentrations of 1, 2.5, 5, 10 and 20 mg RE ml^{-1}, respectively) (Fig. 1a).

B. bassiana strains 9205 and 9112 were isolated from Lepidopteran hosts (Supplementary Table S1). *B. bassiana* strain 3113 was isolated from soil but is an endophyte able to colonize corn plants and provide protection against lepidopteran pests (Pingel & Lewis, 1996; Wagner & Lewis, 2000). At 1 mg RE ml^{-1}, germination rates of *B. bassiana* strains 9205, 9112 and 3113 were 73, 50.5 and 64 %, respectively. These germination rates were not significantly different from each other, but they were significantly higher than those of *A. niger* and *Metarhizium* strains 4620, 2974 and 4600 (Fig. 1a). Germination rates of *B. bassiana* strains 9112 and 3113 were significantly lower than those of the *M. anisopliae* generalist strains (2105, 2575, 549 and 1080) and *T. harzianum* strain T22. *B. bassiana* strains 9112 and 9205 showed slightly increased germination at 2.5 and 5 mg RE ml^{-1}, but germination by 9211 decreased significantly at 20 mg RE ml^{-1}. Germination rates of *B. bassiana* strain 3113 gradually declined at concentrations >1 mg ml^{-1} and had diverged significantly from the other fungal strains at 10 mg RE ml^{-1} (Fig. 1a).

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^{-1}. Fig. 1(b) shows that at concentrations $\leqslant$0.5 mg ml^{-1} the germination rate of *T. harzianum* was significantly lower than that of *M. anisopliae* 2575. The percentage germination of strain 2105 was 1, 12.5 and 42 % with 0.01, 0.05 and 0.1 mg RE ml^{-1}, respectively, whereas germination rates of strain 2575 were 88.6, 98 and 93 % at the same concentrations. This indicates that strain 2575 of *M. anisopliae* is hypersensitive to RE.

Microarray data analysis

Out of 1748 genes included in the cDNA microarrays, we identified 50 genes (2.9 %) that were differentially expressed across the time points evaluated. Differentially expressed genes were automatically detected using BATS and ranked according to their Bayes factors (BF <0.006). Nucleotide query sequences 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 reorganized into previously classified groups of functionally related genes (Wang *et al.*, 2005) (Fig. 2a). *M. anisopliae* genes that
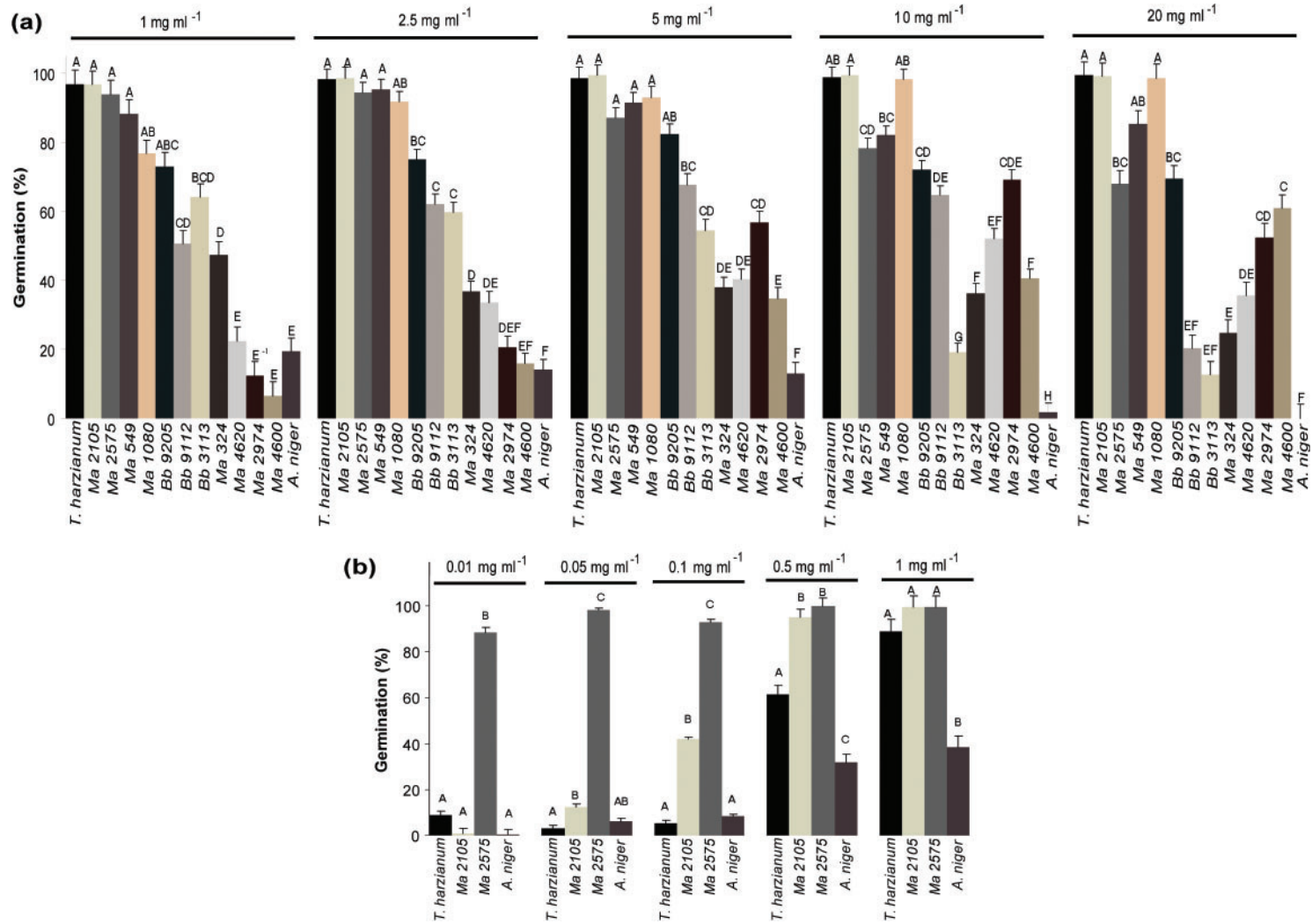
Fig. 1. Germination of *Metarhizium* (Ma) strains, *B. bassiana* (Bb) strains, *T. harzianum* (rhizospheric) and *A. niger* at different concentrations of REs. (a) Concentrations of RE from 1 to 20 mg ml^{-1}. (b) Concentrations of RE <1 mg ml^{-1}. Mean values labelled with the same letter are not significantly different ($P<0.05$).
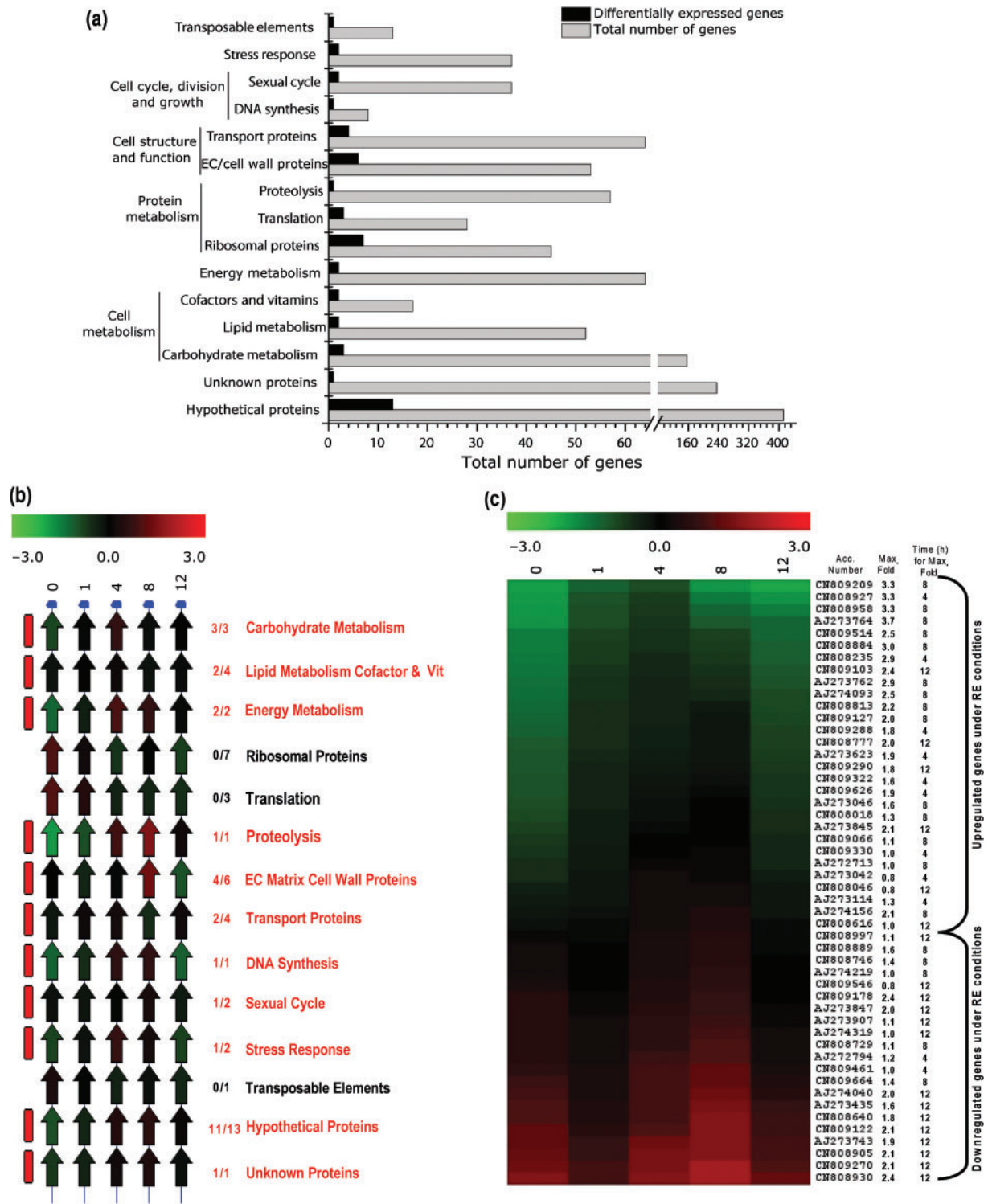
Fig. 2. Fifty genes differentially expressed by *M. anisopliae* 2575 during growth in RE. (a) Differentially expressed genes compared with the total number of genes in each functional group. (b) LEM representing the mean of the differentially expressed genes organized according to functional groups. The vertical red bars show functional groups that contain genes that were upregulated at one or more points. The number of upregulated genes/total number of differentially expressed genes per functional group is also shown. (c) Pattern of expression of the differentially expressed genes after 0, 1, 4, 8 and 12 h growth in RE. GenBank accession numbers, the maximum fold values (Max. fold) and the time (h) when the maximum fold value occurred are provided.

were differentially expressed under RE conditions over 12 h included 13 out of 413 arrayed hypotheticals (predicted sequences with homologues in other organisms but no experimental evidence of function) (13/413, 3.1 %), an orphan sequence with no homologues in databases (1/236, 0.4 %) and genes involved in 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 %), transposable elements (1/13, 7.7 %), and stress response and defence (2/107, 1.9 %) (Fig. 2a).

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

Fig. 2(b) shows the linear expression map (LEM) with the mean of the $\log_2$ ratio of differentially expressed genes classified by functional groups at each time point. Of the 50 differentially expressed genes, 29 (58 %) were upregulated across all time points when the fungus was transferred from SDB (time 0) to RE. They were involved in carbohydrate metabolism (3/29, 10.4 %), lipid metabolism, cofactors and vitamins (2/29, 6.9 %), energy metabolism (2/29, 6.9 %), proteolysis (1/29, 3.4 %), extracellular matrix/cell wall proteins (4/29, 13.9 %), transport proteins (2/29, 6.9 %), DNA synthesis (1/29, 3.4 %), the sexual cycle (1/29, 3.4 %), stress response (1/29, 3.4 %), hypothetical proteins (11/29, 37.9 %) and unknown proteins (1/29, 3.4 %) (Fig. 2b).

The level of expression of individual genes, the maximum fold change and the time (h) when the maximum fold change occurred are presented in Fig. 2(c). Of the 29 genes that were upregulated in RE, nine (31 %) obtained their maximum fold change after 4 h, 14 (48 %) after 8 h and six (21 %) after 12 h (Fig. 2c).

Supplementary Table S2 shows the $\log_2$ expression ratio through time of the 50 differentially expressed genes. The greatest fold increases were observed in the hypothetical genes. Gene AJ273764 was upregulated 3.7-fold (after 8 h), and genes CN808927 and CN809209 were both upregulated 3.3-fold after 4 and 8 h, respectively. Genes CN808884, CN809514 and AJ274093 were upregulated 3.0-, 2.5- and 2.5-fold, respectively, after 8 h. The expression of the subtilisin-like serine protease (Pr1A) gene (CN808958) increased through time in RE, being upregulated 3.3-fold after 8 h. Genes involved in energy metabolism, dihydrolipoyl dehydrogenase (AJ273762) and oxidoreductase (CN808777), were upregulated 2.9- and 2.0-fold after 8 and 12 h, respectively. A stress response heat-shock protein gene (CN808235) was upregulated 2.9-fold after 4 h in RE. Transport proteins, including an ABC transporter ATP-binding protein (CN809103) and a GABA (γ-amino-n-butyrate) permease (CN808046) were upregulated 2.4- and 0.8-fold, respectively, after 12 h. Genes

required for the metabolism of carbohydrates such as glycosyl hydrolase (CN808813), β-glucosidase (AJ273623) and ferulic acid esterase A (*faeA*) (AJ273114) were also upregulated 2.2-fold (after 8 h), 1.9-fold (after 4 h) and 1.3-fold (after 4 h), respectively. RE also increased the expression of genes encoding cell wall proteins and the associated extracellular matrix, including the hydrophobin-like protein precursor (AJ274156) and the predicted cell wall protein AJ273845, which were both upregulated 2.1-fold at 8 and 12 h. Also, the cell wall MAD2 adhesin (CN809626), which is required by *M. anisopliae* to adhere to plant surfaces (Wang & St. Leger, 2007), and a close homologue (CN809322) were upregulated 1.9- and 1.6-fold, respectively, after 4 h. 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 cofactors and vitamins (amidase protein, AJ273042) were also upregulated between 2.0- and 0.8-fold after 4 and 8 h in RE. Although listed as a sex cycle gene, the gene for EsdC is involved in cell wall morphology and is expressed in asexual as well as sexual processes in *Aspergillus* (Han *et al.*, 2008).

Twenty-one differentially expressed genes (42 %) were downregulated in RE (Fig. 2c, Supplementary Table S2). Two genes (9.5 %) were downregulated ~ 1.2-fold after 4 h; 5 genes (23.8 %) were downregulated 1.0 to 1.6-fold after 8 h and 14 genes (66.7 %) were downregulated 0.8 to 2.4-fold after 12 h (Fig. 2c). Downregulated genes included ribosomal proteins, the cold acclimation-induced protein 2-1 (CN808997), the carrier protein ADP/ATP translocase (CN809461) and hypothetical protein AJ272794. Expression of hydrophobin genes (AJ273847 and CN809178) was initially downregulated, but recovered so that by 8 h these genes were upregulated 1.5- and 1.6-fold, respectively. The C-3 sterol dehydrogenase protein AJ274219, the potassium channel protein CN808889 and the hypothetical protein CN808746 were upregulated during the first 4 h of incubation in RE before slowly declining, suggesting a role in the early stages of adaptation to RE.

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 the microarray results (Fig. 3).

DISCUSSION

Root-mediated rhizosphere interactions are classified as being either positive or negative for the plant (Bais *et al.*,

2006). Mycorrhizal associations between fungi and plant roots are clearly positive, being essential for the survival of both partners. While the fungus obtains carbohydrates such as glucose and sucrose (final products of photosynthesis), the plant benefits from the increased uptake of water and nutrients, including nitrogen and phosphorus (Harman & Shoresh, 2007). REs represent a significant carbon cost to the plant (Bais *et al.*, 2006), but there is clearly coevolutionary potential in favouring populations of mycorrhizal fungi and also, presumably, populations of pathogens of herbivorous insects. It would not therefore be surprising if entomopathogenic fungi benefitted from components of REs when adapting to soil environments.

REs 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 (Hale *et al.*, 1978; Rovira, 1965). Our results show that generalist strains of *M. anisopliae* (ARSEF 2105, 2575, 549 and 1080) are as good at germinating in RE as the model rhizosphere-competent fungus *T. harzianum* strain, 22. Specialist strains including strain 324 have lost genes compared with generalists, including genes for carbohydrate metabolism that may be required for a saprophytic existence on plant roots (Wang *et al.*, 2009). Consistent with this, some strains of *M. anisopliae* with reduced rhizosphere competence need higher concentrations of REs to germinate (4620, 2974 and 4600). However, high concentrations of RE inhibit growth of *M. acridum* 324 and generalist strains (2105, 2575, 549 and 1080) to varying extents (Fig. 1a),
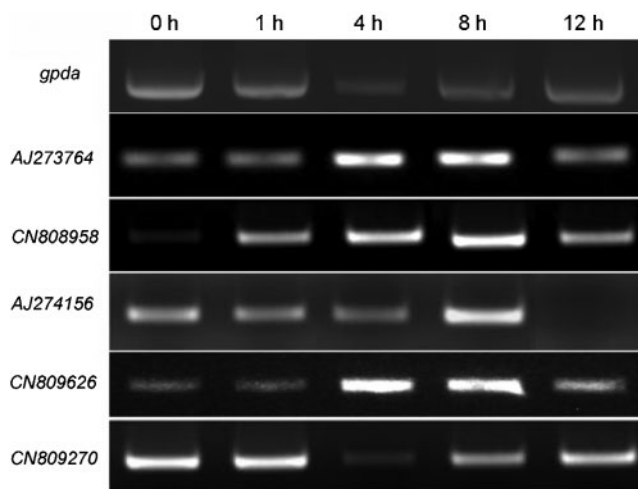
indicating that strains respond differently to the inductive and repressive components of RE. The non-rhizospheric fungus *A. niger* was the most sensitive to the inhibitors in RE, indicating that is not adapted to resist antimicrobial exudate components. Fungi capable of rhizosphere competence presumably need to evolve resistance to these compounds, which are thought to have a role in defending the rhizosphere against pathogens (Bais *et al.*, 2006).

M. anisopliae is found in most soils worldwide, and is able to survive for long periods of time in cultivated grassland soils without an insect host (Bidochka *et al.*, 1998; Milner, 1992; Quesada-Moraga *et al.*, 2007; St. Leger, 2008). Our results show that this fungus contains a set of differentially expressed 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 is mainly downregulated after 24 h at a low concentration of RE (0.1 mg ml^{-1}). Here we demonstrated that the upregulation of genes under RE conditions occurs at earlier time points (mainly 4 and 8 h post-inoculation into RE), and that these upregulated genes have fold increases between 0.8 and 3.7 (Fig. 2c).

The category with the highest percentage of RE-induced upregulated genes (41.4 %) encodes hypothetical or unknown proteins, indicating that many previously uncharacterized *Metarhizium* genes may have functions related to saprophytic survival. Some of these genes have orthologues in plant pathogens and other soil fungi, so whatever role they play in adapting to soil conditions may be conserved. The presence of at least 10 sugars (including glucose, fructose, sucrose, raffinose, 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, *β*-glucosidase AJ273623 and ferulic acid esterase AJ273114) were upregulated during the first 8 h under RE conditions. A formidable array of hydrolytic enzymes is secreted during the pathogenic life cycle of *M. anisopliae*, and these include the subtilisin protease Pr1A (CN808958) (Bagga *et al.*, 2004; Wang *et al.*, 2009). Pr1A was upregulated within 4 h of incubation in RE, and high-level expression was maintained throughout the time-course, demonstrating an important long-term role in proteolysis for Pr1A in the utilization of RE. Cell wall proteins (e.g. adhesin proteins CN809626 and CN809322) were also upregulated in the first 4 h. CN809626 encodes MAD2 (*Metarhizium* adhesin-like protein 2). Its detection in a screen of genes upregulated by RE helps validate the principle of using RE to identify genes involved in rhizosphere interactions. Strains of *Metarhizium* disrupted in *Mad2* show a 90 % reduction in adherence to plant cells (Wang & St. Leger, 2007). In this study, we found an EST sequence in the root library CN809322, originally designated an unknown protein, but with 98 % similarity (E value 2e-136) to the MAD2 transcript (CN809626). Although expressed at lower levels than MAD2, it had an identical pattern of gene expression in RE (Supplementary Table S2).

Fig. 3. RT-PCR to validate microarray gene expression of *M. anisopliae* 2575 growing in REs. 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). The glyceraldehyde-3-phosphate dehydrogenase (*gpda*) gene was used as the reference gene.

This suggests that CN809322 is also involved in attachment to plant surfaces, and it is perhaps responsible for the 10 % of *Mad2* mutant spores that are still adherent. An EST from the root library (CN809103) that was originally designated an unknown protein showed most (46 %) similarity to an ABC transporter (ATP-binding protein) from the symbiotic nitrogen-fixing actinobacterium *Frankia alni* (Supplementary Table S2). 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 defence 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). A putative ABC transporter gene (CN809103) of *M. anisopliae* was upregulated after 1 h under RE conditions with a 2.4-fold increase after 12 h. This indicates that the protein may be required to facilitate the efflux of compounds present in RE that may be toxic to the fungus.

The rhizosphere competence of *M. anisopliae* and knowledge of the genes involved as it adapts and persists in soil environments is important when considering the potential commercial use of biological control agents against root herbivores and soil-borne plant pathogens. The long-term genetic adaptations and evolution of this fungus in cultivated soils (turf plots) are also being studied in our laboratory (O'Brien, 2008). Comprehensive knowledge of short- and long-term 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, and 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. The inability of mutants disrupted in MAD2 to adhere to plant roots provided a clear test of the importance to the fungus of root interactions, and confirms that the most informative approach for analysing the hypothetical proteins will involve comparisons with mutant fungi. Also, comparison of gene expression among rhizosphere-competent and non-rhizosphere-competent strains of *Metarhizium* would complement studies on the physiology of this fungus.

ACKNOWLEDGEMENTS

REFERENCES

Angelini, C., Cutillo, L., De Canditiis, D., Mutarelli, M. & Pensky, M. (2008). BATS: a Bayesian user-friendly software for Analyzing Time Series microarray experiments. *BMC Bioinformatics* **9**, 415.

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

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S. & Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* **57**, 233–266.

Bidochka, M. J., Kasperski, J. E. & Wild, G. A. M. (1998). Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Can J Bot-Rev Can Bot* **76**, 1198–1204.

Bidochka, M. J., Kamp, A. M., Lavender, T. M., Dekoning, J. & De Croos, J. N. A. (2001). Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? *Appl Environ Microbiol* **67**, 1335–1342.

Freimoser, F. M., Screen, S., Bagga, S., Hu, G. & St Leger, R. J. (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* **149**, 239–247.

Hale, M. G., Moore, L. D. & Griffin, G. J. (1978). Root exudates and exudation. In *Interactions between Non-Pathogenic Soil Microorganisms and Plants*, p. 475. Edited by Y. R. Dommergues & S. V. Krupa. Amsterdam: Elsevier Scientific Publishing Company.

Han, K. H., Kim, J. H., Moon, H., Kim, S., Lee, S. S., Han, D. M., Jahng, K. Y. & Chae, K. S. (2008). The *Aspergillus nidulans esdC* (early sexual development) gene is necessary for sexual development and is controlled by *veA* and a heterotrimeric G protein. *Fungal Genet Biol* **45**, 310–318.

Harman, G. E. (2006). Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**, 190–194.

Harman, G. E. & Shoresh, M. (2007). The mechanisms and applications of symbiotic opportunistic plant symbionts. In *Novel Biotechnologies for Biocontrol Agent Enhancement and Management*, p. 374. Edited by M. Vurro & J. Gressel. Dordrecht, The Netherlands: Springer.

Hu, G. & St. Leger, R. J. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl Environ Microbiol* **68**, 6383–6387.

Kerry, B. R. (2000). Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-parasitic nematodes. *Annu Rev Phytopathol* **38**, 423–441.

Milner, R. J. (1992). Selection and characterization of strains of *Metarhizium anisopliae* for control of soil insects in Australia. In *Biological Control of Locusts and Grasshoppers*, pp. 200–207. Edited by C. J. Lomer & C. Prior. Wallingford, UK: CAB International; International Institute of Tropical Agriculture.

O'Brien, T. R. (2008). *Metarhizium anisopliae's persistence as a saprophyte, genetic basis of adaptation and role as a plant symbiont.* PhD thesis, University of Maryland.

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

Prior, C. (1992). Discovery and characterization of fungal pathogens for locust and grasshopper control. In *Biological Control of Locusts and Grasshoppers*, pp. 159–180. Edited by C. J. Lomer & C. Prior. Wallingford, UK: CAB International.

Quesada-Moraga, E., Navas-Cortés, J. A., Maranhao, E. A. A., Ortiz-Urquiza, A. & Santiago-Álvarez, C. (2007). Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. *Mycol Res* **111**, 947–966.

Rovira, A. D. (1965). Plant root exudates and their influence upon soil microorganisms. In *Ecology of Soil-Borne Plant Pathogens, Prelude to Biological Control*, pp. 571. Edited by K. F. Baker, W. C. Snyder, R. R. Baker & others. Los Angeles: University of California Press.

SAS Institute Inc (2006). *SAS/Procedures guide version SAS® 9.1.3.* Cary, NC, USA: SAS Institute, Inc.

St. Leger, R. J. (2007). *Metarhizium anisopliae* as a model for studying bioinsecticidal host pathogen interactions. In *Novel Biotechnologies for Biocontrol Agent Enhancement and Management*, pp. 179–204. Edited by M. Vurro & J. Gressel. New York/Heidelberg: Springer.

St. Leger, R. J. (2008). Studies on adaptations of *Metarhizium anisopliae* to life in the soil. *J Invertebr Pathol* **98**, 271–276.

Wagner, B. L. & Lewis, L. C. (2000). Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. *Appl Environ Microbiol* **66**, 3468–3473.

Wang, C. S. & St. Leger, R. J. (2007). The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. *Eukaryot Cell* **6**, 808–816.

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

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

Wolfger, H., Mamnun, Y. M. & Kuchler, K. (2001). Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. *Res Microbiol* **152**, 375–389.

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

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci Technol* **17**, 879–920.

Edited by: W. Achouak