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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

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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.

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:

* 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).

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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).