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Metarhizium seed treatment mediates fungal dispersal *via* roots and induces infections in insects

Chad A. KEYSER[a,*], *Kristian THORUP-KRISTENSEN*[b], *Nicolai V. MEYLING*[a]

[a]*Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark*

[b]*Department of Plant and Environmental Sciences, University of Copenhagen, Hojbakkegård Alle 13, 2630 Taastrup, Denmark*

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ABSTRACT

The study aimed to evaluate the extent to which *Metarhizium* spp. applied as conidia to seeds will disperse with the growing root system and maintain pathogenicity after dispersion. *Tenebrio molitor* larvae were exposed to roots of wheat plants that had been grown from seeds inoculated with conidia of either *M. brunneum* (KVL 04-57 and KVL 12-37) or *M. robertsii* (ARSEF 2575 and KVL 12-35) in both laboratory and greenhouse settings. All four *Metarhizium* isolates tested maintained pathogenicity towards *T. molitor* larvae for up to 4 weeks after being dispersed by roots through both an artificial growth substratum and non-sterile soil. Based on these results we propose that a plant–root association benefits entomopathogenic fungi with mobility in the soil and an increased likelihood of encountering a susceptible insect host.

Introduction

The soil-inhabiting mitosporic entomopathogenic fungal genus *Metarhizium* (Hypocreales: Clavicipitaceae) has a cosmopolitan distribution and a wide range of arthropod hosts (Zimmermann, 2007). As early as 1888 the potential for a biological control agent was recognized and a *Metarhizium* spp. isolate was mass produced and applied to control a pest insect (Krassilstschik, 1888; Roberts and St. Leger, 2004). Both the need and hope for a reliable biological control agent have fueled *Metarhizium* research for more than a century. Emphasis placed on developing a biological control agent has, however, resulted in a neglect of research concerning basic *Metarhizium* ecology which may be essential to its success for biological control (Bruck, 2005; Meyling and Eilenberg, 2007; Vega et al., 2009).

To fill this gap of knowledge, several studies have recently focused on the fundamental ecology of these fungi both in their natural habitats and in the ecosystems where they are applied. These have shown that genotypic groups of *Metarhizium* spp. can associate closely with habitat and plant species (Bidochka et al., 2001; Fisher et al., 2011; Wyrebek et al., 2011). Rhizosphere competence, the ability of a microorganism to proliferate and function in the rhizosphere

* *Corresponding author*. Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, Stair 2, 3rd Floor, B321, 1871 Frederiksberg, Denmark.
E-mail address: chadaltonkeyser@gmail.com (C.A. Keyser).
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(St Leger, 2008), was observed in *Metarhizium* spp. isolates (Bruck, 2005; Hu and St Leger, 2002). Furthermore, *Metarhizium* spp. applied to the rhizosphere have been shown to benefit plant growth and aid in plant-nutrient acquisition (Behie and Bidochka, 2014; Behie et al., 2012; Khan et al., 2012; Sasan and Bidochka, 2012), and it is now clear that the ecological complexity and importance of the fungal genus *Metarhizium* extend beyond that of 'entomopathogen'.

One central question that remains to be explored is what benefits *Metarhizium* derives from plant associations. Thus far the focus of endophytic interaction between plants and *Metarhizium* has been characterized by how the plant has benefitted, eg. through nutrient acquisition (Behie et al., 2012), while if and how the fungus benefits have largely been ignored. *Metarhizium* spp. can process and utilize plant-derived carbohydrates available in the rhizosphere for growth (Fang and St Leger, 2010; Pava-Ripoll et al., 2011) but it is possible that *Metarhizium* spp. acting as plant symbionts might also increase their exposure to prospective host insects. For example, actively growing plant roots could provide non-mobile microorganisms, such as *Metarhizium* spp. with a means of dispersal, thus increasing their chance of finding a host insect. It can be assumed that there is an increased likelihood of encountering a root feeding herbivore near its food source (Park and Tollefson, 2005; Prystupa et al., 1988).

The first step towards answering these questions is to determine if *Metarhizium* retains its pathogenicity while associating with plant roots. Bruck (2005) found that root cuttings inoculated with *Metarhizium* conidia (actually *M. brunneum*) resulted in successful infections of root feeding black vine weevil (*Otiorhynchus sulcatus*) larvae. Kabaluk and Ericsson (2007) discovered *Metarhizium* infected wire worm larvae (*Agriotes obscurus*) after sowing *Metarhizium*-treated (*M. brunneum*) corn seeds in a field trial. They also observed an increased yield in corn production when the seeds were treated with the fungus and speculated that the observed yield increase was due to seedlings being protected from insect attack either by insects being infected or repelled by the fungus. However, further studies are needed to determine the effectiveness of seed treatments in infecting insects.

The hypothesis tested in this study was that *Metarhizium* spp. applied as seed treatment will disperse with roots in non-sterile soil and retain the ability to infect insects. To test this hypothesis, *Tenebrio molitor* larvae were exposed to the roots of wheat plants that had been grown from seeds inoculated with *Metarhizium* spp. conidia in both laboratory and greenhouse settings. In addition a traditional infection bioassay was performed to determine the virulence of the fungal isolates included in this study.

Materials and methods

Fungal preparation

Four isolates of *Metarhizium* spp. were included in this study. Two isolates of *M. robertsii* (*Mr*): ARSEF 2575 obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (USDA Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA) and KVL 12-35, isolated from agricultural soil in Denmark (Steinwender, 2013); and two isolates of *M. brunneum* (*Mb*): KVL 04-57, same genotype as the active ingredient of the commercial isolate Met52 (Novozymes, Salam, Virginia), and KVL 12-37, isolated from agricultural soil in Denmark (Steinwender, 2013). All four isolates are maintained at −80 °C at the University of Copenhagen and were identified to species using DNA sequencing following Bischoff et al. (2009).

Fresh cultures of each isolate were prepared for experiments from a stock culture, each grown for 2–3 weeks on Sabouraud Dextrose Agar (SDA) (Merck KGaA, Darmstadt, Germany) at 26 °C. Conidia were then harvested with a sterile spatula and suspended in 0.005 % Triton X-100 (Sigma Chemical, MO, USA), the suspension was then passed through glass wool to remove hyphae, conidial clumps and loose agar. Each suspension was vortexed and conidial concentrations estimated by counting in a hemocytometer (Fuchs-Rosenthal).

Conidial viability was checked by transferring 20 μl of the suspension onto agar medium [4-ml SDA with 0.002 % Benomyl active ingredient (Sigma–Aldrich, MO, USA)] and counting conidia germination after 48 hr at 26 °C; suspensions were only used if germination was greater than 90 %.

Seed treatment assay

Seed treatments

For each isolate of *Metarhizium* a 1×10^8 conidia ml^{-1} suspension was prepared and then diluted to create a second suspension of 1×10^7 conidia ml^{-1}. Wheat seeds (*Triticum aestivum*) were pre-germinated to ensure viability and then soaked for 1 hr in a fungal treatment or distilled water (control); they were then removed and allowed to dry on filter paper at room temperature. A prescreening of several varieties of wheat indicated that surface sterilizing seeds removed some but not all natural occurrence of microbes; to maintain a realistic seed-borne microbial community the wheat seeds were not surface sterilized.

Paper roll setup

Ten treated seeds were evenly spaced 2 cm from the top edge of 2-ply filter paper (58 × 29 cm) (Frisenette ApS, Denmark) which had been moistened to saturation with ddH_2O. Seeds were then secured by placing a strip of filter paper (7 × 58 cm) along the top of the paper, which was rolled up into a tube and placed standing upright with seeds in the top in a 600 ml beaker with 200 ml ddH_20, placing the seed approx. 22 cm from the surface of the water (Supplementary Fig S1). Each roll was wrapped in a piece of aluminum foil to keep seeds in the dark and reduce evaporation and then placed under a growth lamp with 12/12 hr light/dark regime at room temperature (21 °C ± 2) for 14 d. The resulting data from this setup will be referred to as either **Paper 10^8** or **Paper 10^7** according to the spore concentration. The setup was repeated on four separate occasions.

Soil-pot experiment

In the greenhouse, pots were prepared with sandy non-sterile subsoil taken from the lower horizon (20–60 cm) of an Agrudalf soil at the University research farm in Taastrup, Denmark. The texture of the soil was 3.9 % clay, 4.7 % silt, 33.3 %