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Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*

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ABSTRACT

Endophytic fungi may display preferential tissue colonization within their plant hosts. Here we tested if the endophytic, insect pathogenic fungi (EIPF) *Metarhizium* and *Beauveria* showed preferential localization within plant tissues, in the field and under laboratory conditions. In the field, plants were sampled from three separate sites (Brock University, St. Catharines, Ontario; Pelham, Ontario; and Torngat Mountains National Park, Newfoundland, Canada) and EIPF were isolated from plant roots, the hypocotyl, and stem and leaves. Two genera of EIPF, *Metarhizium* spp. and *Beauveria bassiana*, were isolated from plants sampled, as well as the nematophagous fungus, *Pochonia chlamydosporium*. *Metarhizium* spp. were almost exclusively found in roots, whereas *B. bassiana* and *P. chlamydosporium* were found throughout the plant. The *Metarhizium* species were identified by RFLP and 95 % were *Metarhizium robertsii*, 4.3 % were *M. brunneum*, and 0.7 % were *M. guizhouense*. Lab studies with *M. robertsii* and *B. bassiana* reflected observations found in the field, that is, *Metarhizium* was restricted to the roots of plants while *B. bassiana* was found throughout the plant. Insect infection by these EIPF is preferential with respect to above and below ground insects, and the present study correlates above and below ground insect infections with endophytic colonization by these EIPF.

Introduction

Endophytic fungi play important roles in plant ecology, community structure, plant evolution, and soil interactions (Carroll, 1988; West et al., 1993; Saikkonen et al., 1998). Endophytes display remarkable diversity in phylogeny, lifestyle (i.e. colonization and dissemination), plant host specificity, and colonization within plant tissue (i.e. roots, stem, leaf) (Porras-Alfaro & Bayman, 2011). Our understanding of the ecology of certain endophytic fungi (e.g. *Epichloe*) is quite broad (Leuchtmann, 1997), however, in other endophytes knowledge remains limited (Pancher et al., 2012).

A specialized group of fungal endophytes are those that possess insect pathogenic capabilities. These endophytic, insect pathogenic fungi (EIPF) include species of *Metarhizium* and *Beauveria* (Sasan and Bidochka, 2012; Posada and Vega, 2005). The endophytic capability and insect pathogenicity of *Metarhizium* and *Beauveria* are coupled so that the fungus acts as a conduit to provide insect-derived nitrogen to plant hosts. Several *Metarhizium* spp. as well as *Beauveria bassiana*, are able to infect insects and transfer insect-derived nitrogen to the host plant (Behie et al. 2012; Behie & Bidochka, 2014). *Metarhizium* and *Beauveria* have global distributions, infect over 200 species of insects (Roberts & Hajek, 1992), and have been

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extensively studied as biological control agents (Ngumbi et al., 2011). While most *Metarhizium* species and *Beauveria* are generalist insect pathogens, they have preferential infection of below ground or above ground insects. In agricultural fields, *Metarhizium* predominantly infected arthropods below ground while above ground arthropods were predominantly infected by *Beauveria* (Meyling et al., 2011).

Here, we wanted to determine if the previously reported partitioning of fungal infection in above or below ground arthropod cadavers coincided with the localization of *Beauveria* and *Metarhizium* within plant tissues. That is, we hypothesized that *Metarhizium* preferentially resides in plant roots, while *Beauveria* colonized above ground tissues. Insects would, therefore, be infected by these EIPF due to their proximity to, or herbivory on, fungal colonized plant tissues. Here we tested the preferential colonization of *Metarhizium* and *Beauveria* on root, hypocotyl and leaf tissues under laboratory and field conditions.

Materials and methods

Fungal isolates

Metarhizium robertsii (ARSEF 2575) and *B. bassiana* (ARSEF 252) were obtained from the United States Department of Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, New York. These fungi were transformed for green fluorescent protein (GFP) expression as previously described (Fang et al. 2006). Cultures were grown on potato dextrose agar (PDA; Difco Laboratories, BD, Sparks, MD, USA) at 27 °C for 10 d to obtain conidia.

Endophytic colonization by Beauveria *and* Metarhizium *in haricot beans in the laboratory*

Phaseolus vulgaris (haricot beans) seeds were obtained from OSC seeds (Waterloo, Ontario, Canada) and used under axenic conditions. Seeds were surface-sterilized (Sasan & Bidochka, 2012) by immersion in 4 % sodium hypochlorite for three 10 min cycles followed by a sterile distilled water rinse. The seeds were then immersed in 30 % hydrogen peroxide for 15 min with gentle agitation and thoroughly rinsed with sterile distilled water to remove hydrogen peroxide. Surface disinfected seeds were then kept overnight at 4 °C. These seeds were tested for fungal and bacterial contamination by plating onto PDA and nutrient agar. No contamination was found and seeds were further used for experimentation.

Petri dishes containing soil were used to establish association between fungi and bean plants, as described by Sasan & Bidochka (2012). Soil (Schultz Potting Mixture, Brantford, ON, Canada) was sterilized with three cycles of autoclaving at 121 °C for 1.5 h each. Petri dishes were filled with sterilized soil and moistened with sterile distilled water. The soil was inoculated (towards the soil surface), with a fungal plug (1 cm^2 each) from an actively growing, GFP-expressing, fungal colony and maintained at 27 °C for 10 d. Controls contained agar plugs without fungi. The side of the Petri dish containing fungal growth was punctured with a hot scalpel to accommodate insertion of a plant root. A root of a previously germinated (axenic) single seedling was inserted through this hole such that leaves remained outside of the Petri dish. These dishes were then covered with aluminum foil and incubated vertically in a chamber with photoperiod of 16 h (white fluorescent tubes, 8 h dark, 25 °C) for up to 60 d (Kottke et al., 1987). Plants were monitored regularly and moistened with sterile distilled water when required for up to 60 d. Root sections were excised for fluorescent microscopic analysis of fungal GFP to confirm endophytic association.

To recover *Metarhizium* and *Beauveria* from bean roots, plants were washed gently with sterile distilled water to remove adhered soil particles from roots. Washed plants were then surface sterilized by three 5 min washes with 4 % sodium hypochlorite (Wyrebek et al. 2011). Plants were then thoroughly washed with sterile distilled water. Plants were divided into three parts; stem and leaves, the hypocotyl and root tissues. Roots were further divided into three parts (terminal, subterminal and nearest to hypocotyl) and stem and leaves were equally divided into two parts (terminal and subterminal). Plant tissues were homogenized in 5 ml distilled water using a Tissue Tearor (Greiner Scientific) homogenizer. The homogenate was serially diluted and 100 μl was plated onto selective PDA media (containing 0.5 g l^{-1} cyclohexamide, 0.2 g l^{-1} chloramphenicol, 0.5 g l^{-1} 65 % dodine, and 0.01 g l^{-1} crystal violet) and incubated at 27 °C for 20 d. *Metarhizium* and *Beauveria* isolates were identified based on growth on selective media, colony morphology and microscopic identification of conidia.

An additional method was used to visualize fungal colonization of plant tissues. At 60 d, plants were harvested and roots were washed in sterile distilled water to remove soil. The roots and leaves were separately crushed between two sterile glass plates. Crushed plant sections were then placed on selective PDA media on large Petri dishes (14 cm in diameter) for 2 h. The plants tissues were then removed and cultures were allowed to grow for 7 d. The fungal colonies were photographed with a light array at 480 nm for GFP imagery.

Endophytic localization of fungi in plants collected in the field

Sites near Brock University (St. Catharines, ON) and Pelham, ON were selected for plant sampling. Plants were sampled in open field habitats. Each area contained diverse species of grasses and forbs. A third site in Torngat Mountains National Park (TMNP; NL, Canada) was also used. Ninety-seven plants were randomly sampled from the three sites. Of these, 56 were from the Brock site, 26 from Pelham and 15 from TMNP.

Plants and their roots were dug out of the earth with a spade and excess dirt removed prior to storage of the entire plant in re-sealable plastic bags at 4 °C for no more than 5 d. Plants were photographed and identified using appropriate field guides.

Excess soil was removed from plant roots with several washes of sterile distilled water. Plants were sectioned into three parts: (1) roots, (2) hypocotyl, and (3) stem and leaves. Each plant tissue (roots, hypocotyl, and stem and leaves) was rinsed with distilled water, weighed, and then placed into 5 ml of sterile distilled water and homogenized using a Tissue Tearor (Greiner Scientific).

The homogenate (100 μl) from each plant tissue was plated in duplicate onto selective PDA media. The plates were incubated