Do alterations of arbuscular mycorrhizal fungal communities change interactions between an invader Hieracium lepidulum and two co-occurring species? A glasshouse study

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Abstract

Recent work has highlighted the potential role of soil microbial and arbuscular mycorrhizal fungi in facilitating plant invasions by changing competitive balance between species. This glasshouse study investigated the extent to which arbuscular mycorrhizal fungi (AMF) can reverse competitive interactions between Hieracium lepidulum, an invasive Asteraceae, and two co-occurring species in New Zealand (Agrostis stolonifera and Poa colensoi). Previous glasshouse competition and performance studies conducted without AMF inoculation indicated relatively poor competitive performance of the invader, and we hypothesised that AMF may alter competitive interactions among plant species allowing the invader to dominate. Plants were grown both alone and in competition, and in substrates inoculated with field soils in which microbial communities were altered. AMF and microbial communities were altered through sterilisation, AMF spore addition, fungicide application and non-treatment. AMF root infection facilitated juvenile growth of H. lepidulum individuals with nutrient addition, but only where other soil microbes were removed (sterilised soil) and AMF spores introduced. AMF and soil microbes suppressed growth of Agrostis stolonifera individuals in the absence of fertilisation. No change in P. colensoi performance occurred irrespective of soil treatment. In contrast to plants grown in isolation, AMF and soil treatments did not cause differences in competitive outcomes between species pairs. Our results do not support the hypothesis that AMF facilitates Hieracium invasion through the competitive suppression of these co-occurring species during the initial juvenile plant growth phase. This work has not eliminated the possibility that competitive interactions are reversed once plants are mature, or in the presence of a full complement of AMF species from field soils.

Key words: invasive species; competitive augmentation; arbuscular mycorrhizal fungi; plant co-existence; facilitation

Introduction

There are many theories relating to increased invasiveness of exotic species in environments to which they have been introduced. These include facilitative mutualisms (Richardson et al. 2000), escape from natural enemies (Keane & Crawley 2002; Mitchell & Power 2003), exploitation of vacant niches (Hierro et al. 2005), evolution of novel invasive traits (Shea & Chesson 2002), allelopathy (Wardle et al. 1998; Hierro et al. 2003), novel weapons (Callaway & Ridenour 2004), exploitation of disturbance or enrichment (fluctuating resource) (Davis et al. 2000; Thompson et al. 2001; Huston 2004), propague pressure (Tilman 1997; Rouget & Richardson 2003; Lockwood et al. 2005), and those relating to invaded community resistance (Wiser et al. 1998; Stohlgren et al. 1999; Hierro et al. 2005). To date, the majority of research in invasion ecology has focused on the role of above-ground interactions (Louda et al. 2003; Fitter 2005). This is despite the pivotal role of below-ground processes and interactions in plant mineral nutrition and physiology (Bever et al. 1996; Smith & Read 1997), community ecology (Grime et al. 1987; Reynolds et al. 2003) and plant invasions (Callaway et al. 2004b; Kulmatiski et al. 2006).

Recently, a series of studies on invasive spotted knapweed, Centaurea maculosa, in the USA has revealed that the presence of arbuscular mycorrhizal fungi (AMF) in soil can potentially reverse competitive outcomes among invasive and resident species (Marler et al. 1999; Callaway et al. 2003; Callaway et al. 2004a; Carey et al. 2004). During these studies competitive performance was reversed between C. maculosa and several co-occurring native species through the presence of AMF root infections, with the invader gaining an advantage in the presence of the AMF (Marler et al. 1999; Callaway et al. 2004a). This work suggests a new mechanism for plant invasions (Grime et al. 1987; Reinhart & Callaway 2004; 2006) and supports increasing attention being paid to microbial interactions with invasive species. Callaway’s research supports AMF as a pivotal mechanism in the structuring of invaded plant communities (Callaway et al. 2004a) and supports an increasing literature showing the fundamental importance of AMF and soil microbial communities in determining the structure of plant communities generally (van der Heijden 1998; Klironomos 2003; Fitter 2005).

The generality of AMF facilitation of plant invaders has not yet been widely investigated. This may partly be due to the general assumption, until recently, that AMF species were non-host specific, and that AMF-plant relationships were generally mutualistic and not parasitic (Richardson et al. 2000). However, with many studies now revealing substantial hidden intra- and inter-specific AMF taxonomic and functional diversity (Munkvold et al. 2004; Fitter 2005), AMF community divergence among adjacent plants in the field (Vandenkoonhuysse et al. 2002), beneficial and parasitic plant–AMF relationships (van der Heijden et al. 1998; Hart et al. 2003; Klironomos et al. 2003) and environmentally induced alterations of plant-AMF interaction (Johnson et al. 1993) there is an urgent need for re-evaluation of AMF roles in plant invasions.

Hieracium species are among a long list of otherwise competitively subordinate plant species that have become invasive and dominant in parts of their introduced range.
Hieracium spp. have become invasive in large areas, both in New Zealand and the USA (Connor 1992; Harris & Mark 1992; Fornasari 1996; Espie, 2001), while being subordinate components of vegetation in native soils (Grime et al. 1988; Fornasari 1996) and in sterile glasshouse studies in the presence of exotic competitors (Radford et al. 2007). With high AMF dependency documented for a number of Hieracium species (Grime et al. 1987; van der Heijden et al. 1998; Hart et al. 2003; Klironomos et al. 2003), Hieracium is a useful group to test the generality that AMF facilitation can act as a mechanism for invasion by these species. The degree to which subordinate or canopy dominant species are dependent on mycorrhiza can affect plant community structure either by increasing or decreasing plant coexistence (Hart et al. 2003; Urcelay & Diaz 2003). The observed loss of native plant communities in the presence of Hieracium species in New Zealand (Scott et al. 1990; Harris & Mark 1992; Rose et al. 2004) could potentially be attributed to AMF facilitation of Hieracium spp. leading to suppression of less AMF dependent associated species.

To gain insights into the generality of AMF facilitation in plant invasions it is necessary to conduct experimental tests across a range of invasive taxa. In this study we test whether the presence of field soil AMF inoculation alters growth and competitive outcomes between H. lepidulum (Stenström) Omang (Asteraceae), an invasive species in southern New Zealand, and two co-occurring species, a native tussock grass, Poa colensoi and an exotic grass, Agrostis stolonifera, from the same subalpine environments. A series of edaphic screening and competition studies under sterile hydroponic conditions previously showed that H. lepidulum performed relatively poorly in terms of growth and competition compared to these species (Radford et al. 2006, 2007). AMF root infection was found not to be present in roots of plants used in these ecological screening studies (Downs & Radford 2005). The potential for different components of the soil biota, including pathogens, bacteria and higher consumers (nematodes, amoeba) (Reynolds et al. 2003; Callaway et al. 2004; Eppinga et al. 2006) to influence and alter H. lepidulum performance, either alone or in series, was assessed through additional soil treatments.

Materials and methods

Experimental design

To investigate the role AMF have in facilitating H. lepidulum (Stenström) Omang invasion, performance across four different soil treatments was assessed individually, and in the presence of Poa colensoi Hook f. or Agrostis stolonifera L. These species were chosen to represent one co-occurring exotic and one co-occurring native species found at the same sites as H. lepidulum (Radford et al. 2007). The basic experimental unit was 2 pots, one with a single plant, and a pot with a single plant of the same species (the target species) flanked by 2 plants representing one of the other species as competitors. Paired pot combinations were positioned together throughout the experiment. Biomass data from only the target species were utilised.

The four soil treatments were used to test whether AMF and microbial community changed growth or competition performance of each target species. Treatments were 1) AMF spores added to sterilised soil (for removal of other microbes) 2) Fungicide addition to selectively remove AMF and other fungi but not other soil microbes 3) soil sterilisation to remove all soil microbes 4) control (soil with no additional treatment). In order to establish the effect of nutrient status on competitive outcomes, 2.5 g of slow release fertilizer (0.22 g nitrate; 0.21 g ammonium; 0.04 g P (0.03 g water soluble); 0.22 g water soluble K; traces of S, Mg, Bo, Cu, Fe, Mn, Mo, Zn) was applied to a duplicate sample set. There were 5 replicates of each treatment and 320 pots in total.

Soil treatments

Soil was collected from Mt Pisa station, central Otago, in New Zealand’s subalpine pastoral rangelands, using a restricted randomisation method (Greg-Smith 1983). Soil had 0.287% total N, 24.08 Olsen available P, 10.87 ammonium acetate extractable K, 9.7 Na, 3.55 Ca, 16.67 Mg, 12.79% organic content, 7.42 C% and 24.17 C:N ratio. This site has extensive H. lepidulum populations and presumably AMF species facilitative to the invader. Soil from the site was mixed with cleaned river sand (Fairfield Quarries Ltd, Fairfield), and unfertilized commercial potting mix (Wallis’ Nurseries Limited, Mosgiel) in a 2:3:3 ratio and placed into 320 1 L plastic pots. The potting mix did not contain any soil, and consisted of peat, humus and silica sand. Use of identical media resulted in zero AMF root colonisation in a previous study (Downs & Radford 2005). 1 L pots were used to limit the amount of time required for competition to occur.

Soil in 160 of the pots were microwave sterilised in polypropylene bags for 5 minutes each after the addition of 200 ml water per pot. Soil from the centre of eight pots was plated on PDA agar and incubated at 24°C to confirm sterility. No microorganisms were present after one month. Batch control showed the lack of response was not due to poor quality agar. Sterilised soil was well watered and dried twice before planting to minimize the chance of a nutrient flush (Callaway et al. 2004b). Of the sterilised growing medium, 80 pots were inoculated with AMF extracted from field soil just prior to planting (1); the remaining 80 sterilised pots were planted with no additional treatment (2). The fungicide benomyl was added to 80 of the pots containing unsterilised growing medium at a rate of 50 ml per pot to reduce AMF abundance in the soil (3). The use of benomyl is commonly utilised in AMF experiments (Paul et al. 1989), although a proportion of non-target fungi can be affected, the effects of benomyl on plant responses are comparable with complete-soil sterilisation (Marler et al. 1999). The remaining 80 pots containing unsterilised growing medium were left untreated (4).

AMF spore extraction

The aim of this extraction was to add AMF spores into otherwise sterile soil so that we could include an AMF soil treatment independent of a general microbial treatment. It should be noted that differing rates of sporulation between AMF species means that only a subset of AMF species from the community may be sampled. Methodology was modified from http://invam.caf.wvu.edu/methods/spores/extraction.htm. In a commercial blender (Waring), field soil samples were blended at high speed (1:1 field soil/water) for five seconds, and immediately poured through three sieves with openings of 300 µm, 250 µm and 45 µm respectively. Material from the 45 µm sieve was transferred into 50 mL conical tubes containing a 40% sucrose solution. Tubes were centrifuged at 960×g for 3 minutes. The supernatant from each tube was quickly decanted into a small 45 µm sieve to collect the AMF spores and washed for two minutes under tap water. AMF spores are much larger than non-mycorrhizal fungus spores (Callaway et al. 2004a), therefore the sieving process to separate AMF spores from other soil microbes was considered sufficient for the purpose of this experiment. The supernatant that passed through the sieve...
Table 1  F and P statistics for Analyses of Variance of differences in individual plant growth (biomass/time) among soil and fertiliser treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DF</th>
<th>F statistic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants grown individually</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. lepidulum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil Treatment (AMF)</td>
<td>3</td>
<td>19.39</td>
<td>0.000</td>
</tr>
<tr>
<td>Fertiliser treatment (N)</td>
<td>1</td>
<td>179.25</td>
<td>0.000</td>
</tr>
<tr>
<td>AMF*N</td>
<td>3</td>
<td>4.04</td>
<td>0.012</td>
</tr>
<tr>
<td>A. stolonifera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil Treatment (AMF)</td>
<td>3</td>
<td>3.90</td>
<td>0.018</td>
</tr>
<tr>
<td>Fertiliser treatment (N)</td>
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<td>76.45</td>
<td>0.000</td>
</tr>
<tr>
<td>AMF*N</td>
<td>3</td>
<td>0.84</td>
<td>0.482</td>
</tr>
<tr>
<td>P. colensoi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil Treatment (AMF)</td>
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<td>1.33</td>
<td>0.283</td>
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<tr>
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</tr>
<tr>
<td>AMF*N</td>
<td>3</td>
<td>2.52</td>
<td>0.075</td>
</tr>
</tbody>
</table>

was checked for AMF spores, which were found to be absent. The AMF spores were stored in batches at 4°C for up to two weeks prior to re-inoculation. Each batch was equivalent to 250 g of field soil.

**Plant seed propagation**

AMF are only known to be present in the roots of living plants or as spores in the soil, therefore to ensure all plants were free of AMF prior to planting seeds were germinated in soil-free potting mix. *H. lepidulum* seeds were collected from Mt Pisa Station in Jan 2003, *P. colensoi* seeds (BP1527) were obtained from the Margot Forde Forage Germplasm Centre, New Zealand, and *A. stolonifera* seeds were obtained from RT Skinner, Nov 2002 batch.

**Seedling establishment**

Seedlings were inserted into the growing medium and depending on soil treatment, given either 5 mL of tap water with or without a batch of AMF spores, before being well covered with soil. To minimize the chance of cross-

Fig. 1a Mean percentage of transects that intersected non-septate AMF hyphae in the roots of individually grown *Hieracium lepidulum* (black bars), *Agrostis stolonifera* (unshaded bars) and *Poa colensoi* (striped bars) within different soil treatments under 100× magnification. Standard error bars are shown. b Mean percentage of transects that intersected non-septate AMF hyphae in the roots of *Hieracium lepidulum* grown in competition with *Poa colensoi* (black bars), *H. lepidulum* grown in competition with *Agrostis stolonifera* (checkered bars), *A. stolonifera* (unshaded bars) and *Poa colensoi* (striped bars) grown in competition with *Hieracium lepidulum* within different soil treatments under 100× magnification. Standard error bars are shown. Letters in brackets represent other species in the pot: Ag = *A. stolonifera*, H = *H. lepidulum* and P = *P. colensoi*.

Fig. 2a Mean biomass (g dry weight) of *Hieracium lepidulum* individuals grown in the four soil treatments, with (black bars) and without (unshaded bars) additional nutrients. Standard error bars are shown. Means with different letters were significantly different in Tukeys pair-wise comparisons. b Mean biomass (g dry weight) of *Agrostis stolonifera* individuals across soil treatments, with (black bars) and without (unshaded bars) additional nutrients. Standard error bars are shown. Means with different letters were significantly different in Tukeys pair-wise comparisons.
Distance between pots was kept as great as space constraints allowed, in an effort to reduce the possibility of microbial and/or spore transmission between pots. Additionally, clear plastic flop-guards were attached to each pot after 3 weeks of growth to prevent shading competition between pots and cross-contamination via above ground biomass. All plants were well watered immediately after planting and daily for the duration of the experiment. Only seedlings that died within the first week were replaced. Competitive effect was calculated in comparison with growth of plants in isolation, with individual plants and those in competition paired randomly prior to the commencement of the experiment. Plants with and without competitors were paired randomly prior to the commencement of the experiment. Plants were grown for 74 days under natural light in a glasshouse maintained at 24°C at the University of Otago, Dunedin, from 15 Dec 2004 to 27 Feb 2005.

Harvesting

All plants were harvested together on 27 Feb 2005. Dry weights of the above ground biomass were obtained following drying in brown paper bags for 72 hours at 60°C. Only live and attached leaves were processed. Below ground biomass was superficially cleared of soil and stored in clear plastic bags at 4°C until processed for AMF quantification.

Root preparation and quantification of AMF infection

The methodology for the preparation and quantification of the roots follows that of Downs & Radford (2005). Fine root segments (c. 20 mm long and <0.5 mm diameter) were washed in distilled water and 0.05% solution of TritonX100 (Sigma), cleared in 10% (w/v) KOH at 90°C for 1 hr, covered with 1% HCL for 3 min, stained in a 1:1 solution 0.05% trypan blue/lactophenol heated to 90°C for 15 minutes and de-stained in lactophenol. Due to rapid deterioration of the roots, only one replicate from each treatment was successfully viewed. Prepared root segments were mounted in 50% glycerol. Root infection by AMF was quantified using a compound microscope (Olympus CH2) at 100× magnification using transect intersect method. Each root was viewed by moving transect across the cross section of an entire root length, with each transect intersect by non-septate mycorrhizal hyphae recorded. Confirmation that hyphae were non-septate was made at 400× magnification. The number of grid movements was multiplied by the number of transects to give percentage root length infected by AMF. The presence of vesicles was used to give additional confirmation that hyphae were AMF. Digital images of the fungal structures were taken using a Leica DC 300 camera together with a Leica DMR compound microscope and Leica IM50 software version 1.2 (Leica Microsystems AG, Switzerland).

Statistical analyses

Statistical analyses were conducted using Minitab version 14. Prior to analysis, data were tested for normality and homogenous variation (Levene’s Test). Factorial Analysis of Variance was used to test for differences in growth/biomass of single plants, and for competitive effects of microbial treatments. Competitive effect was calculated by CE = (W-c)/W where CE = competitive effect; W = the weight of the plant grown alone; and c = the weight of the paired plant grown in the presence of a competitor.

Results

Percent AMF colonisation

AMF root colonisation rates differed among soil treatments, species and plants grown alone versus those grown in the presence of another plant (Fig. 1). As would be expected highest AMF colonisation was observed in non-treated soil (Fig. 1a, b). When plants were grown in
pots individually, non-treated soils had much higher infection rates (>10%) than that in all other treatments (<1%) including the AMF addition to sterile soil. Cross-pot AMF contamination appears to have occurred for one replicate of *A. stolonifera* in the sterilised treatment (Fig. 1a). Plants grown in the presence of AMF (non-treated and AMF treatments) and in the same pot as another plant had greater AMF infection rates (Fig. 1a) than plants grown alone (Fig. 1a). Soil treatments were also more effective in the presence of another plant, with AMF exclusion treatments (sterilisation and fungicide) resulting in complete absence of AMF in roots, while both non-treated and AMF added soils resulted in consistent AMF infection (Fig. 1b). In contrast to plants grown alone, higher rates of AMF root infection were observed in the AMF added treatment for plants grown together in pots with another plant for *H. lepidulum* and *A. stolonifera* (Fig. 1a, b). Colonisation by AMF was observed in the roots of *P. colensoi* and *H. lepidulum* grown individually in the fungicidal soil; in all instances colonisation was less than 2%; which is considered a sufficient level of suppression to elicit a plant response (Wilson & Harnett 1997) (Fig. 1a). Therefore, the sterilised and fungicidal soil treatments were considered effective.

High AMF infection rates in competition pots compared to individually grown plants confirm the competitive soil environment influences AMF colonisation more than inoculation method or spore density. Fungi were confirmed as AMF by the presence of non-septate hyphae and vesicles. Arbuscules are also considered useful morphological diagnostic characters, but these are short-lived within the cells (Smith & Read 1997), and were not observed in this study.

**Species grown individually (growth response)**

*Hieracium lepidulum*

Within the unfertilised treatments there was no significant difference in *H. lepidulum* growth between soil treatments (Fig. 2a). However, with fertilisation, presence of AMF and an absence of other soil biota (AMF added to sterilised soil, Fig. 1a) lead to significantly higher growth rates (Table 1; *P*<0.05) in *H. lepidulum* (Fig. 2a). *A. stolonifera* showed a significant growth advantage in sterilised soils only (Table 1; *P*<0.05) in the absence of fertiliser, but not in fertilised soil (Fig. 2b; Table 1). *Poa colensoi* growth (ca. 0.15 – 0.40 g DW) was substantially lower than that of *A. stolonifera* (Fig. 2b) or *H. lepidulum* (Fig. 2a), and did not differ significantly (Table 1; *P*>0.05) among AMF or fertility treatments.

**Competitive effect**

Significant competition effects were measured with nutrient treatments, between species pairs and for species pair*competitor interactions (Table 2). Overall, nutrient addition reduced the competitive effect and there was a greater competitive effect in competition with *Agrostis* (Fig. 3). *Agrostis* had a greater competitive effect than *Poa* on *Hieracium* while *Hieracium* had a greater effect on *Poa* than *Agrostis* (Fig. 3). There was no significant competitive effect of mycorrhizal/microbial treatment, or of any treatment interaction with this, during the experiment (Table 2, Fig. 3).

**Discussion**

Our results did not provide evidence to support the hypothesis that AMF root associations alter competitive hierarchies between an invader, *H. lepidulum*, and associated species in New Zealand. Indeed there is reduced (though not significant) competition by *Hieracium* in the presence of *Agrostis* associated with AMF inoculation (Fig. 3). However, this study does confirm successful establishment of AMF networks in *H. lepidulum* roots, and also a role for AMF infection in facilitation of *H. lepidulum* growth in isolation (Fig. 2a). This supports results from previous research showing benefits of AMF root associations to *Hieracium* spp. (Grime et al. 1987; van der Heijden et al. 1998; Hart et al. 2003; Klironomos et al. 2003) and specifically to *H.*
lepidulum (Downs & Radford 2005) under some conditions.

An unexpected finding is that this benefit was relatively small compared to other Hieracium species studied (van der Heijden et al. 1998; Hart et al. 2003; Klonomos et al. 2003) and occurred only in the absence of other soil microbes (sterilised soil treatment). This suggests that non-AMF microbial interactions are mainly negative to this invader, at least in association with the soil disturbances applied in this study. Negative New Zealand soil microbial associations with H. lepidulum is inconsistent with the recently raised hypothesis that release from soil pathogens is an important mechanism underlying plant invasions (Beckstead & Parker 2003; Eppinga et al. 2006).

Lack of AMF benefit to H. lepidulum in terms of competitive interactions may be due to methodological soil treatment effects on AMF communities, rather than a lack of competitive augmentation. Field soil samples used in this experiment were highly modified and disturbed, being physically removed from field locations, mixed and homogenised, sieved and placed in a relatively warm air-conditioned glasshouse. Such treatment may alter the physical and chemical properties of soils collected, and to selectively change microbial communities to the detriment of plant species. In addition, AMF addition treatments would have selected only sporulating AMF species, thereby potentially reducing the range of AMF associations and interactions. Little work has been undertaken on the direct effects of soil disturbance on microbial communities, largely because of the methodological problems inherent in any sampling based on physical soil disturbance or removal. New techniques involving low impact sampling of soil communities, or in situ experimentation, are required before we can begin to test for soil microbial community impacts under standard soil sampling techniques.

An additional possibility explaining a lack of AMF-plant invader response might be that AMF facilitation is growth stage dependent and that sampling was too early to detect AMF facilitation in this study. Bethlenfalvay et al. (1982) showed that the early growth of soybean was inhibited in the presence of AMF. They attributed this response to disproportionate carbohydrate demand on the host by the AMF, relative to the reward received. This response was later reversed after the ninth week of the experiment, when mycorrhizal enhancement of growth was observed and was associated with increased P uptake. Had the duration of this experiment been extended beyond the initial juvenile growth phase, it is possible that a positive mycorrhizal response may have been observed for Hieracium plants.

Like Centaurea maculosa, for which AMF competitive augmentation has been identified (Callaway et al. 2004a), H. lepidulum is an apparently competitively subordinate species that has achieved dominance of many invaded habitats in New Zealand subalpine vegetation communities. It is this apparent lack of strong competitive abilities, and evidence of AMF associations from previous studies that raised the possibility that some external factor may have been operating to facilitate greater impacts on native communities than expected based on competitive performance in glasshouse studies alone. Recent work has identified nutrient stress of plant competitors on degraded and disturbed sites as a possible mechanism allowing H. lepidulum to dominate vegetation despite the lack of strong competitive ability (Radford et al. 2009). However, it remains unknown whether AMF associations could aid this invader in augmenting nutrient supply at these nutrient stressed sites, or whether this association could inhibit nutrient supply to competitor species in situ. Future studies need to test the mechanism of AMF competitive augmentation more widely among invasive species and co-occurring species. These tests should preferably be undertaken in the field with the minimum of soil disturbance to enhance the functioning of all mycorrhizal interactions.

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