Persistence of Brazilian isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *M. robertsii* in strawberry crop soil after soil drench application

Thiago Castro[a,c,*], Johanna Mayerhofer[b], Jürg Enkerli[b], Jørgen Eilenberg[c], Nicolai V. Meyling[c], Rafael de Andrade Moral[d], Clarice Garcia Borges Demétrio[d], Italo Delalibera Jr.[a]

[a] Department of Entomology and Acarology, ESALQ- University of São Paulo, Av Padua Dias, 11–P.O. Box 9–13418-900, Piracicaba, SP, Brazil
[b] Molecular Ecology, Institute for Sustainability Sciences, Agroscope, Reckenholzstrasse 191, 8046 Zurich, Switzerland
[c] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[d] Department of Exact Sciences, ESALQ-University of São Paulo, Av Padua Dias, 11–P.O. Box 9–13418-900, Piracicaba, SP, Brazil, Brazil

ABSTRACT

Establishment, persistence and local dispersal of the entomopathogenic fungi *Metarhizium anisopliae* (ESALQ1037) and *M. robertsii* (ESALQ1426) (Ascomycota: Hypocreales) were investigated in the soil and rhizosphere following soil drench application in strawberries between 2012 and 2013 at a single location in Inconfidentes, Minas Gerais, Brazil. *Metarhizium* spp. isolates (n = 108) were collected using selective agar media and insect bait methods, and characterized by sequence analyses of the 5'–end of the translation elongation factor 1-α and the MzFG543igs intergenic region and by multilocus simple sequence repeat analysis. Both applied fungal isolates were frequently recovered from bulk soil and rhizosphere samples of the treated plots, suggesting that they were able to establish and disperse within the soil. Persistence within the soil and strawberry rhizosphere for both fungal isolates was observed up to 12 months after application with frequencies of 25% of haplotypes similar to isolate ESALQ1037 and 87.5% of haplotypes similar to isolate ESALQ1426, respectively. Overall, *M. robertsii* was the most abundant species in the agroecosystem studied representing 77.8% of the isolates recovered across all sample dates.

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1. Introduction

The world's strawberry production was approximately 7.8 million tons in 2013 and Brazil produced more than three thousand tons in 2013, with a yield of 8500 kg/ha (FAOSTAT, 2016). Strawberries (*Fragaria x ananassa* Duch.; Rosales: Rosacea) produced for direct consumption are a growing and promising market in Brazil with profit margins at the point of sale around 20% with a current price of the final product of approx. USD 4.4/kg (AGRA-FNP-Pesquisas, 2015). However, damages from pests and diseases cause significant losses for farmers (Wilson and Tisdell, 2001).

The high load of chemical pesticides used in the Brazilian conventional strawberry production system is of concern, especially regarding negative impacts on the environment and natural enemies of agricultural pests (Sato et al., 2007). Several studies have demonstrated that fungicides, and in some instances herbicides, can significantly reduce the germination and growth of entomopathogenic fungi (Samson et al., 2005; Yáñez and France, 2010; D'Alessandro et al., 2011). Strawberry producers have experienced problems with the efficacy of chemical products used in pest control, likely due to a selection favoring the development resistant pest populations after prolonged cyclical application (Sato et al., 2005). In addition, the use of chemical pesticides increases the risk of pesticide residues in the harvested fruits, and it may cause health problems for farm workers and contamination of the environment (Maredia, 2003).

A viable alternative to chemical pesticides in strawberry production is biological control, such as the use of entomopathogenic fungi—in particular species from Ascomycota, which often have a broad host range. Much effort has been put into the research and development of *Metarhizium* spp. (Hypocreales:

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* Corresponding author at: University of São Paulo-ESALQ Rua Padua Dias, 11, Agronomia, Piracicaba 13418-900, Brazil.
  E-mail address: thiago.castro@usp.br (T. Castro).

Clavicipitaceae) as biological control agents for inundative biological control in agriculture and forestry (Vega et al., 2012). Brazil has a long history of using isolates of *Metarhizium* as biocontrol agents especially against spittlebugs (Hemiptera: Cercopoidea) in sugarcane, it is estimated that around two million hectares are treated annually (Parra, 2014). However, limited knowledge is available about the indigenous communities of *Metarhizium* spp. in Brazilian agroecosystems (Lopes et al., 2013a, b) and the potential impact of inundative fungal applications on these communities still needs to be investigated.

The genus *Metarhizium* contains species which occur naturally within the soil environment (Meyling and Eilenberg, 2007; Vega et al., 2009; Rudeen et al., 2013; Steinwender et al., 2014), from where they can be recovered. It has been suggested that *Metarhzium* spp. use the soil as a reservoir for long-term persistence, even when crops and insects are not present in the field (Klingen and Haukeland, 2006). Recently, a number of studies have demonstrated that *Metarhizium* spp. benefit plant growth (Behie et al., 2012; Khan et al., 2012; Sasan and Bidochka, 2012; Behie and Bidochka, 2014) and are antagonistic towards pests and diseases (Sasan and Bidochka, 2013). Soil inoculation of *Metarhizium* spp. in strawberry has the potential to promote plant growth and to control below-ground pest of strawberries such as *Agrotis* spp. (Lepidoptera: Noctuidae) and *Naupactus tremolerasi* Hustache (Coleoptera: Curculionidae). Understanding the persistence of applied isolates of *Metarhizium* spp. in the soil and rhizosphere of agroecosystems is important to predict the possible duration of the beneficial effects of soil inoculation. The species *Metarhizium robertsii* is the most abundant *Metarhizium* species to occur naturally in Brazilian agricultural soils but fungal biological control products are currently used for spittlebug control, including all commercial products in the country, belong to *M. anisopliae* Mani 2 subclade. Recent studies indicate that Brazilian *M. robertsii* may be better adapted as an entomopathogen in the soil environment than *M. anisopliae* (Rezende et al., 2015) suggesting that isolates of *M. robertsii* could be more suitable for biological control of soil dwelling pests than the currently used *M. anisopliae* based agents. Molecular markers with high discriminatory power are required for identification of individual fungal isolates, for instance when studying behavior and fate of an applied isolate in the field. Simple sequence repeats (SSR or microsatellite markers) have proven to be very useful for consistent and explicit assessment of isolate identity and persistence (Pilz et al., 2011; Kepler et al., 2015; Mayerhofer et al., 2015a,b; Steinwender et al., 2014, 2015). On the other hand, analyses of DNA sequence and subsequent comparison with sequences of references strains are used to determine species affiliation. In the genus *Metarhizium* the gene translation elongation factor 1-α and the MzFG543igs intergenic region are the loci targeted for such purposes (Kepler et al., 2015; Steinwender et al., 2014, 2015).

The aim of this study was to evaluate the establishment, persistence and local dispersal of two Brazilian isolates of *Metarhizium* (*M. anisopliae* and *M. robertsii*) over one year after experimental application in a strawberry cropping system in Minas Gerais State in Brazil. The two applied isolates were discriminated from the indigenous *Metarhizium* spp. community by SSR marker and phylogenetic analyses of DNA sequence data, which in addition provided information on the diversity of this important group of entomopathogenic fungi in the agroecosystem.

2. Material and methods

2.1. Experimental field description

The experiment was performed in a strawberry crop at the campus of the Federal Institute of Education, Science and Technology (IF Sul de Minas Gerais) in Inconfidentes city, state of Minas Gerais (MG) Brazil (22°19′2″S; 46°19′42″W; 904 m altitude). The field was cultivated with organic strawberry in the previous year and before that, the area had not been used for cultivation. Thus, the area has no recent history of application of chemical pesticides. During the experimental period (May 2012–August 2013) no chemical pesticides were applied. Fertilizers were applied according to soil analysis and drip irrigation was performed. One thousand five hundred and twenty strawberry seedlings (San Andreas variety) were planted in 12 beds, each 15 m long, with three rows per bed, with 35 cm between each plant in May 2012.

2.2. Treatment application

Two Brazilian *Metarhizium* isolates were used:1) *M. anisopliae* ESALQ1037, isolated in March 1992 in Porto Alegre, Rio Grande do Sul state, from *Solenopsis* sp. (Hymenoptera: Formicidae) and 2) *M. robertsii* ESALQ1426, isolated from soybean crop soil (selective agar medium) in December 2007 in Londrina, Paraná state. Both isolates are deposited at the Collection of Entomopathogenic Microorganisms of the Laboratory of Pathology and Microbial Control of Insects (LPCMI) of the Escola Superior de Agricultura "Luiz de Queiroz" at University of São Paulo (ESALQ-USP). ESALQ1037 is widely used to control the root spittlebug, *Mahanarva fimbriolata* in Brazil and *M. robertsii* ESALQ1426 has been recently investigated as plant growth promotor.

Aerial conidia were produced on parboiled rice by the plastic bag method (Jaronski and Jackson, 2012), and were then mechanically harvested from a fungus-rice mixture using an electrically vibrating sieve containing a set of three 20 cm round sieves of 32 mesh (pore size 500 μm) (Bertel Indústria Metalúrgica Ltd., Brazil). Different batches of dried conidial powder (<13% w/w final moisture) were vacuum-sealed and stored at −20 °C until use. The conidia viability was determined using the direct count method as described by Oliveira et al. (2015); briefly, the counting of germinated and un-germinated conidia on 4 mL of PDA amended with 0.001% (v/v) Derosal® 500 SC (Carbendazim, Bayer CropScience, SP, Brazil) on Rodac® plates.

Immediately after the initial soil sampling in September 2012 (see below) for baseline characterization of resident *Metarhizium* haplotypes, the two *Metarhizium* isolates were applied to the experimental strawberry field as a randomized block design, containing four blocks with the three treatments (1. *M. anisopliae* ESALQ1037; 2. *M. robertsii* ESALQ1426 and 3. Control, water), yielding 12 plots (beds) in total. The unformulated fungal suspensions amended with 0.01% of Tween 80 (Oxiteno, Brazil) were applied by drenching 100 mL of 1×10^8 viable conidia/mL on the soil surface around each of the 43 strawberry plants in the center row of the beds (after intense homogenization of the fungal suspensions). In beds with the control treatment 100 mL water was applied to each plant in a similar manner. The application was done between 6 and 7 pm, to minimize detrimental UV effects on conidial viability.

2.3. Sampling dates

Soil samples were taken across four sampling time points: 1) 4 September, 2012 (Before), prior to fungal application in order to characterize the indigenous Metarhizium spp. community in the soil, 2) 9 January, 2013; 3) 16 April, 2013, and 4) 21 August, 2013; to evaluate the persistence of the inoculated isolates at the experimental field site (After).

2.4. Soil sampling

Each soil sample was taken to a depth of 10 cm using a cylindrical sampler (Sondaterra, Brazil) with defined volume (70 cm^3). The samples were stored in sterile plastic bags and kept in Styrofoam boxes at approximately 5 °C during transport to the laboratory as described by Inglis et al. (2012).

Each sample consisted of five sub-samples of soil collected in four center row beds of each treatment per sampling occasion, at 2 m intervals and 10 cm away from the strawberry roots. These five combined sub-samples were manually homogenized in the plastic bag, forming one composite sample per bed on each of the four sampling occasions, yielding 48 composite samples per treatment for the total experimental period. In addition, for each sampling occasion, four composite samples were taken from the soil 10 cm away from the roots of five plants of *Bidens pilosa* (Asterales: Asteraceae)—the most abundant species of spontaneous herb found close to the experimental field (maximum 3 m distance from strawberry plants). This sampling thus generated in total 20 composite samples from the crop margins.

To evaluate the rhizosphere colonization of the inoculated *Metarhizium* spp. isolates, we also conducted a root sampling of the strawberry crop on the last sampling date by digging up five randomly selected whole strawberry plants from the center row of each bed using a garden shovel. Surplus soil was shaken off so that samples represented the entire root system and adhering rhizosphere soil. These samples were stored as mentioned for the soil samples above.

2.5. Isolation of entomopathogenic fungi

The isolation of entomopathogenic fungi from the soil was carried out using selective agar media and insect baiting technique.

2.5.1. Selective agar media

The selective agar media was prepared using Potato Dextrose Agar (PDA) with 0.002% Dodine (Dodex 450 SC—Sipcam Isagro, Brazil) and 0.05 g/L Gentamicin (Amresco Inc., USA) to reduce the growth of contaminant fungi and bacteria that normally occur in the soil, as described by Fernandes et al. (2010). Each soil sample was homogenized by hand and 10 g of soil from each composite sample was added to a Schott tube (250 ml) containing 90 mL of sterile distilled water and 0.01% of Tween 80. The solutions were homogenized on a Vortex-type stirrer and serially diluted (10^{-1}, 10^{-2}, 10^{-3}). Volumes of 0.1 ml of each dilution were then inoculated in two replicates into Petri dishes (90×15 mm) containing selective media and incubated in a climatic chamber at a temperature of 25 ± 1 °C, relative humidity of $70 \pm 10\%$ and photoperiod of 12 h for seven days. After this period, Petri dishes were assessed daily for the presence of *Metarhizium* colonies which were identified by morphological characteristics as described by Humber (2012). The determination of *Metarhizium* colonies was based on colony morphology: conidial chains, hyphae, phialides, and conidia were confirmed by light microscopy ($\times 400$). Colonies on plates were counted and the numbers of Colony Forming Units (CFU) per gram of humid soil were calculated. Pure *Metarhizium* isolates of colonies were obtained by streaking small quantities of conidia using platinum sterile loops in PDA medium plates (Inglis et al., 2012).

2.5.2. Insect baiting

The insect baiting method applied was described by Inglis et al. (2012). Soil samples were homogenized by hand using a metal sieve with pore size of 4 mm. One hundred grams of the sieved soil were transferred to transparent plastic pots with 200 mL capacity with perforated lids. Ten 3rd or 4th instar larvae of *Tenebrio molitor* (Coleoptera: Tenerionidae) from a laboratory colony (LPCMI—ESALQ/USP) were transferred to the surface of the soil in plastic pots. Soil samples were moistened with sterile distilled water using a manual spray whenever deemed necessary.

The pots were stored in a climatic chamber at 25 ± 1 °C, $70 \pm 10\%$ in darkness and each container was turned up-side down every day during the first week to induce the movement of the larvae through the soil. From the 5th day onwards, inspections of dead larvae were made every three days for a period of three weeks. Dead larvae were surface sterilized to prevent the growth of external contaminant saprophytic fungi by immersion in sodium hypochlorite 2% for 30 s, and washed three times in sterile distilled water and then individually placed in 24 wells cell culture plates with a lid on. Moistened cotton pieces with sterile distilled water were added to ensure high relative humidity. After observation of external fungal growth on the insect cadavers, small quantities of conidia were transferred using platinum sterile loops in PDA medium plates to obtain pure colonies.

2.5.3. Isolation from strawberry rhizospheres

For the isolation of rhizosphere fungi, the method described by Wyrebek et al. (2011) was employed. The roots were first shaken manually to remove any loose soil. Roots were then washed with sterile distilled water and manually cut into small pieces (approx. 0.5 cm) using laboratory scissors. Ten grams of the five plant root cuttings from the composite sample were placed together in 20 mL of distilled water plus 0.05% Tween 80 in a 40 mL flat bottom glass tube. The tubes were vortexed for 10 min and 100 µL of this solution was serially diluted (10^{-1}, 10^{-2}, 10^{-3}) and 0.1 mL of each dilution were plated in two replicate plates on selective media as described above. After seven days, *Metarhizium* colonies were identified morphologically and the number of CFU's per gram of humid roots was calculated. *Metarhizium* isolates with different colony morphology from each sample as above, representing each isolation method and sampling occasion were cryopreserved in the Collection of Entomopathogenic Microorganisms of ESALQ-USP and included for further molecular characterization as described below.

2.6. Molecular identification

2.6.1. DNA extraction

DNA was extracted using ABI PrepMan Ultra protocol as described by Kepler et al. (2014) from vegetative hyphae and conidia grown 5–10 days on sterile filter paper strips overlaid on PDA plates. Mycelium and conidia were transferred to a 2 mL sterile tube containing 300 mL Prepman extraction buffer and zirconia-silica beads. Cells were disrupted in a FastPrep-2 5G Instrument (MP Biomedicals) with two 10 s cycles at a speed setting of 5.5. The tubes were incubated in boiling water for 10 min and subsequently centrifuged for 10 min at 14,000g. One hundred and seventy-five µL of the supernatant were transferred to a fresh tube and stored at -20 °C.

2.6.2. SSR marker analyses and gene sequencing

SSR marker analyses were performed as described by Mayerhofer et al. (2015a,b) using 11 markers: Ma307, Ma145, Ma165, Ma416, Ma2097, Ma164, Ma2098, Ma2065, Ma2089, Ma2063, Ma2054 (Enkerli et al., 2005; Oulevey et al., 2009). SSR loci was PCR amplified as described by Mayerhofer et al. (2015a,b) and products analyzed on an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). Fragment sizes (allele sizes) were determined using the GenMarker v1.51 (SoftGenetics LLC, State College, PA) software and GeneScan ROX400 (Applied

Biosystems) as internal size standard. SSR data were analyzed using GenAlEx 6.5 (Peakall and P.E, 2006, 2012).

Individual multilocus SSR haplotypes were assigned to species by sequencing the 5' end of nuclear encoded translation elongation factor 1-alpha (5'-TEF1-α) (Bischoff et al., 2009) and a nuclear intergenic marker MzFG543igs (Kepler and Rehner, 2013). PCR amplifications were performed for one representative isolate of each SSR haplotype with primers EF2F (5'-GGAGGACAAGACTCA-CATCAACG- 3') and EFjR (5′-TGYTCNCGRGTYTGNCCRTCYTT-3') and MzFG543igs_1F (5'-ATTCATTCAGAACGCCTCCAA-3') and MzFG543igs_4R (5'-GGTTGCGACTGAGAATCCATG-3'). PCR products were purified using the geneMAG-PCR Kit (Chemicell, Germany) and sequenced using the same primers mentioned above. Sequencing was performed by Beckman Coulter Genomics (United Kingdom).

Sequences were edited and aligned with 17 reference sequences obtained from GenBank, representing ex-type cultures or taxonomically confirmed isolates of *M. anisopliae*, *M. brunneum*, *M. guizhouense*, *M. lepidiotae*, *M. pingshaense* and *M. robertsii* (Bischoff et al., 2009; Steinwender et al., 2014; Rezende et al., 2015) (cf. Table A.1) using MAFFT with the FFT-NS-i alignment option in Geneious 7.1.8. software (Kearse et al., 2012). The sequence of *M. lepidiotae* (ARSEF7412) was included as outgroup and jModelTest 2.1.7 (Darriba et al., 2012) was used to calculate the evolution model for the phylogenetic tree (best-fit models of nucleotide substitution). The most parsimonious tree was calculated based on Bayesian (GTR-GAMMA) and Maximum Likelihood (GTR-GAMMA) model parameters using 1000 bootstrap replicates with the software Geneious 7.1.8 (Kearse et al., 2012) of the combined single gene alignments (5'-TEF1- α and MzFG543igs) made in the software Mesquite 3.04 (Maddison and Maddison., 2015).

2.7. Statistical analyses

CFU counts of *Metarhizium* spp. in each plot were analyzed by fitting a linear mixed model to the proportion of *Metarhizium* CFU data with a full interaction between sample dates and plot treatment with a linear predictor, including random intercepts and slopes for each plot. Sub-models were tested using likelihood-ratio (LR) tests (McCullagh and Nelder, 1989).

The proportions of *T. molitor* larvae killed by *Metarhizium* in soil samples were analyzed by fitting a binomial generalized linear mixed model with a full interaction between sampling time points and treatment linear predictor and including random intercepts and slopes for each plot, as well as an observation-level random effect to model overdispersion. Submodels were tested using LR tests (Demétrio et al., 2014). All analyses were performed using the R statistical software environment (R Development Core Team, 2015)

3. Results

3.1. Isolation of entomopathogenic fungi

3.1.1. Selective agar media

Metarhizium spp. occurred naturally in the soil of the experimental agroecosystem with CFUs detected in samples at all sampling occasions also before application (Fig. 1). No significant differences were found between the densities of *Metarhizium* spp. in the different treated soils (soil from strawberry plots inoculated with *M. anisopliae* or *M. robertsii*, uninoculated control plots and margin crop area) at each sampling time point, except for September 2012 ($3.5 \pm 1.0 \times 10^3$ CFU/g) where the concentration in the *M. robertsii* treatment was lower than the concentration in the other treatments (LR = 18.35, d.f. = 9, p = 0.0313) (Fig. 1). The abundance of *Metarhizium* spp. in soil over time, within each treatment, differed only in plots where *M. anisopliae* and *M. robertsii* were applied. The highest CFU density was observed in the latter treatment in August 2013 ($1.4 \pm 0.6 \times 10^4$ CFU/g).

3.1.2. Insect bait mortality

The percentages of *T. molitor* larvae killed by *Metarhizium* spp. exposed to different soil samples were not significantly different among treatments, except in August 2013, when the values were higher in *M. anisopliae* treated plots ($77.5 \pm 6.3\%$) than all other treatments (LR = 1.50, d.f. = 3, p = 0.6813). Overall, larval mortality proportions in the soil samples increased significantly over time (LR = 28.70, d.f. = 1, p < 0.0001). In strawberry plots inoculated with *M. anisopliae* the mortality proportion was significantly higher in August 2013 than the previous three sampling occasions (Fig. 2). For *M. robertsii*, the proportions were at comparable levels after inoculation and no mortality was found before inoculation. In the uninoculated control plots larval mortality was significantly higher in August and April 2013 compared with the mortality measured in the two previous sampling occasions. At the last sampling occasion in August 2013, the percentages of larvae killed by *Metarhizium* spp. were $50.0 \pm 15.8\%$, $45.0 \pm 6.5\%$ and $36.0 \pm 12.1\%$ in treatment
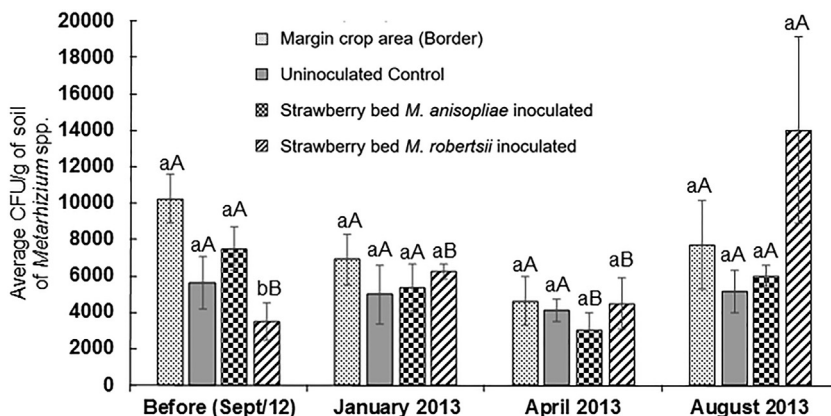

Fig. 1. Average densities (±SE) [Colony forming units (CFU) per g of humid soil] of *Metarhizium* spp. in strawberry crop soil. Different lower-case letters indicate differences among treatments at the same date; different upper-case letters indicate differences of same treatments among sampling dates. Data were analyzed by linear mixed models and with full interaction between sampling time point and treatment as the linear predictor (P ≤ 0.05).
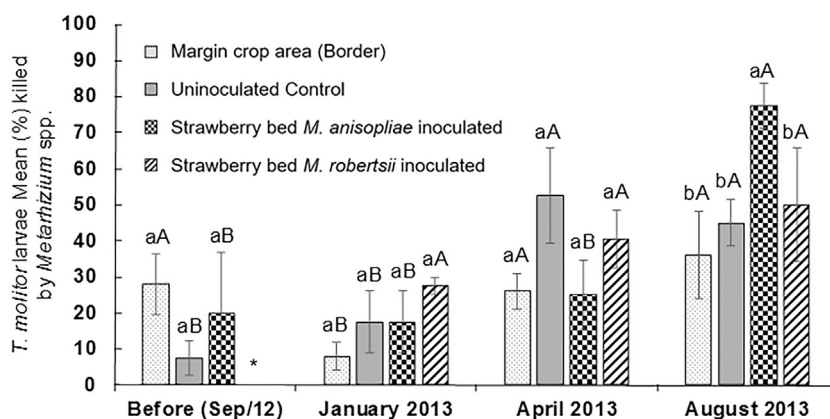
Fig. 2. Mortality (Mean ± SE) of *Tenebrio molitor* larvae used in the isolation of *Metarhizium* spp. by the insect bait method. Different lower-case letters indicate differences among treatments at the same date; different upper-case letters indicate differences of same treatments among sampling dates both using binomial generalized linear mixed models and with a full interaction between sampling time point and treatment as the linear predictor (P ≤ 0.001). * = No mortality of larvae in this treatment.

plots inoculated with *M. robertsii*, uninoculated control and border plants, respectively (Fig. 2).

3.2. SSR marker analyses and gene sequencing

Of the 108 isolates recovered from all 12 plots during the experimental period and included in SSR marker analyses, 60.2% was recovered using the insect bait method and 39.8% with selective media method; 27.8% were found in the margins of the crop associated with the border herb plants, 25.0% in the uninoculated control plots, 20.4% in *M. anisopliae* treated plots, 16.7% in the *M. robertsii* treated plots, 5.5% were recovered from strawberry rhizospheres in *M. robertsii* treated plots and 4.6% were from rhizospheres in *M. anisopliae* treated plots (Table 1).

Eleven SSR markers were applied in this study and all of them were polymorphic revealing 2–3 alleles per locus (Table 2). The analyses resolved 11 haplotypes among the 108 isolates investigated.

Species assignment based on sequence alignments and subsequent phylogenetic analyses of 5'-TEF1-α and MzFG543igs sequences revealed four *Metarhizium* species among the 11

haplotypes clustering with *M. anisopliae*, *M. robertsii*, *M. brunneum* and *M. pingshaense* reference isolates (Fig. 3). Sequence alignments consisted of 517 positions for 5'-TEF1-α (Table A.3) and 693 position for MzFG543igs (Table A.4) and after combining them, sequence differences were compared in an alignment of 1210 positions of 5'-TEF1-α and MzFG543igs concatenated dataset (Table 3). *M. robertsii* intraspecific differences ranged up to 3 positions, while lower intraspecific variation was observed within *M. anisopliae* with just 1 position. Base pair differences between *M. robertsii* and *M. brunneum* haplotypes ranged from 44 to 46 positions, *M. anisopliae* and *M. brunneum* haplotypes ranged from 50 to 51 positions representing the largest differences found among the haplotypes studied. The fourth species recovered was *M. pingshaense* represented by a single haplotype with a maximum difference of 24 base pair differences of *M. anisopliae*, 29 positions of *M. robertsii* and 46 positions of *M. brunneum*.

Three haplotypes were found among the 31 *M. anisopliae* isolates found in this study (Table 1), which were referred to as haplotypes A (representing the applied isolate ESALQ1037), B and C; six haplotypes among 75 *M. robertsii* isolates: D, E, F, G, H and I ("I" includes the applied isolate ESALQ1426); and the single

Table 1
Occurrence of each *Metarhizium* haplotype in strawberry crop soil before the application and at three subsequent sampling time points. (Ma = *Metarhizium anisopliae*, Mr = *M. robertsii*, Mb = *M. brunneum* and Mp = *M. pingshaense*).

Strawberry Plots															Margins					Roots	Total
		M. anisopliae				*M. robertsii*				Control					Border plants						
	application	Before	After			Before	After			Before	After				Before	After				After	
Species	Haplotype	Sep/12	Jan/13	Apr/13	Aug/13	Sep/12	Jan/13	Apr/13	Aug/13	Sep/12	Jan/13	Apr/13	Aug/13		Sep/12	Jan/13	Apr/13	Aug/13		Aug/13	
Ma	A[a]	0	6	9	1	0	0	0	1	0	3	3	0		0	1	0	0		5	29
	B	0	0	0	0	0	0	0	0	0	0	0	1		0	0	0	0		0	1
	C	0	0	0	0	0	0	0	0	0	0	0	0		1	0	0	0		0	1
Mr	D	0	0	0	0	0	0	0	0	0	0	1	0		0	0	0	0		0	1
	E	1	0	0	3	0	0	1	0	1	4	7	3		2	4	5	2		3	36
	F	1	0	0	0	0	0	0	0	0	0	0	1		0	3	2	0		0	7
	G	0	0	0	0	0	0	0	0	0	0	0	0		0	0	1	0		0	1
	H	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	1		0	1
	I[a]	0	0	0	0	0	2	7	7	0	0	0	2		0	1	4	3		3	29
Mb	J	0	0	1	0	0	0	0	0	0	0	0	0		0	0	0	0		0	1
Mp	K	0	0	0	0	0	0	0	0	0	1	0	0		0	0	0	0		0	1
Total n isolates		2	6	10	4	0	2	8	8	1	8	11	7		3	9	12	6		11	108

[a] Haplotype of applied isolates (*M. anisopliae*—ESALQ1037 and *M. robertsii*—ESALQ1426).

Table 2
Number of alleles, allele size range [base pairs] for 11 Simple Sequence Repeat loci for each of the eleven haplotypes of *Metarhizium* spp. identified with DNA sequence based analyses.

Locus	*M. brunneum* n = 1 and *M. pingshaense* n = 1	*M. anisopliae* n = 31	*M. robertsii* n = 75
Ma145	1 [106]	1 [106]	2 [107;108]
Ma164	2 [116;121]	1 [116]	2 [116;117]
Ma165	2 [140;141]	2 [140;141]	1 [143]
Ma307	2 [112;161]	2 [161;164]	3 [149–161]
Ma416	2 [115;116]	1 [115]	1 [126]
Ma2054	2 [218;238]	1 [218]	2 [217;220]
Ma2063	2 [144;143]	2 [144;146]	1 [137]
Ma2065	2 [131;146]	1 [131]	2 [129;131]
Ma2089	2 [196;199]	2 [196;198]	1 [194]
Ma2097	2 [189;191]	2 [191;195]	3 [181–185]
Ma2098	2 [171;179]	1 [171]	3 [171–197]
Resulting number of haplotypes	2 (J, K)	3 (A[a]–C)	6 (D–I[a])

[a] Haplotype of applied isolates (*M. anisopliae*—ESALQ1037 and *M. robertsii*—ESALQ1426).
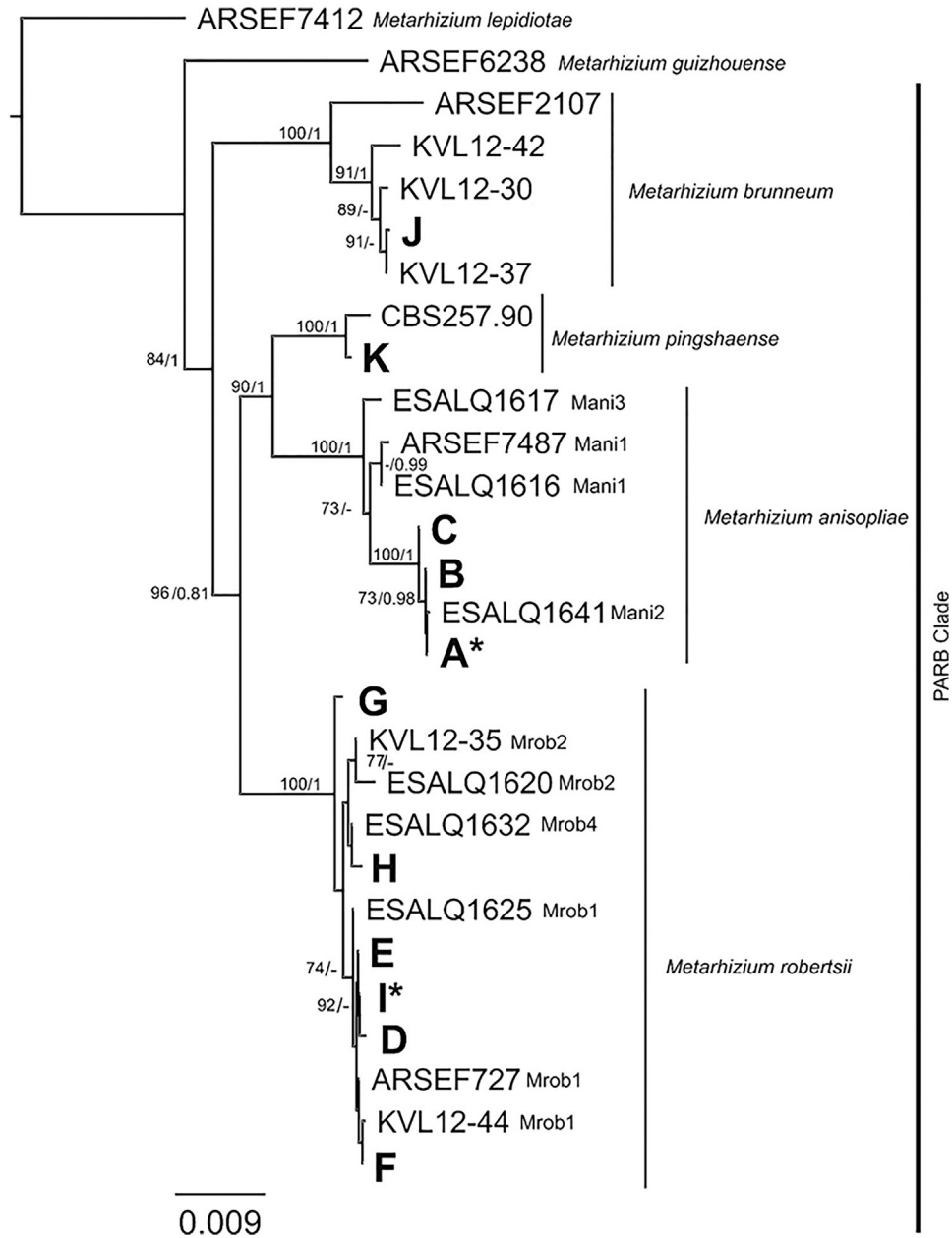
Fig. 3. Maximum likelihood (ML; GTR-gamma model)/Bayesian (B; GTR-gamma model) phylogeny of the combined data set of 5' TEF-α and MzFG543igs sequences of 11 isolates representing individual haplotypes A–K identified in the experiment crop, including 17 taxonomically validated reference strains accessioned in ARSEF (ARS Entomopathogenic Fungal Culture Collection, CBS (Fungal Biodiversity Centre) and ESALQ (Collection of Entomopathogenic Microorganisms of LPCMI-ESALQ-USP). Bootstrap support values ≥ 70% are presented near nodes of each type of analyses (ML/B).

isolates of *M. brunneum* and *M. pingshaense* were referred to haplotypes J and K, respectively (Fig. 3).

3.3. Species and haplotype frequencies

Of the three most frequently isolated haplotypes, two were assigned to *M. robertsii* (E, I) and one assigned to *M. anisopliae* (A). *Metarhizium robertsii* was the most frequent species comprising 69.4% of all isolates, followed by 28.7% for *M. anisopliae*, and a single isolate for both *M. brunneum* and *M. pingshaense*. Among the two *M. robertsii* haplotypes, haplotype E (33.3% of the *Metarhizium* spp. isolates) represented an indigenous haplotype which was detected in all treatments and I (26.8%) representing the applied *M. robertsii* isolate, were the most frequently isolated. Among the *M. anisopliae*, haplotype A, representing the applied isolate, was most frequent (Table 1). Except for *M. robertsii* haplotype F (6.5%), all remaining haplotypes were represented by single isolates only.

The haplotypes (A and I) of the two applied isolates were not detected in soil of any of the treatments before application. However, at different sampling time points post inoculation, isolates with these haplotypes were recovered in plots where they were not applied (Table 1). The inoculated *M. anisopliae* haplotype A was found four months after inoculation in all treatments except in *M. robertsii* treated plots; in this latter treatment haplotype A appeared after one year. The haplotype frequency of the applied *M. anisopliae* isolate (A) increased 4 months after application to 0.40 but decreased after 8 and 12 months to 0.29 and 0.20, respectively. The haplotype frequency of the applied *M. robertsii* isolate (I) increased from not detected before inoculation to 0.12, 0.29 and 0.40, after 4, 8 and 12 months, respectively. The inoculated *M. robertsii* haplotype I was detected after four months in the plots where it was applied and in the soil around border plants. After one year (August 2013) this haplotype was also detected in the uninoculated control plots while it was never recovered in the *M. anisopliae* treated plots. The number of haplotypes detected after the inoculation (not including haplotypes of the inoculated isolates) was lower in the *M. robertsii* (n = 1) and *M. anisopliae* (n = 2) treated plots than in the uninoculated control plots (n = 5) and around border plants (n = 4). More *M. robertsii* isolates (n = 22) where obtained at the last sample occasion than *M. anisopliae* isolates (n = 3). The haplotype frequency of most common indigenous haplotype (E) before the inoculation of the treatments was 0.67 and was stable after this period (0.32, 0.31 and 0.31 after 4, 8 and 12 months).

Three haplotypes of *M. anisopliae* and *M. robertsii* were recovered from the rhizosphere samples. Besides the inoculated isolates (haplotype A and I), only the most common indigenous isolate in the soil (*M. robertsii* haplotype E) was found from the rhizosphere (Table 1).

4. Discussion

In this study, we demonstrate that two *Metarhizium* isolates applied in the strawberry field showed establishment, persistence and local dispersion in the soil and strawberry rhizospheres over a 1-year period. The inclusion of SSR markers allowed for a reliable and transparent identification of the applied fungal isolates among the indigenous *Metarhizium* haplotypes. The applied isolates were recovered most frequently in their specific treatment plots for up to one year after application, but they were also found in uninoculated strawberry control plots and in soil of field margins. Both isolation methods recovered the applied isolates and previous studies have demonstrated that the two methods will recover the same *Metarhizium* haplotypes (Steinwender et al., 2014; 2015). The recovered CFU densities of the in vitro method were relatively high compared to reports of other studies; Scheepmaker and Butt (2010) concluded by reviewing the literature that 830 CFU/g soil, as defined by the 95th percentile of the geometric mean, represents natural background levels. The indigenous *M. robertsii* haplotype E was most abundant and widely distributed across samples while haplotype I representing the applied *M. robertsii* isolate was recovered from both inoculated and uninoculated plots (control and border plants) and rhizosphere samples. These results indicate that *M. robertsii* is the most naturally abundant species at the field site and this species was represented by five indigenous haplotypes indicating significant diversity. Rezende et al. (2015) proposed the hypothesis that Brazilian *M. robertsii* haplotypes may be better adapted for persistence in the soil environment as opposed to being solely an entomopathogenic fungus staying above ground. The inoculated *M. robertsii* isolate ESALQ1426 seemed to persist better than the other applied isolate *M. anisopliae* ESALQ1037. So far, most characterized isolates originating from insect hosts in Brazil belong to the *M. anisopliae* Mani2 clade (Rezende et al., 2015), including the inoculated isolate ESALQ1037 of the present study. The apparent soil-based ecology of *M. robertsii* also included recovery from plant roots as has been reported in other studies (Behie et al., 2012; Steinwender et al., 2015).

However, haplotype A representing the applied *M. anisopliae* isolate was recovered from the rhizosphere of the strawberry plants at the final sampling occasion one year after application, indicating that a member of Mani2 clade can be associated with roots. Rhizosphere soils are potential reservoirs for *Metarhizium* spp., since these soils can be seen as the soil/root interphase where plants, insects and microbes interact (Hu and St. Leger, 2002). Persistence of *M. brunneum* in the strawberry rhizospheres has been hypothesized to depend on local adaptations to the prevailing abiotic conditions at the field site (Klingen et al., 2015), and rhizosphere compatibility of *Metarhizium* spp. in Brazil could be an important trait of a biocontrol agent for long term persistence and prolonged biocontrol efficacy. Further studies are needed to evaluate if rhizosphere colonization of strawberries are beneficial to plant health as has been seen in laboratory settings for other crops (Sasan and Bidochka, 2012, 2013).

The implementation of SSR markers to study entomopathogenic fungi into the soil produced a detailed data set, revealing the natural occurrence of indigenous haplotypes and persistence over time of each specific applied isolate in strawberry crops. Vieira Tiago et al. (2012) studied the persistence of *M. anisopliae* in vases with sugarcane soil in a greenhouse using PCR-DGGE techniques, and detected the fungus for up to 60 days. The extrapolation of these laboratory data into a field situation is, however, difficult. A

Table 3
Base pair difference among the 11 *Metarhizium* haplotypes in sequences of the 5' end of Elongation Factor 1-α and MzFG543igs concatenated dataset (total positions 1210 bp). (*Ma = Metarhzium anisopliae, Mr = M. robertsii, Mb = M. brunneum* and *Mp = M. pingshaense*).

Species	Haplotype	Ma A[a]	Ma C	Ma B	Mp K	Mr D	Mr E	Mr I*	Mr F	Mr G	Mr H	Mb J
Ma	A[a]											
Ma	C	1										
Ma	B	0	1									
Mp	K	24	23	24								
Mr	D	36	35	36	29							
Mr	E	34	33	34	27	2						
Mr	I[a]	35	34	35	28	1	1					
Mr	F	35	34	35	28	1	1	0				
Mr	G	35	34	35	28	3	3	2	2			
Mr	H	34	33	34	27	2	0	1	1	3		
Mb	J	51	50	51	46	46	44	45	45	45	44	

[a] Indicates haplotypes of the applied isolates (*M. anisopliae*—ESALQ1037 and *M. robertsii*—ESALQ1426).

high persistence of the *M. brunneum* isolate Bipesco5 and *M. anisopliae sensu lato* isolate 2277 in corn field soil in Hungary for up to 15 months was demonstrated by Pilz et al. (2011) using some of the same SSR markers as in the present study. These data corroborate our findings regarding the long-term field persistence of inoculated *Metarhizium* isolates.

The applied isolates were detected also in plots where these fungal isolates were not applied 4–12 months post application, indicating the potential for horizontal dispersal of the isolates. Activity of soil arthropods and/or rain may be responsible for the dissemination of fungal propagules (Dromph, 2001; Meyling and Eilenberg, 2007), but it cannot be excluded that agronomic manipulations have contributed to the dispersion given the limited distances between experimental plots. Horizontal dispersal may represent an important mechanism for establishment of a biocontrol agent in a habitat or a crop; however, specifically designed experiments would be required to assess this aspect in detail.

Although the fungus treated plots had a greater post-inoculation abundance of the experimentally applied haplotypes than in neighboring control plots, there was a relatively high degree of diversity of indigenous haplotypes in all plots. The fact that seven of the eleven haplotypes were detected in one isolate only indicates that sampling was not saturated. For an in depth study of genetic diversity of *Metarhizium* in these plots additional sampling would be required. Nevertheless, data suggest that the application of biocontrol fungal isolates did not significantly alter the *Metarhizium* community composition within the one-year time frame of this study.

Earlier studies in Brazil have recovered several naturally occurring *Metarhizium* species. Lopes et al. (2013a) found in small agricultural habitats (commercial banana fields) *M. anisopliae*, *M. pingshaense* and what is likely to be an undescribed *Metarhizium* species. Lopes et al. (2013b) further found *M. lepidiotae* in maize cropping systems, while *M. brunneum* was reported infecting a hemipteran species in southern Brazil (Lopes et al., 2014). Rocha et al. (2013) studied the *Metarhizium* diversity in soils in central Brazil and found a large number of isolates of *M. anisopliae, M. robertsii* and *M. pemphigi*. Rezende et al. (2015) focused on the *Metarhizium* diversity associated with sugarcane agriculture and identified two new taxonomically unassigned linages besides the ones found in the studies above. In the present study we corroborate that *M. robertsii* and *M. anisopliae* are most abundant in agroecosystems in Brazil, including in strawberry field soil and rhizospheres.

Besides the haplotypes representing the two applied *Metarhizium* isolates, we identified five indigenous haplotypes in the strawberry field margins and the control plots, indicating that the strawberry cultivation regimes did not negatively affect fungal diversity directly. High *Metarhizium* spp. densities were found in soil samples collected in agricultural sites in Switzerland from low-input permanent grassland and improved field margins compared to arable, intensively cultivated fields, indicating that both semi-natural habitat types may provide potential refuges for *Metarhizium* spp. (Schneider et al., 2012). Clifton et al. (2015) proposed that the abundance of *M. anisopliae s.l.* in field margins was negatively affected by proximity to conventional fields, suggesting that cropping practices within a field could affect soil-borne microorganisms outside the field. In our study, the margin and border plants yielded comparable abundance and diversity of *Metarhizium* spp. as compared to uninoculated control plots. The lack of chemical control measures in the experimental strawberry fields could have been an important contributor to this observation. The persistence and dispersal of *M. anisopliae* and *M. robertsii* applied through soil drenching in strawberry soils demonstrated in this study provides valuable information regarding fate and efficacy of biocontrol agents in the field.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2016.09.031.

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