

Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity

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Here we tested the hypothesis that species of the soil-inhabiting insect-pathogenic fungus *Metarhizium* are not randomly distributed in soils but show plant-rhizosphere-specific associations. We isolated *Metarhizium* from plant roots at two sites in Ontario, Canada, sequenced the 5' EF-1 α gene to discern *Metarhizium* species, and developed an RFLP test for rapid species identification. Results indicated a non-random association of three *Metarhizium* species (*Metarhizium robertsii*, *Metarhizium brunneum* and *Metarhizium guizhouense*) with the rhizosphere of certain types of plant species (identified to species and categorized as grasses, wildflowers, shrubs and trees). *M. robertsii* was the only species that was found associated with grass roots, suggesting a possible exclusion of *M. brunneum* and *M. guizhouense*. Supporting this, *in vitro* experiments showed that *M. robertsii* conidia germinated significantly better in *Panicum virgatum* (switchgrass) root exudate than did *M. brunneum* or *M. guizhouense*. *M. guizhouense* and *M. brunneum* only associated with wildflower rhizosphere when co-occurring with *M. robertsii*. With the exception of these co-occurrences, *M. guizhouense* was found to associate exclusively with the rhizosphere of tree species, predominantly *Acer saccharum* (sugar maple), while *M. brunneum* was found to associate exclusively with the rhizosphere of shrubs and trees. These associations demonstrate that different species of *Metarhizium* associate with specific plant types.

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INTRODUCTION

Metarhizium is an insect-pathogenic fungus currently used as a biological control agent against various insect species (Lomer *et al.*, 1997, 2001; Milner & Pereira, 2000; Hunter *et al.*, 2001; Maniania *et al.*, 2003; Shah & Pell, 2003). Recent studies suggest that another ecological role of this fungus is as a plant rhizosphere associate. A green fluorescent protein (GFP)-expressing *Metarhizium* applied in an agricultural setting was not randomly distributed in the soil but showed preferential association with the plant rhizosphere (Hu & St Leger, 2002). The rhizosphere has been identified as a potential reservoir for *Metarhizium*, with propagules persisting in the inner rhizosphere and decreasing over time in bulk soil (Hu & St Leger, 2002). Furthermore, populations of *Metarhizium* have been shown to increase significantly over time within the

rhizosphere (Bruck, 2005). Also, a plant adhesin (MAD2) has been shown to enable attachment to plants (Wang & St Leger, 2007).

Notably, *Metarhizium* is phylogenetically related to the fungal grass endosymbionts *Claviceps* and *Epichloë* (Spatafora *et al.*, 2007), suggesting a possible origin of plant association in *Metarhizium*. In a recent review article, Bruck (2010) suggested that rhizosphere competence in insect-pathogenic fungi was dependent upon the host plant. Additionally, Vega *et al.* (2009) suggested that future research on entomopathogenic fungi should concentrate on understanding the ecology of the fungi, focusing on roles such as rhizosphere colonization, in order to use them more effectively. The use of *Metarhizium* as a biocontrol agent has mostly fixated on its ability to kill insects, with little consideration of plant associations or rhizosphere ecology, thus limiting our ability to fully exploit this fungus for biocontrol efforts.

A common paradigm in insect pathology suggests that the host insect is the predominant influence on the population genetics of insect-pathogenic fungi. As such, the population genetics of *Metarhizium* has been assumed to be influenced primarily by host insect taxa (Riba *et al.*, 1986; St Leger *et al.*, 1992; Bridge *et al.*, 1993, 1997; Fegan *et al.*,

The GenBank/EMBL/DDBJ accession numbers for the 5' EF-1 α sequences of *Metarhizium* isolates are HM748301 (HKB1-1b), HM748302 (B18-ai), HM748303 (M31-ai), HM748304 (G90-bi), HM748305 (G55-ai), HM748307 (B77-ai), HM748308 (B34-aiii) and HM748309 (B35-avi).

A supplementary figure, showing RFLP analysis of *ntl* and *pr1* using *RsaI* for isolates of *Metarhizium*, is available with the online version of this paper.

1993; Leal *et al.*, 1994, 1997; Tigano-Milani *et al.*, 1995). However, this paradigm is being challenged. For example, in Ontario, the fungus previously known as *Metarhizium anisopliae* was found to comprise two cryptic species, namely Ontario group 1 (OG1) and Ontario group 2 (OG2) (Bidochka *et al.*, 2001, 2005). These genetically distinctive, non-recombining groups were found to be strongly associated with habitat type, with isolates from agricultural/open field habitats belonging predominantly to OG1, and OG2 isolates found in forested soils. Interestingly, no differences in insect host specificity or virulence were found between these groups (Bidochka *et al.*, 2001, 2005).

Here we further explored the ‘habitat hypothesis’ (i.e. agricultural/open fields or forests) in defining the distribution of *Metarhizium* species (Bidochka *et al.*, 2001) and suggest that *Metarhizium* species show plant-specific rhizosphere associations within a habitat. Our objectives were to randomly collect plant root samples along a linear transect from an agricultural/open field habitat through to a forested habitat, at two independent sites, and to isolate and identify the *Metarhizium* species associated with these plant roots. *Metarhizium* was isolated from plant roots using a selective agar, and the 5′ EF-1 α gene was sequenced in a selection of isolates and compared with previously deposited nucleotide sequences for confirmation of *Metarhizium* species (Bischoff *et al.*, 2009). The genus *Metarhizium* has recently been subdivided into different species according to the sequences of several genes (Bischoff *et al.*, 2009). The sequence for the 5′ region of EF-1 α was deemed to be a diagnostic marker for *Metarhizium* species identification (Bischoff *et al.*, 2009). We assessed the sequence data and designed an RFLP analysis of 5′ EF-1 α in order to rapidly identify, at the *Metarhizium* species level, a large number of isolates. We also utilized EF-1 α to identify *Metarhizium* species for representative isolates that we had previously referred to as OG1 and OG2.

METHODS

Root sample collection. A site near Brock University (St Catharines, ON), as well as a site in Guelph, ON, were selected using a geographical information system (GIS) (Google Earth). Locations were chosen that had a large, established forest area adjacent to a large open field with a strong delineation between habitats. The Brock site (43° 06′ 50.95″ N 79° 14′ 53.21″ W) forest was mature and contained mostly maple, ash and beech, with the open field dominated by goldenrod species. The mature forest at the Guelph site (43° 29′ 34.34″ N 80° 13′ 33.57″ W) contained mostly cedar, ash and maple, while the open field consisted of aster, goldenrod and wild carrot. Digital photographs of the sites are available from the authors upon request.

One hundred plant root samples were randomly collected approximately every metre along a linear transect that ran from the field to the forest, with the first 50 samples primarily from field plants, including grasses and wildflowers, and the last 50 predominantly from forest plants, trees and shrubs. Plant roots were dug out with a spade and excess soil was tapped from the roots before storage in separate,

pre-labelled plastic reclosable bags. For mature trees and larger shrubs, fibrous roots found between the larger woody roots were collected. Additionally, representative foliage from each plant was photographed and collected in a pre-labelled envelope and was used to identify plant species using botanical keys and appropriate field guides.

***Metarhizium* isolation.** Roots were washed with sterile distilled water to remove excess soil. Soil that adhered closely to the root was kept as representative of the rhizosphere. The roots were cut into 0.5 cm pieces, placed in 5 ml distilled water and homogenized using a rotary homogenizer (Greiner Scientific). Samples (100 μ l) of homogenate were spread, in duplicate, onto selective media, containing 39 g potato-dextrose agar (PDA) l⁻¹ (Difco), 0.5 g cycloheximide l⁻¹, 0.2 g chloramphenicol l⁻¹, 0.5 g 65% dodine l⁻¹ and 0.01 g crystal violet l⁻¹. The plates were incubated at 27 °C for 20 days. *Metarhizium* isolates were identified by colony morphology, namely white mycelia with green conidia, as well as microscopic identification of conidial morphology. Morphologically differing colonies were individually isolated from the selective plate of each plant root sample and grown on PDA plates at 27 °C for 10 days. Morphologically similar colonies were also isolated multiple times from the same plant root sample.

Isolates HKB1-1b and 43a-2i (Bidochka *et al.*, 2001) were used as representative isolates of OG1 and OG2, respectively. 43a-2i has been previously identified as *M. brunneum* and accessioned as ARSEF 8680 (Bischoff *et al.*, 2009). ARSEF 2575 is the ex-type for *M. robertsii* (Bischoff *et al.*, 2009).

The construction of GFP expressing plasmids, transformation, and creation of transgenic fungal lines were performed as previously described (Fang *et al.*, 2006) in order to produce a GFP-expressing transformant of ARSEF 2575 (*M. robertsii*). The transformant did not show any differences in growth, insect virulence or colony morphology in comparison to the wild-type.

DNA extraction. Morphologically different isolates were inoculated into 50 ml 0.2% (w/v) yeast extract, 1% peptone, 2% glucose broth (YPD) in flasks. The flasks were shaken at 200 r.p.m. at 27 °C for 3–4 days. The mycelia were removed by vacuum filtration onto Fisherbrand P8 filter paper and washed with distilled water. The samples were then crushed in liquid nitrogen using a mortar and pestle, and DNA was extracted using either a DNeasy Plant Mini kit (Qiagen) or a Plant/Fungi DNA Isolation kit (Norgen).

PCR amplification and RFLP analysis. The subtilisin-like protease 1 (*pr1*) and neutral trehalase (*ntl*) genes were amplified for all isolates according to previously described conditions and digested with *RsaI* as previously described (Leal *et al.*, 1997; Bidochka *et al.*, 2001; Small *et al.*, 2004). Each isolate was identified as belonging to either OG1 or OG2 based on RFLP banding pattern (Bidochka *et al.*, 2001; Small *et al.*, 2004).

The 5′ region of the EF-1 α gene was also amplified according to previously described conditions (Rehner & Buckley, 2005; Bischoff *et al.*, 2006, 2009). 5′ EF-1 α products were sequenced for five isolates of OG1 and two isolates from each of the possible genetic groups present in OG2, including representative isolates HKB1-1b (OG1), 43a-2i (OG2) and ARSEF 2575 (OG1). Putatively unique nucleotide polymorphisms were identified for the 5′ EF-1 α product of each species, and restriction site analysis of the 5′ EF-1 α sequences revealed that Ontario isolates could be differentiated based on RFLP banding patterns of the 5′ EF-1 α amplification product double digested with *MseI* and *XhoI*. This was performed in a total volume of 20 μ l, which included 5 μ l 5′ EF-1 α PCR product, 2 μ l 10 \times NE Buffer 4 (NEB), 4 μ l 10 \times BSA, 10 units *MseI* (NEB) and 20 units *XhoI* (NEB). Reactions were incubated at 37 °C for 16 h.

All amplified DNA sequences and RFLP products were visualized by electrophoresis on a 1% agarose gel, run at 70 V for 45 min in 0.5 × TBE buffer.

Sequencing. Prior to sequencing, DNA samples were purified using a QIAquick PCR Purification kit (Qiagen). The 5' EF-1 α PCR products were sent to the core molecular biology lab at York University, ON, for sequencing.

Phylogenetic identification of *Metarhizium* isolates Molecular phylogenetic analysis of the 5' EF-1 α sequences was conducted in order to identify the *Metarhizium* species of sequenced isolates. Alignments were made with CLUSTAL_X 2.1 (Larkin *et al.*, 2007) using the default settings. A maximum-parsimony phylogenetic tree was constructed using PHYLIP 3.69 (Felsenstein, 2009). Non-parametric bootstrapping was conducted using 1000 pseudoreplicates, with 10 random addition replicates per parsimony run and subtree pruning and regrafting (SPR) branch swapping.

Seed sterilization, conidial inoculation and microscopy.

Phaseolus vulgaris (haricot bean) and *Panicum virgatum* (switchgrass) seeds were surface-sterilized using a modification of the method of Miché & Balandreau (2001). First, seeds were immersed in sterile distilled water for 30 min. The seeds were then immersed in 4% sodium hypochlorite solution for 2.5 h. The fluid was decanted and the seeds were washed with sterile distilled water. The seeds were then immersed in 15% hydrogen peroxide for 30 min followed by washing three times with sterile distilled water. Sterilized seeds were kept at 4 °C overnight to allow for synchronization of growth. Switchgrass seeds were immersed in an inoculum of 10⁷ spores ml⁻¹ of GFP-expressing *Metarhizium* (ARSEF 2575) for 1 h, plated in sterile soil, and kept at 25 °C for 14 days with a photoperiod of 12 h. For haricot bean, sterilized seeds were allowed to develop into seedlings, and then inoculated. The treated bean roots were kept at 25 °C for 14 days with a photoperiod of 12 h. Sterile water was added regularly to avoid drying of the samples.

Roots were rinsed with sterile distilled water without a fixing reagent. A Leica DM RBE laser scanning confocal microscope equipped with an argon/krypton laser was used for the observation of GFP-expressing *Metarhizium* on roots.

Root exudate collection. Surface-sterilized switchgrass seeds were soaked in water in Petri dishes, kept on an orbital shaker and provided with light. Once 90% of the seeds had germinated, samples were kept on the shaker for an additional 4 days, after which root exudate was collected by vacuum filtration.

Conidial germination assay. Conidial suspensions (10⁷ conidia ml⁻¹) of ARSEF 2575 (*M. robertsii*), 43a-2i (*M. brunneum*) and B77-ai (*M. guizhouense*) were inoculated (1% v/v) into switchgrass root exudate and incubated at 15 °C or 27 °C. Positive controls for conidial germination were also done in YPD. Conidial germination was assessed microscopically (Leitz DIAPLAN light microscope) at 12, 24 and 48 h.

RESULTS

Metarhizium species in the sampling sites

After plant root samples were collected, the *Metarhizium* colonies were isolated and DNA was extracted, RFLP analysis of *ntl* was initially used to assign each isolate to OG1 or OG2 (see Supplementary Fig. S1A, available with the online version of this paper). RFLP analysis of the *pr1*

gene showed that isolates assigned to OG2, based on RFLP analysis of *ntl*, displayed two possible banding patterns for *pr1*, which suggested the presence of two distinct genetic groups within OG2 (Supplementary Fig. S1B).

In order to resolve the exact species composition of each of these distinct genetic groups, the 5' region of EF-1 α was amplified and sequenced for four isolates of OG1 and two isolates from each of the possible genetic groups present in OG2, including representative isolates HKB1-1b (OG1), 43a-2i (OG2) and ARSEF 2575 (OG1). Using molecular phylogenetic identification, isolates initially assigned to OG1 were identified as *M. robertsii* (Fig. 1). OG2 was determined to comprise two species, namely *M. brunneum* and *M. guizhouense* (Fig. 1).

Restriction enzymes *MseI* and *XhoI* were used to cleave amplified portions of the 5' EF-1 α gene, which resulted in RFLP patterns that differentiated Ontario isolates of *Metarhizium* (Fig. 2). All species displayed a distinct RFLP pattern, with the exception of the *M. brunneum* isolates, for which two distinct RFLP patterns were identified while screening isolates. Sequence analysis of the 5' EF-1 α gene for another *M. brunneum* RFLP pattern showed six single-nucleotide polymorphisms, including one within an *MseI* site, resulting in a polymorphic RFLP pattern. This alternate *M. brunneum* 5' EF-1 α gene shared 99.2% identity with the other *M. brunneum* 5' EF-1 α sequence (705/711 bp).

Using this RFLP analysis, screening of all isolates collected demonstrated that *M. robertsii* is the most prevalent species of *Metarhizium* in the two sampling sites in Ontario (Fig. 3), with 49 and 32 isolates collected from the Guelph and Brock sites, respectively. *M. brunneum* was also present at

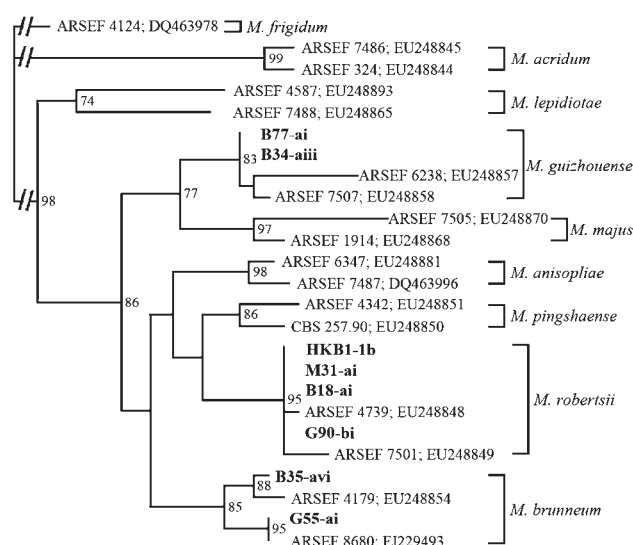


Fig. 1. Maximum-parsimony phylogenetic tree of 5' EF-1 α sequences of *Metarhizium* isolates. Bootstrap values are based on 1000 pseudoreplicates; values >70% are shown.

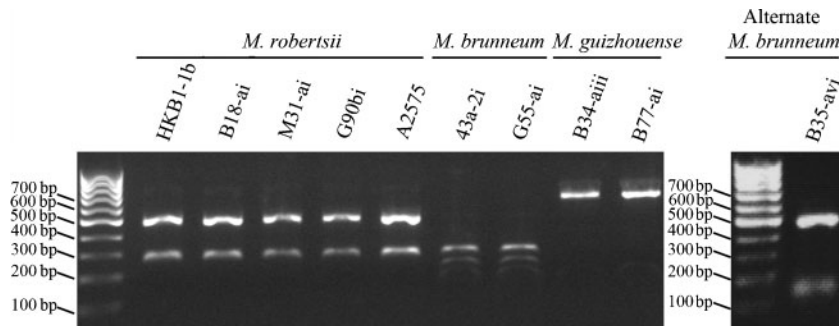


Fig. 2. RFLP analysis of 5' EF-1 α using *MseI* and *XhoI* for *Metarhizium* isolates.

both sites, but at lower frequency, with 13 and 2 isolates collected from the Guelph and Brock sites, respectively. Interestingly, *M. guizhouense* was only isolated from the Brock site, with 11 isolates collected, but not at the Guelph site. Morphologically similar isolates from the same plant root sample were always identified as the same species, and thus were only counted as a single isolate.

Plant-specific associations

Of all 200 plant root samples collected, 102 (51%) resulted in isolations of *Metarhizium*. *Metarhizium* was found to associate with 63%, 62%, 55% and 35% of grass, wildflower, shrub and tree root samples, respectively. Generally, *Metarhizium* was found most abundantly from wildflower species.

Three species of *Metarhizium* were isolated from the rhizosphere of plant root samples collected during this study, namely *M. robertsii*, *M. brunneum* and *M. guizhouense*. After identifying the species of *Metarhizium* isolates collected, an association between *Metarhizium* species and habitat was observed (Fig. 3), confirming previous results (Bidochka *et al.*, 2001). That is, *M. robertsii* was found predominantly in the agricultural or open field habitats, while *M. brunneum* and *M. guizhouense* (at the Brock Site) were found predominantly in the forested habitats.

Additionally, it was found that within these habitats, the three species of *Metarhizium* associated with specific plant types (Fig. 3). There were significant associations of *Metarhizium* species with plant type at the Brock and Guelph sites (Yates' $\chi^2=19.808$, $P<0.005$ and $\chi^2=21.192$, $P<0.001$, respectively). Notably, *M. robertsii* was the only species isolated from grasses. It was also commonly isolated from wildflowers. It is also notable that *M. brunneum* and *M. guizhouense* were only isolated from wildflowers when *M. robertsii* was also isolated from that same plant root sample.

With the exception of one co-occurrence with *M. robertsii* on the wildflower *Solidago altissima* (late goldenrod), *M. guizhouense* was isolated exclusively from trees, suggesting a specific association with this plant type. Additionally, it was observed that *M. guizhouense* was predominantly isolated

from species of the *Acer* (maple) genus, primarily *Acer saccharum* (sugar maple) (Fig. 3). Of the 11 different occurrences of *M. guizhouense* at the Brock site, seven were isolated from trees of the genus *Acer*. Six of these were from *A. saccharum* and one was from *Acer nigrum* (black maple). At the Guelph site, three *A. nigrum* and one *Acer saccharinum* (silver maple) roots were sampled, from which a single *M. brunneum* isolate was obtained from *A. nigrum* (Fig. 3). Additionally, *M. guizhouense* was the only species to be isolated from root samples of *Fraxinus americana* (white ash), with one of nine samples at the Brock site resulting in isolations of this species. Neither of two ash root samples at the Guelph site had *M. guizhouense* (Fig. 3).

M. brunneum was uncommon at the Brock site and was isolated on its own from one tree, *Fagus grandifolia* (American beech). It was also found to co-occur with *M. robertsii* on the wildflower *Barbarea vulgaris* (wintercress). At the Guelph site, *M. brunneum* was isolated exclusively from shrub and tree species, with the exception of one co-occurrence with *M. robertsii* on the wildflower *Trifolium hybridum* (alsike clover) (Fig. 3). *M. brunneum* was also found to co-occur with *M. robertsii* on the shrub *Cornus alternifolia* (alternate-leaved dogwood) and the tree *Betula alleghaniensis* (yellow birch). Additionally, *M. brunneum* was the only species to be isolated from root samples of *Thuja occidentalis* (eastern white cedar), with three of 11 samples at the Guelph site resulting in isolations of this species.

In vitro microscopy

In vitro experiments demonstrated the close association of a GFP-expressing *Metarhizium* isolate (ARSEF 2575) with *Phaseolus vulgaris* (haricot bean) root (Fig. 4a) and *Panicum virgatum* (switchgrass) root (Fig. 4b). Hyphae can be seen growing on the root surfaces, as well as around the root hairs in switchgrass. Plants appeared healthy even after long-term (up to 20 days) root colonization by *Metarhizium*.

Conidial germination assays

The level of conidial germination in switchgrass root exudate was monitored over 48 h for an isolate of each

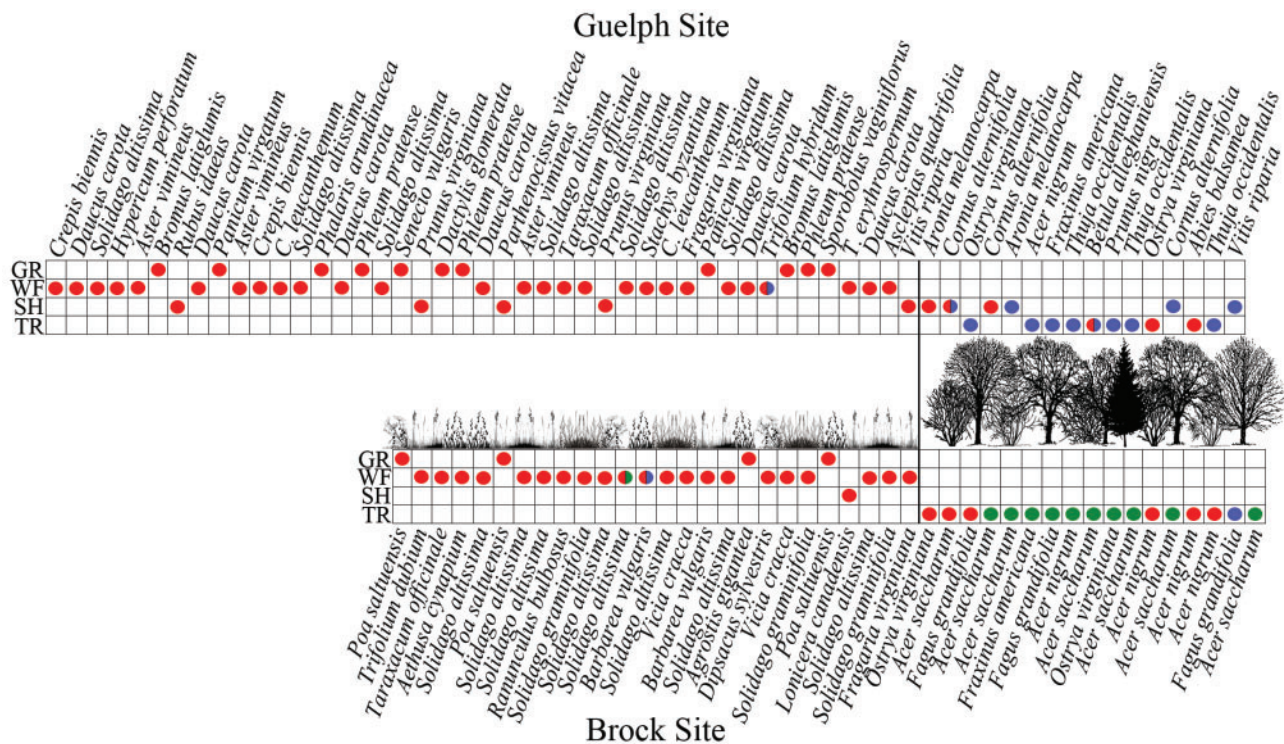


Fig. 3. Plant species, plant type (GR, grass; WF, wildflower; SH, shrub; TR, tree) and *Metarhizium* species (red, *M. robertsii*; blue, *M. brunneum*; green, *M. guizhouense*) isolated from plant root samples collected at the Guelph and Brock sites. Samples on the left represent transect samples in the agricultural forage field, and samples on the right represent the transect samples in a forest. Plant root samples collected resulting in no *Metarhizium* isolations were as follows. Guelph: GR – *Phalaris arundinacea*, *Agrostis perennans*; WF – *Cirsium arvense*, *Convolvulus arvensis*, *Solidago altissima* (2), *Aster vimineus*, *Daucus carota*, *Thaspium trifoliatum*; SH – *Rubus idaeus*, *Salix bebbiana*, *Aronia melanocarpa* (2), *Parthenocissus vitacea*, *Frangula alnus*; TR – *Thuja occidentalis* (8), *Abies balsamea* (5), *Fraxinus americana* (2), *Acer saccharinum*, *Tsuga canadensis* (2), *Betula alleghaniensis* (3), *Acer nigrum* (2), *Fraxinus nigra*, *Prunus nigra*, *Ostrya virginiana*. Brock: GR – *Poa saltuensis* (4), *Schizanthus purpurascens*, *Glyceria canadensis*, *Agrostis gigantea*; WF – *Solidago altissima* (3), *Dipsacus sylvestris* (4), *Daucus carota* (3), *Barbarea vulgaris* (3), *Chrysanthemum leucanthemum*, *Hieracium pratense*, *Lepidium campestre*, *Solidago graminifolia* (2), *Petasites frigidus*, *Taraxacum officinale*, *Dalibarda repens*, *Plantago major*; SH – *Prunus virginiana* (2), *Cornus stolonifera*, *Sambucus racemosa* subsp. *pubens*; TR – *Acer saccharum* (7), *Fraxinus americana* (8), *Fagus grandifolia* (6), *Acer nigrum* (2), *Ostrya virginiana*.

species: ARSEF 2575 (*M. robertsii*), 43a-2i (*M. brunneum*) and B77-ai (*M. guizhouense*) (Fig. 5). Differences in germination and hyphal growth were observed between the *Metarhizium* species grown in switchgrass root exudate, relative to growth in YPD as a positive control. *M. robertsii* demonstrated a high level of germination in the root exudate. A high level of hyphal growth was also seen after 24 h. Additionally, the germination rate of *M. robertsii* conidia in switchgrass root exudate was higher than the rate in YPD after 48 h at 27 °C and 15 °C. Conversely, *M. brunneum* and *M. guizhouense* showed minimal germination in switchgrass root exudate, relative to YPD. Similar results were obtained at 27 °C and 15 °C. The differences in relative rates of germination between *M. robertsii*, and *M. brunneum* or *M. guizhouense* were statistically significant at all time intervals (12, 24 and 48 h) and both temperatures (27 °C and 15 °C) (*t* tests $P \leq 0.0005$). Conidia failed to germinate in water for all three species.

DISCUSSION

Here we identified three sympatrically occurring species of *Metarhizium* in Ontario that were associated with the rhizosphere of specific plant types. We suggest that plant-rhizosphere-specific associations within a habitat determine the distribution of *Metarhizium* species. *M. robertsii* preferentially associated with grasses and wildflowers. *M. brunneum* preferentially associated with shrubs and trees, and *M. guizhouense* was isolated almost exclusively from trees, predominantly *A. saccharum*.

Field data demonstrated that *M. robertsii* is the most prevalent *Metarhizium* species in our two sampling sites in Ontario (Fig. 3), a finding supported by bulk soil sampling (Bidochka *et al.*, 2001). There may be some selective advantage to this species, which may not be due to superior virulence or ability to infect a host (Bidochka *et al.*, 2001), but rather to an ecological ability to survive or inhabit a

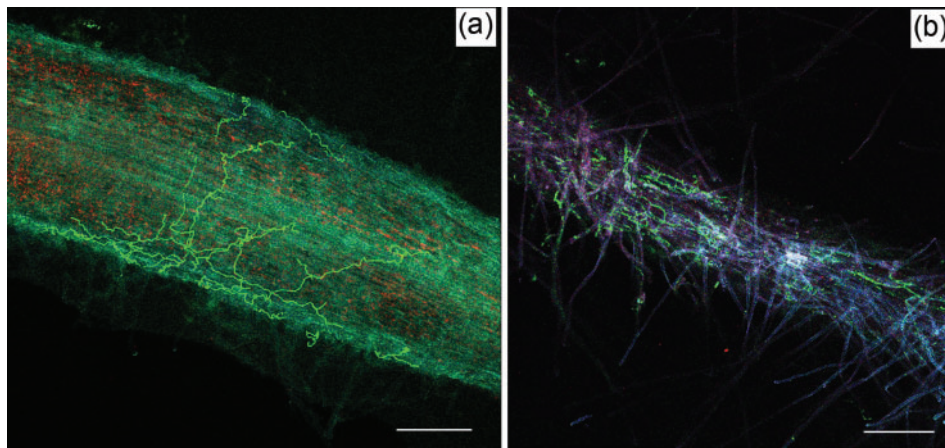


Fig. 4. Confocal images of a GFP-expressing *Metarhizium* (ARSEF 2575) associating with *Phaseolus vulgaris* (haricot bean) root (a) and *Panicum virgatum* (switchgrass) root (b). Scale bars, 200 μm.

certain plant rhizosphere. Moreover, this ecological ability may be mechanistically unrelated to pathogenicity, as Ontario species have demonstrated no significant differences in host specificity or virulence. A similar hypothesis has been proposed for the plant pathogen *Fusarium graminearum*, in which one lineage was found to be more prevalent than others, while all lineages had similar pathogenicity (Lee *et al.*, 2009).

In addition to being the most prevalent species found in Ontario soils, *M. robertsii* also demonstrated an ability to

associate with a wider range of plant types and species (Fig. 3). In particular, *M. robertsii* was prevalent among wildflower species and was the only species of *Metarhizium* to associate with wildflowers on its own. On the other hand, *M. brunneum* and *M. guizhouense* were only found to associate with wildflowers when in conjunction with *M. robertsii*. The exact nature and mechanisms of these associations are unknown, and there is a paucity of research conducted on plant associations of similar rhizosphere-competent fungi. However, examples of specific mycorrhizal associations are known. Synergistic colonization has been suggested in arbuscular mycorrhizal fungi, in which *Gigaspora rosea* and *Scutellospora castanea* colonization was significantly increased in the presence of *Glomus* species, particularly *Glomus mosseae* (van Tuinen *et al.*, 1998). In the case of *S. castanea*, when inoculated on its own, root colonization frequency was zero after 8 weeks, but increased to approximately 35% in the presence of *Glomus* species.

While *M. robertsii* was found to co-occur in the rhizosphere with other *Metarhizium* species, *M. brunneum* and *M. guizhouense* were never isolated from the rhizosphere of the same plant. There may be competition between *M. brunneum* and *M. guizhouense*, wherein one species out-competes the other with respect to root colonization. In the ectomycorrhizal fungus *Rhizopogon*, the timing of colonization, as well as the proportion of the root system colonized, are key factors in competitive success (Kennedy *et al.*, 2009). In arbuscular mycorrhizal fungi, colonization of a plant by one fungus has been shown to alter root exudation, which may inhibit development of subsequent mycorrhizal interactions (Piniot *et al.*, 1999; Vierheilig *et al.*, 2003).

Host plant specificity has been suggested to be responsible for differences in ectomycorrhizal community composition and diversity (Molina *et al.*, 1992; Kernaghan *et al.*, 2003). This finding may have implications in *Metarhizium* colonization of the plant rhizosphere, where we found that this is not a loose association but rather that

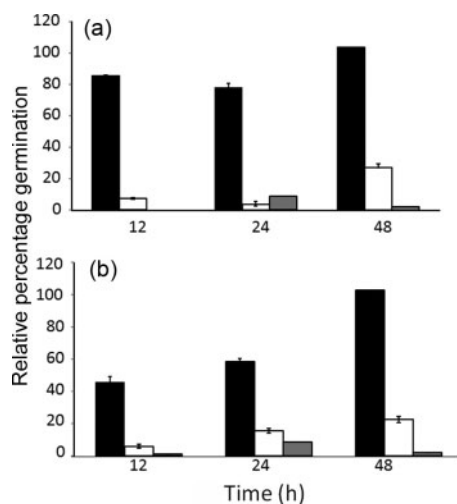


Fig. 5. Relative germination rates of *M. robertsii* (black bars), *M. brunneum* (white bars) and *M. guizhouense* (grey bars) conidia inoculated into *Panicum virgatum* (switchgrass) root exudate at 27 °C (a) and 15 °C (b). Germination rates were relative to YPD as the positive control. Conidia failed to germinate in water for all three species. Germination was scored from 100 conidia; means \pm SD are shown ($n=3$). The experiment was repeated twice with similar results.

Metarhizium is attracted to and associates intimately with root surfaces (Fig. 4). In the field, *M. robertsii* was the only species found to associate with grass roots. Additionally, the *in vitro* conidial germination experiments demonstrated that switchgrass root exudate was a favourable medium for germination of *M. robertsii* conidia compared with *M. brunneum* or *M. guizhouense* (Fig. 5).

The *in vitro* experiments complement the field data and demonstrate that this exclusion is a plant-associated effect, rather than an artefact of the habitat association or due to a specific insect host. While the mechanism for this exclusion is unknown, it may be due to compounds in the grass root exudate. Phenolics, and other compounds such as isothiocyanates, have been implicated in the inhibition of root colonization by some fungi (Piotrowski *et al.*, 2008; Wolfe *et al.*, 2008; Bainard *et al.*, 2009). Grass species may exude a compound that is toxic or inhibitory to the growth of *M. brunneum* and *M. guizhouense*. Alternately, *M. brunneum* (Guelph site) was isolated from *T. occidentalis*, and *M. guizhouense* was isolated from *Fraxinus americana*, to the exclusion of other *Metarhizium* species. The associations observed in this study are unlikely to be due to chance alone, and indicate specific rhizosphere associations.

Specific rhizosphere associations have been noted in the ectomycorrhizal fungi *Tuber borchii*, *Tuber melanosporum* and *Lactarius quietus* (Courty *et al.*, 2008; Martin & Nehls, 2009). Conversely, arbuscular mycorrhizal fungi are largely not host-specific (Klironomos, 2000). In most instances, these associations have been shown to be beneficial to the host plant, and are critical for nutrient cycling in sustainable ecosystems (Estrada-Luna *et al.*, 2000; Rai *et al.*, 2001; Sirrenberg *et al.*, 2007; Felten *et al.*, 2009; Martin & Nehls, 2009; Baldi *et al.*, 2010). Specific rhizosphere associations, with respect to rhizosphere competence, have also been demonstrated in *Metarhizium*, wherein certain isolates are only rhizosphere-competent with certain plants (Bruck, 2010).

Our findings of plant-rhizosphere-specific associations in *Metarhizium* have significant implications for the use of *Metarhizium* as a biological control agent. The use of a rhizosphere-competent isolate of *Metarhizium* provided nearly 80% control against a target insect (Bruck, 2005). Plant-rhizosphere specificity must be taken into account in order to optimize delivery against a target insect and ensure sustainability in the plant rhizosphere.

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