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ENVIRONMENTAL BIOTECHNOLOGY

Application of the entomogenous fungus, *Metarhizium anisopliae*, for leafroller (*Cnaphalocrocis medinalis*) control and its effect on rice phyllosphere microbial diversity

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Abstract Microbial pesticides form critical components of integrated pest management (IPM) practices. Little, however, is known regarding the impacts of these organisms on the indigenous microbial community. We show that *Metarhizium anisopliae* strain CQMa421 was highly effective in controlling the rice leafroller, *Cnaphalocrocis medinalis* Guenee. In addition, *M. anisopliae* distribution and its effects on phyllosphere microbial diversity after application in field trials were investigated. Phylloplane specific distribution of the fungus was observed over time, with more rapid declines of *M. anisopliae* CFUs (colony-forming units) seen in the top leaf layer as compared to lower layers. Application of the fungus resulted in transient changes in the endogenous microbial diversity with variations seen in the bacterial observed species and Shannon index. Notable increases in both parameters were seen at 6-day post-application of *M. anisopliae*, although significant variation within sample replicates for bacteria and fungi were noted. Application of *M. anisopliae* increased the relative distribution of bacterial species implicated in plant growth promotion and organic pollutant degradation, e.g., *Methylobacterium*, *Sphingobium*, and *Deinococcus*. These data show minimal impact of *M. anisopliae* on endogenous microbial diversity with transient changes in bacterial abundance/diversity that may result in added benefits to crops.

Keywords *M. anisopliae* · Distribution · Phyllosphere · Microbial diversity · Microbial community

Introduction

The use of microbes is increasingly being recognized as a viable option for insect control. Over 50 different genera of entomopathogenic viruses, bacteria, fungi, and nematodes are currently commercially available and are being used as part of integrated pest management practices (IPM) (Glare et al. 2012; Lacey et al. 2015). Interest in the use of these organisms has grown as few new chemical pesticides are being approved and increased health concerns prompt regulatory restrictions on synthetic chemical pesticides (Ansell 2008; Bielza et al. 2008; Marx-Stoelting et al. 2011). However, widespread adoption and use of microbial biological control agents, especially in agriculture and for staple crops, remains lagging in part due to issues of cost and effectiveness. In addition, little is known concerning the effects of foliar application of the significant numbers of microbial cells needed for effective pest control on indigenous microbial communities.

Entomopathogenic fungi play a crucial natural ecological role in controlling insect populations (Roberts and St Leger 2004; Gillespie and Claydon 2006). Most insect pathogenic fungi belong to the orders *Entomophthorales* and *Hypocreales*

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(Hussain et al. 2014). Presently, over 7000 isolates of insect-pathogenic fungi, mostly *Metarhizium* and *Beauveria* spp., are available and can service as mycoinsecticides targeting over several hundred different insect pests worldwide (Hussain et al. 2014; Lacey et al. 2015). Although a wide range of formulations have been developed, most contain one of several infectious propagules including aerial conidia, blastospores, or in the case of some *Metarhizium* spp., sclerotia (Mascarin and Jaronski 2016; Wraight et al. 2016). A number of environmental and ecological considerations have also been considered, particularly non-target effects on beneficial insects and indigenous microbes and persistence issues (Hajek and Goettel 2007; Babendreier et al. 2015; Lacey et al. 2015). As agricultural applications require dispersal of the fungal agent on plants where indigenous microbial communities, resident within the phyllosphere exist, an examination of the effects of the fungus on these communities is important. Persistence of the biological agent after application also affects the efficacy of target pest control (Collins et al. 2003).

Phyllosphere microbiota are highly diverse and include many different bacteria, filamentous fungi and yeasts, algae, and various protists and other single-celled eukaryotes (Lindow and Brandl 2003). The phyllosphere community composition is dependent upon a wide range of factors, including the plant species, temperature, humidity, nutrient availability (on the plant surface), sun/UV exposure levels, and even the underlying soil geochemistry (Lindow and Leveau 2002). Some phyllosphere microbes are beneficial to plants, e.g., are involved in nitrogen fixation, act as barriers to the growth of plant pathogens, facilitate the acquisition of nutrients, and/or act to degrade or eliminate harmful pollutants (Kvasnikov et al. 1974; Murty 1984; Hirano and Upper 2000; Krechel et al. 2002; Krimm et al. 2005; Schreiber et al. 2005; Sandhu et al. 2007). However, resident phyllosphere microbes also include plant pathogens that upon favorable conditions can result in plant disease. Farming practices often dramatically alter phyllosphere microbial populations, including increasing the levels of some human pathogens, ultimately compromising food safety (Henis and Bashan 1986; Lindow and Leveau 2002). Ideally, agricultural practices that seek to limit insect pests should also be compatible with efforts to limit harmful microbes while promoting the growth and/or activities of beneficial ones.

Rice is a worldwide staple food, consumed almost daily by more than 1 billion people in China alone, where more than 30% of the world's rice production occurs (Yang et al. 2008; Knief et al. 2012). Chemical pesticides are important tools for protecting rice harvests from a wide variety of insect pests. However, many chemical pesticides have unwanted environmental effects including altering the composition of microorganisms within the phyllosphere, potentially decreasing those beneficial to plants and/or increasing harmful ones (Lal and Saxena 1982; Zhang et al. 2009). Use of chemical pesticides may decrease microbial biomass due to direct toxicity (Ko and Lockwood 1986), but in some cases may increase microbial biomass due to the active and/or inert ingredient acting as a nutrient source selecting for specific microbes (Chinalia and Killham 2006). A number of studies have assessed the effects of chemical pesticides on microbial communities in a range of environments (Salonlus 1972; Walter et al. 2007; Vig et al. 2008; Moulas et al. 2013), and in some instances, indirect effects, e.g., decreases in microbial predator populations, or only minor effects, have been reported (Pandey and Chauhan 2007). Regarding the latter, strawberry phyllosphere microbiota were not greatly affected by application of the bacterium, *Bacillus subtilis* (Wei et al. 2016), and no significant effects were observed on fungal and bacterial communities of strawberry leaves after application of the fungal agents *Aureobasidium pullulans* or *Beauveria bassiana* (Sylla et al. 2013).

In this study, we investigated the pest control efficacy, distribution, and effects on phyllosphere microbiota in field trials of *Metarhizium anisopliae* (CQMa421) applied as a foliar spray targeting the rice leafroller, *Cnaphalocrocis medinalis* Guenee. Phyllosphere microbial diversity and community structure were examined during the reproductive (booting, ~52 days after sowing) stage of rice (*Oryza sativa*). Microbial diversity was probed using next-generation sequencing (NGS) technology of the V4 hypervariable region of the 16S ribosomal RNA (rRNA) gene for bacterial identification and the rRNA first internal transcribed spacer (ITS1) region for fungal identification (Fierer et al. 2012; Edwards et al. 2015; Prober et al. 2015).

Materials and methods

Cultivation of fungi and field trials

M. anisopliae var. *anisopliae* strain CQMa421 (strain accession number: CGMCC NO. 460) used in the field trials was provided by the Genetic Engineering Research Center, College of Life Science at Chongqing University, China. Application of *M. anisopliae* strain CQMa421 in oil miscible suspension formulation (8×10^{10} spores/ml) was prepared as described (Peng et al. 2008). Briefly, mycelia were first produced in a liquid fermentation reactor and then inoculated into autoclaved rice with 40–50% water in volume for producing conidia. Conidia were harvested after 15 days of growth, allowed to air-dry, and suspended in soybean oil. The fungal oil miscible suspension formulation was made by mixing the soybean-*M. anisopliae* oil formulation to the indicated dilution with a mixture comprising 40% water, 56% soybean oil, and 4% (*v*/*v*) mixed emulsifiers (Span-80 and Tween-20). The formulation was thoroughly mixed at high speed in a beater. The concentration of the *M. anisopliae* oil formulation was adjusted to 8×10^{10} spores/ml by serial dilution and verified

by spore counting using a Petroff–Hausser counting slide. Field trail experiments were performed in plots separated by nylon net cages (80 mesh, 5–10% shade) 180 cm in height. The experimental plot was located at the Genetic Engineering Research Center, Chongqing University, China. Rice-germinated shoots (~800 *O. sativa* subsp. QU5, obtained from Chongqing Academy of Agricultural Science) were planted in six 15 m^2 plots with line width of 30 cm × 40 cm on April 13, 2015. The experiment was performed as a randomized complete block in a split–split plot design with three replications. During the growth phase, the daily air temperature high ranged from 30.4 to 38.1 °C (mean 34.5 °C), the average relative air humidity was approximately 90%, and illumination time ranged between 0 and 11.8 h in the field (mean 6.5 h). Plants were watered and fertilized in accordance to local grower practices, including addition of urea (5 kg/120 m^2) as a fertilizer during the early plant tillering stage. Any visible weeds were removed by hand before *M. anisopliae* was applied, and hand weeding was performed once more during the study period.

M. anisopliae application and bioassays for *C. medinalis*

M. anisopliae spores prepared as indicated above were applied to treatment plots and the oil miscible formulation lacking any fungal spores was applied to control plots when rice leaf rolls with *C. medinalis* larvae appeared (typically during the later period of rice tillering). *M. anisopliae* was applied to treatment plots at the rate of 2.5 ml of formulated product mixed 200 ml sterile water per plot. All plots were checked every 3 days after *M. anisopliae* treatment. Time points included 0 day before application and 3, 6, 9, and 12 days after *M. anisopliae* applied. A five-point sampling mode per plot was performed, and two rice clumps per point were checked. The effect of *M. anisopliae* on the leafroller rice pest was assessed via examination of the number of new leaf rolls as this represents the phenotypic consequence of the insect on the rice leaf, i.e., insect activity results in a characteristic rolling of the leaves. Two ratios were measured: (1) percentage of new leaf rolls (%) and (2) survival rate of *C. medinalis* (%). The percentage of new leaf rolls (%) was calculated by measuring [total new leaf rolls in a rice clump 3 days after the previous sampling time point]/[total leaf in the rice clump at the sampling time point]. The survival rate of *C. medinalis* (%) was calculated by measuring [total *C. medinalis* in a rice clump at the sampling time point]/[total new leaf rolls in the rice clump 3 days after the previous sampling time point].

M. anisopliae persistence in the rice phyllosphere

Before sample collection for microbiome assessment, *M. anisopliae* persistence was surveyed in the plots. Time points included 0 day within 2 h post-application, and 3, 6, 9, and 12 days after *M. anisopliae* application. There was no rain during the sampling period. For each sample, 30 whole rice leaves per plot were sheared with sterile scissors and placed in a plastic Ziplock bag (24 cm × 35 cm). Sections of the rice leaves were isolated using a sterilizing punch (1 cm^2), and samples were placed in 50 ml Erlenmeyer flasks containing 30 ml of 0.05% (*v*/*v*) Tween-80 solution. The suspension was shaken (250 rpm) for 20 min at room temperature, and then placed in an ultrasonic cleaning bath (Shanghai Kudos Instrument Co. Shanghai, China) for 2 min. Serial dilutions of the suspension were plated using a spiral plater onto selective media [one fourth SDAY media amended with 0.2 g/L chloramphenicol (Solarbio. Beijing, China) and 0.05 g/L dodine (Sigma-Aldrich. St. Louis, MO)]. Plates were incubated in complete darkness at 28 °C for 4 days. *M. anisopliae* colonies were identified, and the number of colony forming units (CFUs/cm^2) was assessed.

Sample collection of microbial community assessment in the rice phyllosphere

For each plot, about 200 g of the upper, middle, and bottom sections of the rice leaf was cut and immediately placed into sterile polythene bags (approximate time of collection ~6 pm for all samples). Samples were immediately transported to the laboratory (less than a half of hour) and further processed within 24 h. For each sample (treatments and controls), the entirety of the 200 g of leaf material was aseptically transferred into a Ziplock bag (24 cm × 35 cm) containing 500 ml sterile precooled TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5) supplemented with 0.05% Tween-80. Leaf surfaces were washed to collect the microbial population from the leaf material by 5 min (each step) of (1) shaking, (2) vortexing, and (3) sonication of the samples in the TE-buffer with the Ziplock bag kept in ice water (~4 °C) for each processing step. The cell suspension was separated from the leaf material by filtration through a sterile nylon mesh. Sonication was performed at a frequency of 40 kHz in an ultrasonic cleaning bath (Shanghai Kudos Instrument Co. Shanghai, China) to dislodge the microbes from leaf surface. Cell suspensions (after filtration) were placed in six 100-ml tubes/sample, and cells were pelleted via centrifugation at 3150×*g* for 15 min at 4 °C (Delmotte et al. 2009). Cell pellets from multiple tubes were pooled into 1.5-ml reaction tubes and washed twice with TE-buffer with Tween-80. Cell pellets were immediately frozen at −80 °C until DNA extraction.

DNA extraction

DNA extraction was performed using the E.Z.N.A.™ Soil DNA Kit (Omega, Norcross, GA) as described (Knief et al. 2012) with slight modifications. Frozen cell pellets were re-suspended in 1 ml of kit-supplied SLX Mlus buffer with

500 mg of glass beads, and cell lysis was performed at 65 Hz for 90 s. The cell debris suspension was immediately processed using procedures according to the instructions in the kit manual. Finally, total DNA was obtained from the column by two sequential elutions with 100 μL elution buffer.

16S rRNA gene V4 and ITS1 amplification, quantification, and sequencing

A total of 54 rice leaf samples corresponding to sections derived from the top, middle, and bottom of the rice leaf layers and over a time course (0 day, within 2 h, and 6 and 12 days, post-application of *M. anisopliae*) were collected from three separate treatment plots and three blank control plots were collected. Sequencing of the V4 hypervariable region of the 16S rRNA gene was performed for bacterial identification, and the first internal transcribed spacer (ITS1) region of the rRNA operon was used for fungal identification (Fierer et al. 2012; Edwards et al. 2015; Prober et al. 2015). Briefly, 35 cycles of polymerase chain reaction (PCR) amplification of the target marker genes was performed. Error-correcting 12-bp barcoded primers specific to each sample were used to permit multiplexing of samples (David et al. 2014; Edwards et al. 2015). PCR products from all samples were quantified using the PicoGreen dsDNA assay, and pooled together in equimolar concentrations. Each library was submitted to BGI (Shenzhen, China), for 250 × 250 paired end, dual index sequencing on an Illumina MiSeq instrument. Illumina sequence reads have been deposited under the NCBI SRA accession number SRP096089.

Sequence analysis

The sequences obtained from the MiSeq runs were demultiplexed using a custom Python script with quality filtering (Edgar 2013). The sequences were overlapped to form contiguous reads using MOTHUR's command make.contigs (Schloss et al. 2009). Sequences were dereplicated and singleton sequences were removed prior to phylotype determination. The sequences were then clustered into operational taxonomic units (OTUs) by UCLUST (Edgar 2010) based on 97% pairwise identity using QIIME's open reference OTU picking strategy that used the Greengenes 13_5 database and UNITE(v20140703) for 16S and ITS rRNA sequences, respectively (Edgar 2013). Taxonomic classification of the representative sequence for each OTU was done using QIIME's version of the Ribosomal Database Project's classifier against the Greengenes 16S rRNA database (13_5 release) and UNITE: Version 6 20140910 (Abarenkov et al. 2010; Cole et al. 2009). All OTUs identified as belonging to chloroplast or mitochondria were removed from the dataset. The representative sequences for each OTU were aligned using PyNAST in QIIME (Caporaso et al. 2010). Chimeric OTUs were identified using QIIME's implementation of ChimeraSlayer and removed from the OTU table and OTU representative sequences file (Haas et al. 2011).

Statistical analysis

The percentage of new leaf rolls caused by *C. medinalis* was used to assess the biopesticide activity of *M. anisopliae*, and the data was subjected to analysis of variance (ANOVA) for a completely randomized design. Analysis of data from the *M. anisopliae* population ($\log_{10}$[CFUs/cm^2]) and sampling date was performed using the General Linear Model (GLM) procedure to describe the relationship (Prober et al. 2015; Ruiz-Gonzalez et al. 2015). OTU tables for each experiment were normalized by the trimmed mean of M values (TMM) method using the BioConductor package EdgeR in R (Robinson et al. 2010). Rarified OTU subsets were generated to calculate the alpha diversity in QIIME. Species richness with observed species index and species diversity using the Shannon index were used to indicate α-diversity in the R package Vegan (Oksanen et al. 2007). To uncover whether the α-diversity index was significantly influenced by *M. anisopliae*, application time and leaf layer position, Sobs and Shannon indexes were examined through multivariate analysis of variance and linear mixed model fitted by maximum likelihood (Prober et al. 2015; Hong et al. 2016). Weighted and unweighted UniFrac distances were calculated from the normalized OTU tables for each experiment (Lozupone and Knight 2005). To explore the bacterial and fungal similarities and differences between samples, ANOVA tests were performed on the weighted and unweighted UniFrac distances matrix and used to determine whether the weighted and unweighted UniFrac distances among samples were significantly different between control and treatment samples (Koren et al. 2012). All statistical tests were two-tailed and were conducted in SPSS 17.0 software (SPSS Inc. Chicago, USA). Data are presented by mean ± SE. The significance level of all analyses was set at 0.05, except when specified.

Biomarker analyses

A linear discriminant analysis effect size (LEfSe) algorithm (Segata et al. 2011) was used to test significant associations between bacterial or fungi taxa and groups (control vs *M. anisopliae* applied). The LEfSe algorithm was used to discover biomarkers (genes, pathways, or taxa) of different sample groups employing the linear discriminant analysis to approximate the effect size of each biomarker identified. A significant association between bacterial or fungal clades and a specific group can be detected when there is consistently higher relative abundance of the clade in the group's samples.

Results

Control of *C. medinalis* by *M. anisopliae* strain CQMa421

In order to assess the ability of *M. anisopliae* to control the rice leafroller, the effect of the fungus on *C. medinalis* was measured 3, 6, 9, and 12 days after foliar spray application in field conditions (Fig. 1). A single foliar spray was performed using an ultralow volume sprayer at a rate of 2.5 ml of formulated product mixed 200 ml sterile water per plot on rice seedlings ~52 days after planting (late tillering stage) as detailed in the "Materials and methods" section. Plots of ~45 m^2 were treated with fungal spores, corresponding to ~300 rice seedlings. Similar sized control plots were treated with the spray solution lacking any fungal spores. Both the percentage of leaf rolls caused/made by *C. medinalis* (Fig. 1a) and the survival rate of *C. medinalis* (%) (Fig. 1b) significantly declined compared to control plots. As compared to controls, new leaf rolls decreased between 40 and 70% in treated plots, 3 and 12-day post-*M. anisopliae* application, respectively. These data were in good agreement with decreases seen in *C. medinalis* survival that dropped to 5% in *M. anisopliae* treated plots, while remaining >25% in control treated plots (Fig. 1b).

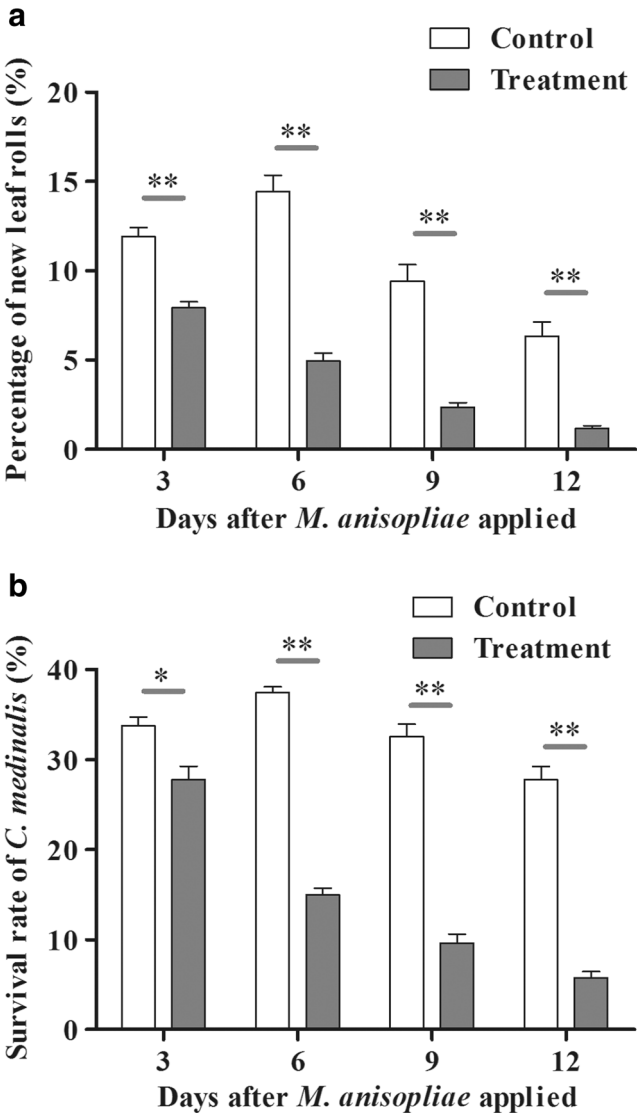


Fig. 1 Control of *C. medinalis* Guenee by *M. anisopliae* in field trials. **a** New leaf rolls (%) in *M. anisopliae* and control plots. **b** Survival rate of *C. medinalis* (%) in *M. anisopliae* and control plots. Analysis of variance (ANOVA) was used to determine whether significant difference between control and treatment samples. *Error bars* represent ±SE. *$p \leq 0.05$; **$p \leq 0.01$

Spatial and temporal distribution of *M. anisopliae* in the rice phyllosphere after application

Two methods were used to assess the spatial and temporal distribution of *M. anisopliae* in the rice field plots after foliar spray. These included plating and counting of recoverable CFUs from leaf samples and quantification of the *M. anisopliae* ITS sequencing signal. The GLM procedure was performed to describe the relationship between data from the *M. anisopliae* population ($\log_{10}$[CFUs/cm^2]) and sampling date. A steady decline in the population density as measured by recoverable CFUs, i.e., ($\log_{10}$[CFUs/cm^2]), of *M. anisopliae* over the 12-day study period was observed (Fig. 2). At the initial time point after application (2 h), spore concentrations for the top, middle, and bottom regions of the rice plant were 4.13, 2.23, and 1.04 ($\times 10^3$ [CFUs/cm^2]), respectively (Fig. 2a). After 3 days, these levels were reduced to 0.33, 0.33, and 0.22 ($\times 10^3$ [CFUs/cm^2]), and by 12 days, *M. anisopliae* levels had decreased to 0.14, 0.94, and 1.81 CFUs/cm^2, at the different foliar levels, respectively (Fig. 2a). The greatest rate of reduction was seen at the top rice leaf layer, with similar decline rates seen for the middle and bottom rice leaf layers (Fig. 2b, $p < 0.001$). The rate of decline of recoverable *M. anisopliae* was 3.75 ($\log_{10}$[CFUs/cm^2]) at the top leaf layer, and 2.83 ($\log_{10}$[CFUs/cm^2]) and 2.31 ($\log_{10}$[CFUs/cm^2]) for the middle and lower leaf layer regions, respectively (Fig. 2b). Although the population density in the top leaf layer at the initial time point was higher than the others (reflecting the dispersal and distribution of the fungal spores due to the spray mode of application), as the rate of CFU loss was also higher, by the last time point examined (12 days), few *M. anisopliae* CFUs could be detected in this layer. Relative abundance was also monitored via ITS1 of the rRNA operon as detailed in the "Materials and methods" sections (Fig. 2c). These data were in general agreement with the recoverable CFU results, showing relative mean abundances of 53.6, 47.32, and 22.21% at 2 h for the top, middle, and bottom rice phyllosphere sampling levels, which decreased to 4.85, 12.66, and 9.77% by 12 days.

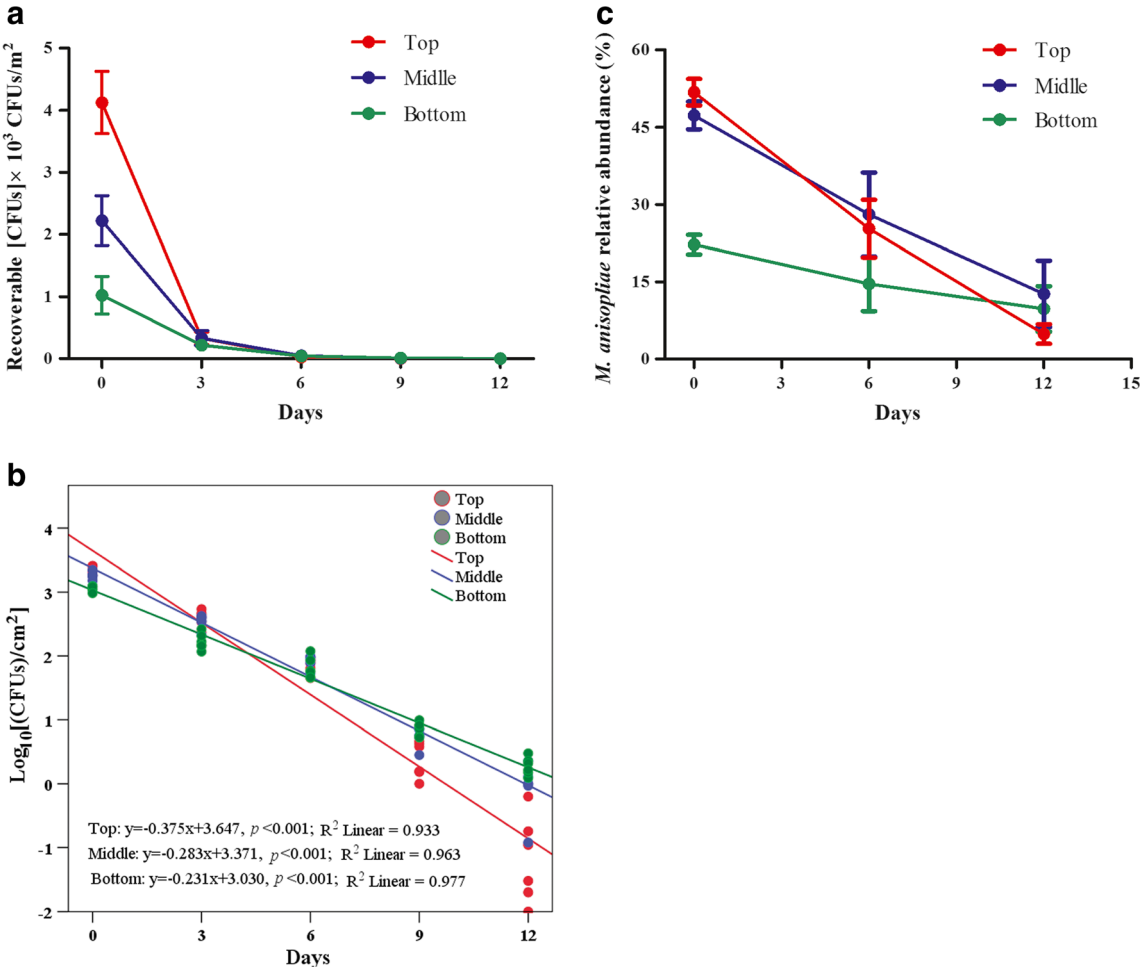


Fig. 2 Distribution of *M. anisopliae* in the rice phyllosphere. **a** Time course of colony-forming units (CFUs) recovered from the top, middle, and bottom of the rice leaf layer after *M. anisopliae* application. **b** General Linear Model (GLM) analysis of *M. anisopliae* density indexed by $Log_{10}[(CFUs)/cm^2]$ in the rice leaf layers. **c** Time course of *M. anisopliae* relative abundance (%) derived from ITS1 sequencing. *Error bars* represent ±SE

Microbiota structure in the rice phyllosphere

In total, 54 samples were collected corresponding to three time points (0-, 6-, and 12-day post-application of *M. anisopliae*) and three rice phyllosphere levels (top, middle, and bottom). Samples were barcoded and pooled for sequencing. Sequencing generated datasets consisting of 1,527,013 and 1,706,024 16S and ITS1 rRNA reads for each target region, respectively. Chloroplast sequences were removed from the bacterial dataset. Relative abundance of *M. anisopliae* sequences ranged from 1.15 to 55.23% in the different samples (Supplemental Fig. S1). As mentioned above, the highest levels of *M. anisopliae* were found at the 0 day top leaf (mean 53.6%) and the lowest at 12-day top leaf (mean 4.85%) (Fig. 2c and Supplemental Fig. S1). Sequences corresponding to *M. anisopliae* signals were removed from the fungal dataset for further analyses. Filtered 16S and ITS sequence reads were then analyzed by PyNAST (Python Nearest Alignment Space Termination) alignment at a threshold of 97% sequence identity. In the resultant analyses, 1,420,259 bacterial and 1,408,872 fungal sequences were grouped into 1026 and 453 OTUs, respectively.

Analyses of the 16S rRNA dataset indicated the dominance of the phylum *Proteobacteria*, comprising ~90% of the bacterial diversity observed across all samples in the rice phyllosphere examined, irrespective of *M. anisopliae* application (Fig. 3a). The remaining bacteria were distributed in the phyla *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, and *Thermi*. Within the *Proteobacteria*, the major bacterial classes observed were *Alphaproteobacteria* (18% of the total *Proteobacteria*), *Betaproteobacteria* (9%), *Gammaproteobacteria* (60%), and *Deltaproteobacteria* (0.5%). Within the *Gammaproteobacteria*, the genera *Acinetobacter* (11% of the total), *Pantoea* (19%), and *Pseudomonas* (10%) appeared to predominate. However, ~30% of the bacterial OTU sequences could not be assigned

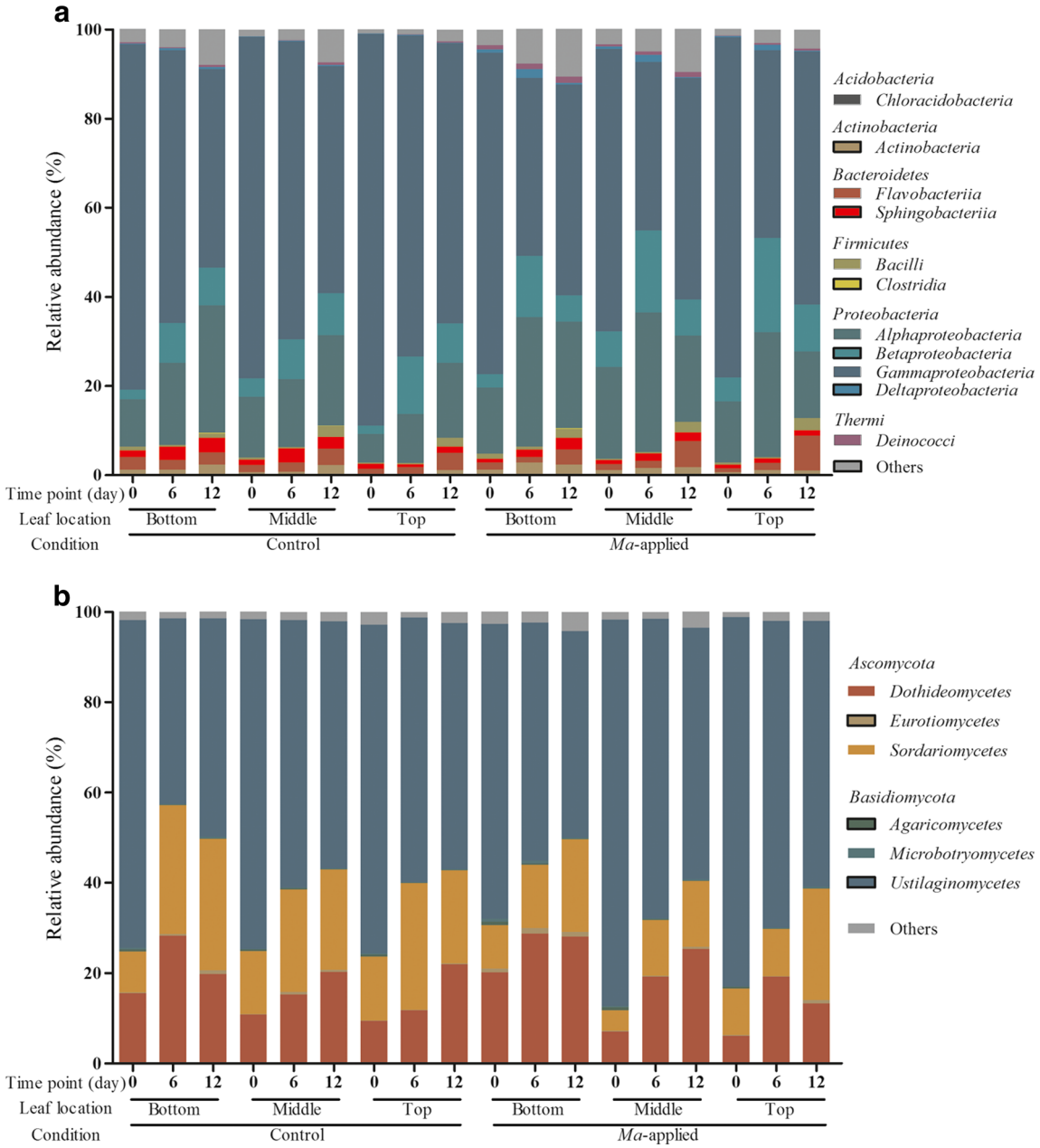


Fig. 3 Microbiota relative abundance histograms. Time course and leaf layer position of the relative abundance of the main bacterial (**a**) and fungal (**b**) classes detected in the rice phyllosphere by 16S rRNA and ITS1 sequencing, respectively. *Ma*, *M. anisopliae*

to known bacterial genera. Significant variation was seen among different time points, and at this level of analyses, no significant differences were found between *M. anisopliae* applied samples and control ones based on relative abundance (Fig. 3a). Analyses of absolute abundance were performed to ensure that the relative abundances were not biased due to potential changes in absolute levels of identified OTUs (Supplemental Fig. S2a). These analyses revealed no significant changes in absolute levels.

Analyses of the fungal ITS1 rRNA dataset revealed the predominance of the phylum *Basidiomycota* (63%) followed by members of the phylum *Ascomycota* (36%) across all samples (Fig. 3b). At the class level, within *Basidiomycota*, OTUs belonging to the *Ustilaginomycetes* were the most predominant (62% of the total *Basidiomycetes* sequences), with *Dothideomycetes* (18%) and *Sordariomycetes* (17%), the major represented OTUs within *Ascomycota*. For the fungal analyses, the absolute abundances of *Ustilaginomycetes* and *Sordariomycetes* seen in the *M. anisopliae* treatment group significantly decreased relative to the control at each time points (Supplemental Fig. S2b; $p < 0.05$). With respect to the relative fungal abundance analyses, significant variation was noted between the various time points (Fig. 3b).

Effects of *M. anisopliae* application on microbial diversity

In order to determine whether species richness and diversity were affected by *M. anisopliae*, multivariate analyses of variance on the dataset was performed on various microbial (Alpha) diversity indices including the observed species index and the Shannon index. The observed species index is used to estimate microbial species richness, and the Shannon index involves the calculation of species diversity as determined by species richness (number of different species present) and evenness (relative abundance of different species) (Schloss et al. 2009 and Tan et al. 2015). These analyses indicated that the bacterial observed species and the bacterial Shannon indices were significantly affected by application of *M. anisopliae*, the time point considered after application, and the leaf layer sample position (Tables 1 and 2, $p < 0.01$; Table 3, $p < 0.01$). This output was confirmed by modeling to a linear mixed model (Table 3, $p < 0.01$). However, there was only a significant interactive effect detected in the bacterial Shannon index for the *M. anisopliae* (*Ma*) × time post-*Ma* application comparison (Table 3, $p < 0.01$). In this case, both overall bacterial species and the bacterial Shannon index increased over the time course of the experiment (from 0- to 6- to 12-day post-*Ma* application; Supplemental Fig. S3a). The phylloplane position of the rice plant, i.e., the top, middle, and bottom layer, also significantly affected bacterial diversity (Table 1, $p < 0.01$; Table 3, $p < 0.01$). Comparison of the top, middle, and bottom layers revealed a significant decrease from the bottom to middle and to the top in both bacterial species and the Shannon indices (Table 1; Supplemental Fig. S3a). In terms of overall bacterial alpha diversity, most of the samples in which *M. anisopliae* applied were similar to control treatment in terms of the number of observed species and the Shannon diversity indices (Supplemental Fig. S3a). However, the observed species at the top-leaf layer sample (6 days) and the Shannon diversity index of the bottom-leaf layer sample (6 days) post-*M. anisopliae* application were significantly higher than controls (Supplemental Fig. S3a, $p < 0.05$).

Table 1 Effects of *M. anisopliae*, *M. anisopliae* post-applied time, and leaf layer on observed species and Shannon index of bacteria in rice leaf using multivariate analysis of variance

Factors	Items	d.f.	*F*	*p*
M. anisopliae	Observed species	1	18.200	0.000
	Shannon	1	12.570	0.001
M. anisopliae post-applied time	Observed species	2	31.800	0.000
	Shannon	2	23.340	0.000
Leaf layer	Observed species	2	16.750	0.000
	Shannon	2	25.090	0.000

d.f. degrees of freedom, *F* mean square/mean square residual, *p* significance level

Table 2 Effects of *M. anisopliae*, *M. anisopliae* post-applied time, and leaf layer on observed species and Shannon index of fungi in rice leaf using multivariate analysis of variance

Factors	Items	d.f.	*F*	*p*
M. anisopliae	Observed species	1	4.533	0.038
	Shannon	1	0.282	0.598
M. anisopliae post-applied time	Observed species	2	0.351	0.706
	Shannon	2	5.104	0.010
Leaf layer	Observed species	2	17.253	0.000
	Shannon	2	4.237	0.020

d.f. degrees of freedom, *F* mean square/mean square residual, *p* significance level

The distribution of fungal species and the fungal Shannon index were found to be significantly affected by the rice leaf layer, i.e., between the top, middle, and bottom-regions of the rice leaf (Table 2, $p < 0.05$; Table 4, $p < 0.05$). Both overall fungal species detected and the fungal Shannon index significantly decreased from the bottom rice leaf level to the middle level, and then to the top rice leaf level (Tables 2 and 4; Supplemental Fig. S3b). Application of *M. anisopliae* also significantly affected the observed fungal species (Table 2, $p < 0.05$), an effect that varied depending on time post-application. The fungal Shannon index progressively increased when comparing 0- to 6- and 12-day post-*M. anisopliae* treatment (Table 2, $p < 0.05$; Table 4, $p < 0.05$; Supplemental Fig. S3b). The observed fungal species and fungal Shannon diversity of the top rice–leaf layer sample at 0 day of post-*M. anisopliae* application were significantly lower than controls; however, the observed fungal species and fungal Shannon diversity at other time-points and phylloplane position were similar to blank controls (Supplemental Fig. S3b).

Effects of *M. anisopliae* application on microbial community

To explore how application of *M. anisopliae* impacted the microbial community structure of the rice phyllosphere, we compared the similarity of weighted (unweighted) UniFrac distances between treatment and control groups (Koren et al. 2012). Although there are some slight fluctuations in individual variation in the community composition for bacteria and fungi between the different leaf layers and sampling time (Supplemental Fig. S4), no significant differences were found between *M. anisopliae* application and controls in these analyses, suggesting no significant effect of *M. anisopliae* (CQMa421) on community structure of rice phyllosphere microbes.

Table 3 Effects of treatment and conditions on bacteria Shannon index using Linear mixed model

	Factors	d.f.	F	Significant contrasts	*p*
Main effects	*M. anisopliae* (*Ma*)	1	13.260	*M. anisopliae* vs blank control	0.001
	Ma. post-applied time	2	26.457	0 and 6 vs 12 days	0.000
	Leaf layer	2	24.614	Bottom and middle vs top	0.000
Interaction terms	*Ma*. × *Ma*. post-applied time	2	6.025	*M. anisopliae* on (0 and 6 vs 12 days)	0.006
	Ma. × leaf layer	2	0.235		0.792
	Ma. post-applied time × leaf layer	4	0.438		0.780
	Ma. × *Ma*. post-applied time × leaf layer	4	0.086		0.986

AIC = 21.919, BIC = 25.086

AIC Akaike information criterion, *BIC* Bayesian information criterion, *d.f.* degrees of freedom

Biomarker analysis

In order to more robustly identify features that are statistically different among biological classes, the linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al. 2011) algorithm was employed to identify specific taxa responding to *M. anisopliae* application as compared to control groups in the bacterial and fungal taxa over the time course of the experiment. These data revealed that for the bacterial taxa, during the 6-day time course, six predominant phenotypes at the class level were identified as high-dimensional biomarkers for separating phyllosphere bacteria seen in *M. anisopliae* treated samples compared to controls (Fig. 4a). Five of these classes (i.e., *Actinobacteria*, *Cytophagia*, *Alphaproteobacteria*, *Deltaproteobacteria*, and *Deinococci*) showed increased representation in *M. anisopliae* treated samples, with one (*Gammaproteobacteria*) lower in the treated samples. At the genus level, seven predominant phylotypes (i.e., *Spirosoma*, *Methylobacterium*, *Sphingobium*, *Sphingomonas*, *Aquabacterium*, *Cystobacter*, *Deinococcus*) were found at high representation, in the *M. anisopliae* 6-day post-treatment sample as compared to controls, although the overall most predominant genera (*Pseudomonas*) were lower in the *M. anisopliae* treated 6-day samples as compared to control groups. However, this difference was not seen in analyses of the 12-day samples (Supplemental Fig. S5). At the species level, *Sphingomonas yabuuchiae* was found to be higher in the *M. anisopliae* 6-day treated group at all leaf layer positions relative to the control (Fig. 4a).

Analyses of the fungal taxa at 6 days revealed *Ascomycota* and *Basidiomycota* as the major phyla/divisions (Fig. 4b). Overall, the families *Ustilaginaceae* (*Basidiomycota*, order *Ustilaginales*) and *Davidiellaceae* (*Ascomycota*, order *Capnodiales*) showed increased representation in *M. anisopliae* treated samples, with *Trichosphaeriaceae* (*Ascomycota*, order *Trichosphaeriales*) lower in treated samples. At the genus level, three predominant phylotypes—*Cladosporium*, *Pseudocercospora* (*Ascomycota*: *Capnodiales*) and *Strelitziana* (*Ascomycota*: *Chaetothyriales*)—were elevated in the *M. anisopliae* (6-day post-treatment) as compared to controls, while *Nigrospora* (*Ascomycota*: *Trichosphaeriales*) showed a decrease in *M. anisopliae* 6-day post-treatment samples (Fig. 4b). *Strelitziana albiziae* was only found to be higher in the *M. anisopliae* 6-day treated groups than control ones at the species level. We also identified high-dimensional biomarkers at 12 days (Supplemental Fig. S6). These analyses showed a slight increase in *Aspergillus flavus*, the predominant species, after *M. anisopliae* application (relative distribution ~0.02 vs 0.01%, respectively; $p > 0.05$, metastats test).

Table 4 Effects of treatment and conditions on fungus Shannon index using Linear mixed model

	Factors	d.f.	F	Significant contrasts	*p*
Main effects	*M. anisopliae* (*Ma*)	1	0.334	*M. anisopliae* vs blank control	0.567
	Ma. post-applied time	2	6.049	0 and 6 vs 12 days	0.005
	Leaf layer	2	5.022	Bottom and middle vs top	0.012
Interaction terms	*Ma*. × *Ma*.post-applied time	2	0.660	*M. anisopliae* on (0 and 6 vs 12 days)	0.523
	Ma. × leaf layer	2	2.946		0.065
	Ma.post-applied time × leaf layer	4	2.088		0.103
	Ma. × *Ma*.post-applied time × leaf layer	4	1.332		0.277

AIC = 12.649, BIC = 9.482

AIC Akaike information criterion, *BIC* Bayesian information criterion, *d.f.* degrees of freedom

Discussion

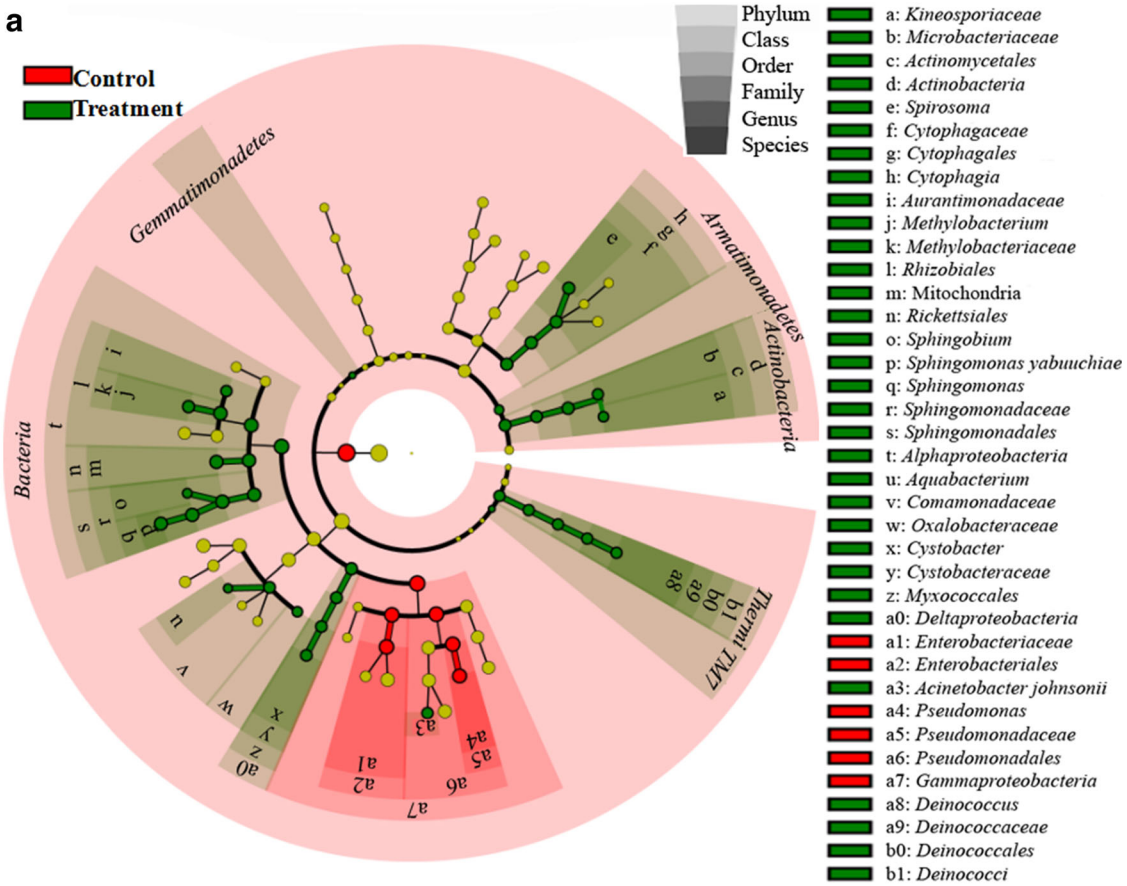
Despite extensive studies on fungal insect pathogens and the many reports on their potential for agricultural biological control, widespread adoption of entomogenous fungi as part of routine agricultural practices remains lagging (Glare et al. 2012). Recent additional environmental roles for many of these fungi including functioning as plant endophytes, acting as antagonists of plant pathogens, forming associations in the rhizosphere, and possibly even helping in plant growth promotion continue to indicate that these agents are viable alternatives to chemical pesticides (Vega et al. 2009; Ownley et al. 2010; Behie et al. 2012). *M. anisopliae* has been applied for the control of forestry and agriculture pests (Remadevi et al. 2010), and a number of field trials evaluating entomopathogenic fungi against rice pests have been reported (Karthiba et al. 2010; Jia et al. 2013; Golshan et al. 2013; Lee et al. 2015; Silva et al. 2015). However, the distribution of *M. anisopliae* in the rice phyllosphere after application and any effects on endemic microbial populations has yet to be reported. These latter aspects are important as any changes in soil and plant microbes can have unanticipated effects on crop health and productivity.

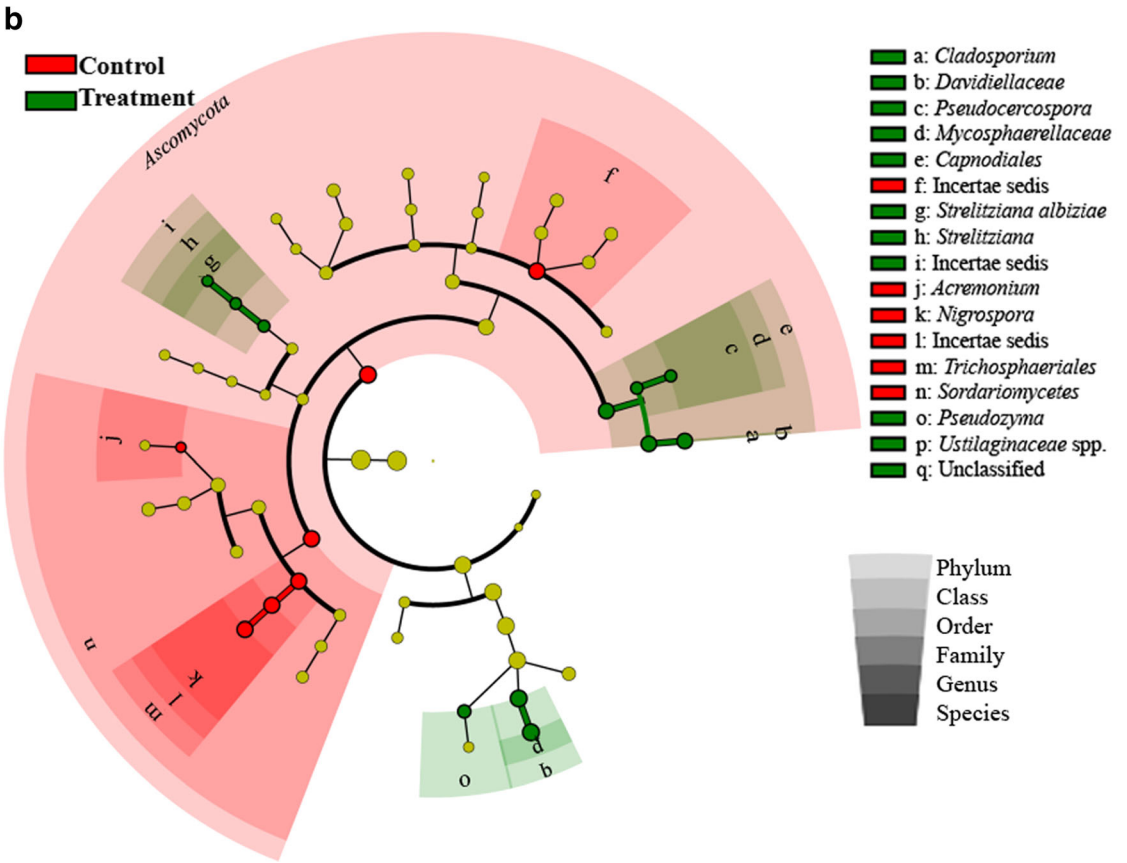
In this report, we have examined the pest control efficacy and environmental fate of *M. anisopliae* (CQMa421) after foliar application, and measured any effects of the fungus on plant microbial populations across the rice phyllosphere, i.e., at the top, middle, and bottom layers of the growing plant. Our data revealed significant variation and only relatively subtle shifts in the bacterial and fungal populations within the examined 12-day window after application of the fungal agent. The ability of *M. anisopliae* to control the rice leaf roller *C. medinalis* was confirmed, and our data are comparable to what has been reported for control of this rice pest using the related insect pathogenic fungus, *B. bassiana* (B2) (Sivasundaram et al. 2008; Karthiba et al. 2010).

Although a high spore density was detected immediately after application, the population density of *M. anisopliae* spores rapidly decreased in the various rice phylloplane sections examined, i.e., the top, middle, and bottom leaf layers of the plant. The most rapid decline was seen in the top leaf layer where *M. anisopliae* CFUs could hardly be detected by day 12. The rate of decline in *M. anisopliae* recovery from the middle and bottom layers was somewhat lower; however, by day 12, similar levels were seen in all samples. The more rapid decline at the top leaf layer may be consistent with greater exposure to solar irradiation. Although *Metarhizium* spp. have been found persist and adapt to the soil and plant rhizosphere after application, overall numbers decrease immediately after application and appear to stabilize somewhat over time (St Leger 2008; Tiago et al. 2014). These studies focused on the soil and plant roots, whereas we report on *M. anisopliae* persistence in leaf layers. A potential reservoir of *M. anisopliae* may exist as the fungus can colonize internal tissues of certain plants. In *Brassica napus*, *M. anisopliae* has been reported in the plant tissues, from where it is subsequently capable of infecting *Plutella xylostella* larvae feeding on the plants (Batta 2013). However, *M. anisopliae* spores were shown to survive less than 3 days when applied on maize leaves, a finding that differs from persistence in the soil, where the fungus could still be detected 15 months after application (Pilz et al. 2011). In this study, we measured the relative abundance of *M. anisopliae* over a time course after application using recoverable CFUs and sequencing. Although semi-quantitative, we found that *M. anisopliae* could survive at least 12 sdays in the rice leaf, albeit significantly decreasing in titer within this time frame. Somewhat variable results have been reported in other studies. Owing to the lateral shading provided by the adjacent canopy of the top leaf layer, *B. bassiana* conidial viability on the melon, *Cucumis melo* leaf undersides remained robust, decreasing only ~9–11% per day (Jaronski 2010). In the upper leaf layer of the melon, a 47% daily decrease in *B. bassiana* conidial recovery after application has been reported (Jaronski 2010). Our analyses included both a DNA-based technique that could potentially detect DNA from dead or non-viable fungal cells in the phyllosphere as well as direct plating of samples, demonstrating a good correlation between these two approaches. Possible reasons for the decrease in *M. anisopliae* population in our samples include the strong solar radiation and high temperatures that occur in the long hot summers of Chongqing, China, and our results that are consistent with a few previous studies in similar conditions (Rastogi et al. 2012; Ortiz-Urquiza et al. 2015). Overall, our data indicate a restricted persistence of *M. anisopliae* (CQMa421) in the rice phyllosphere. However, despite the decrease in CFU/ITS-based sequence recovery across the rice phyllosphere, control of *C. medinalis* remained robust over the time course examined. These findings imply that low levels of the fungal agent after the initial high titer application may be sufficient for pest control and further research examining the ecological mechanisms behind this are warranted.

In terms of the microbial community, our data revealed that members of the *Proteobacteria* were the most abundant bacterial phyla detected across all the rice leaf samples. Metagenome analyses have been used to identify bacterial community members at high taxonomic resolution (Knief et al. 2012). However, some variations have been noted between uses of different experimental methodologies. Phyllosphere bacterial diversity was found to have relatively higher proportions of *Alphaproteobacteria* and *Actinobacteria* based on metagenome datasets, whereas

Fig. 4 Cladograms of key phylotypes of bacterial (**a**) and fungal (**b**) taxa showing significant changes between *M. anisopliae* and control plots 6-day post-treatment. Phylotypes were identified using LEfSe


a
Control
Treatment
Phylum
Class
Order
Family
Genus
Species
Gemmatimonadetes
Armatimonadetes
Actinobacteria
Bacteria
Therm_TM7
a: Kineosporiaceae
b: Microbacteriaceae
c: Actinomycetales
d: Actinobacteria
e: Spirosoma
f: Cytophagaceae
g: Cytophagales
h: Cytophagia
i: Aurantimonadaceae
j: Methylobacterium
k: Methylobacteriaceae
l: Rhizobiales
m: Mitochondria
n: Rickettsiales
o: Sphingobium
p: Sphingomonas yabuuchiae
q: Sphingomonas
r: Sphingomonadaceae
s: Sphingomonadales
t: Alphaproteobacteria
u: Aquabacterium
v: Comamonadaceae
w: Oxalobacteraceae
x: Cystobacter
y: Cystobacteraceae
z: Myxococcales
a0: Deltaproteobacteria
a1: Enterobacteriaceae
a2: Enterobacteriales
a3: Acinetobacter johnsonii
a4: Pseudomonas
a5: Pseudomonadaceae
a6: Pseudomonadales
a7: Gammaproteobacteria
a8: Deinococcus
a9: Deinococcaceae
b0: Deinococcales
b1: Deinococci


b
Control
Treatment
Ascomycota
a: Cladosporium
b: Davidiellaceae
c: Pseudocercospora
d: Mycosphaerellaceae
e: Capnodiales
f: Incertae sedis
g: Strelitziana albiziae
h: Strelitziana
i: Incertae sedis
j: Acremonium
k: Nigrospora
l: Incertae sedis
m: Trichosphaeriales
n: Sordariomycetes
o: Pseudozyma
p: Ustilaginaceae spp.
q: Unclassified
Phylum
Class
Order
Family
Genus
Species

mainly *Alphaproteobacteria* was highly represented in metaproteome datasets (Knief et al. 2012). In the rice phyllosphere, based on our 16S rRNA analyses, *Gammaproteobacteria* were found to be the dominant class in rice phyllosphere, similar to results reported for lettuce (Williams and Marco 2014). With respect to fungal biodiversity, the phyllosphere of grapevine (Perazzolli et al. 2014) and strawberry (Wei et al. 2016), *Ascomycota* and *Basidiomycota* were the most abundant fungal phyla, as also seen in our data for the rice phyllosphere by the analysis of barcoded pyrosequencing of the first internal transcribed spacer (ITS1) region of the rRNA operon. However, no obvious changes in the most abundant bacterial and fungal phyla were seen between *M. anisopliae* treated and untreated samples. These data suggest little to no significant (long term, i.e., >1 month) effects of application of *M. anisopliae* (CQMa421) on the dominant microbial taxa found in the rice phyllosphere.

Our data show that *M. anisopliae* application slightly impacted bacteria diversity and community composition during the first 6 days in the booting stage of rice growth, whereas no significant changes in fungal diversity occurred after application. Not too surprisingly, regardless of *M. anisopliae* application, the bottom rice leaf layers, and to a lesser extent, the middle layer contained greater diversity than the top layer. The effect of *M. anisopliae* on bacterial diversity on the bottom layer was most noticeable at the 6-day time point, in which the diversity was increased. Little to no effects of *B. subtilis* treatment on microbial diversity was seen in the strawberry phyllosphere (Wei et al. 2016); however, significant changes were reported in the pepper phyllosphere after application of chemical insecticides (Moulas et al. 2013). Increases in bacterial abundance and a shift in community composition within the pepper plant phyllosphere following application of cypermethrin have also been reported (Zhang et al. 2009). In the present study, increased diversity of bacterial species was found only in the top rice leaf layer at 6-day post-application, and an increased bacterial Shannon Index was seen for the rice leaf bottom layer at 6-day post-*M. anisopliae* application. These results indicate that *M. anisopliae* application may result in only transient changes in the bacteria microbiota associated with the rice plant leaves. This difference may result from its restricted persistence in rice leaf and are consistent with a previous study in maize leaves (Batta 2013). Similarity of weighted (unweighted) UniFrac distances between groups can be used to explore the extent to which microbial community phylogeny varies (Koren et al. 2012). By measuring weighted (unweighted) UniFrac distances for bacteria and fungi, we found no obvious difference, again likely due to the restricted distribution and persistence of *M. anisopliae*.

At deeper classification levels, there were a few microbial species that were significantly affected by *M. anisopliae* application, primarily occurring at the 6-day post-application time point. Several of these "biomarker" species that increased post-*M. anisopliae* application appeared to be microorganisms beneficial to crops and/or animals/mammals, and included members corresponding to *Aquabacterium*, *Sphingobium*, *Sphingomonas*, and *Methylobacterium*. A number of these (e.g., *Aquabacterium*, *Sphingobium*, and *Sphingomonas*) have previously been found to be able to degrade organic pollutants, a potential useful outcome (Nagata et al. 2010; Tang et al. 2013; Wilson et al. 2016). Some species of *Methylobacterium* and *Pseudomonas* are also plant growth-promoting or display antagonistic effects against plant pathogens, and hence increases in their levels may facilitate plant disease resistance and/or stress response (Naureen et al. 2009; Luo et al. 2011; Spence et al. 2014). At the species level, *S. yabuuchiae*, an organic pollutant degrading strain (Park et al. 2015), was more highly represented in the *M. anisopliae* application group as compared to control ones.

In terms of fungal biodiversity, a number of potential biomarker members were found to have a higher relative distribution at the 6-day post-*M. anisopliae* application. These included members related to *Cladosporium* (Bensch et al. 2012), *Pseudocercospora* (Crous et al. 2013), and *S. albiziae* (Crous et al. 2010). A lower distribution of members of the *Nigrospora* genus was seen for the 6-day post-*M. anisopliae* application. Across the whole experimental period, however, no consistent fungal biomarker genera/species could be identified that could discriminate between *M. anisopliae* application and control plots, suggesting minimal effects on the overall fungal microbial community of the rice phyllosphere. Only *A. flavus* was detected as a potential high-dimensional biomarker on day 12 post-*M. anisopliae* application. This strain may be an opportunistic animal and/or human pathogen (Amaike and Keller 2011); however, its relative distribution was small and only slightly higher after *M. anisopliae* application (0.02 vs 0.01%).

In summary, our data indicate a rapid decrease in overall fungal spore counts within several days after application, with effective control of the rice leafroller, *C. medinalis*, during the critical stage when the rice seedling is most vulnerable to the rice pest. Although alternations in the abundance of rice phyllosphere microbiota, and observed species and Shannon index of bacteria, were noted after *M. anisopliae* application, clear changes were difficult to discern due to large variations, and any effects appeared to be transient. A number of potential bacterial and fungal biomarkers were identified and longer-term studies are needed to better understand any additional effects. Our results support a conclusion that *M. anisopliae* strain (CQMa421) can control the rice pest and represent an "environmental friendly biopesticides" in that little to no changes or adverse effects towards resident microbial populations are likely to result from its application.

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