Lack of belowground mutualisms hinders Pinaceae invasions

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Abstract. Why particular invasions succeed and others fail is not well understood. The role of soil biota has been proposed as important. However, the role of mutualists has received much less attention than that of pathogens. Here we report that lack of adequate ectomycorrhizal fungi hinders invasion by exotic Pinaceae on Isla Victoria, Argentina, by reducing both the probability of establishment and growth of invading individuals. More than one hundred exotic tree species were introduced to this island ca. 80 years ago, but invasive trees are found in high densities only in areas adjacent to plantations. With a series of greenhouse and field experiments we found lower mycorrhizal colonization levels and few fungal species far from original plantings, and key fungal mutualists are confined to areas near plantations, probably owing to dispersal limitations. Low inoculum levels far from the plantations are retarding the invasion. Our experiments indicate that positive interactions belowground can play a key but underappreciated role in invasion dynamics.

Key words: biological invasions; Isla Victoria, Argentina; mycorrhizal fungi; pine invasion; Pinus; soil biota; spore bank.

INTRODUCTION

Though plant invasions threaten biodiversity conservation and incur large economic costs (Mack et al. 2000, Pimentel et al. 2005), why particular invasions succeed and others fail is often not well understood. Recent work suggests soil biota, mainly pathogens, may promote invasion when interactions with exotic plants are less negative than those with native plants (Klironomos 2002, Callaway et al. 2004, Mangla et al. 2008). Conversely, facilitation by beneficial soil microbes may directly control biological invasions (Simberloff and Von Holle 1999, Richardson et al. 2000, Bruno et al. 2003). For example, ectomycorrhizal symbioses can be crucial for plant establishment, as evidence from native ranges suggests (Horton et al. 1999, Weber et al. 2005, Horton and van der Heijden 2008). However, the role of ectomycorrhizal fungi in plant invasion has rarely been explored.

Ectomycorrhizal (EM) fungi can profoundly affect conifer establishment, thereby limiting ability of conifer species to colonize new regions (Thiet and Boerner 2007). For example, Pinaceae depend on ectomycorrhizal symbiosis to survive, and several plantation forests worldwide have failed for want of adequate EM inocula (Hacskaylo 1972, Mikola 1990, Read 1998). Important characteristics of EM fungi that make their absence a likely factor in failure of conifer invasion are that (1) they are not free living and (2) they are host specific to a degree that often prevents native fungi from associating with exotic trees, especially if the exotic tree species is in a family unrepresented in the native forest (Molina et al. 1992, Vellinga et al. 2009). For instance, in New Zealand, exotic Pinaceae do not form symbioses with native fungi associated with Nothofagus (Nothofagaceae) (Orlovich and Cairney 2004). The same phenomenon, failure of Pinaceae to associate with native fungi, has been reported in other regions (Mikola 1970, Tedersoo et al. 2007). Another crucial factor is amount of inoculum in the soil. When EM plants are established, the primary inoculation source is thought to be existing mycelial networks associated with plants already present (Selosse et al. 2006). Establishment of exotic Pinaceae around plantations is then limited to the root zone of plantation trees, where seedlings can tap the preexisting mycelial network. Establishment beyond this root zone is limited by the presence of spore inoculum, but the mere presence of spores in the soil does not guarantee inoculation, because a high density of spores may be needed to colonize conifer roots (Castellano et al. 1985, Read 1998). Also, there is evidence of poor dispersal of fungal species outside Pinaceae plantations (Mikola 1953, Lamb 1979, Allen et al. 1992, Davis et al. 1996), and for long-distance dispersal some species require animal vectors that may be absent in regions of introduction. These characteristics of Pinaceae and EM fungi make absence of compatible symbionts a likely contributor to the lack of Pinaceae invasion in areas where neither the trees nor their associated fungi are native.

Despite the evidence implying an important role for mycorrhizal fungi in conifer invasion, from studies in their native range and from studies in forestry in their exotic range, their potential role has not been studied...
empirically. It is frequently suggested that mycorrhizal fungi cannot limit invasion owing to their supposed ubiquity (Richardson et al. 1994, Reinhart and Callaway 2006). The goal of this research is to test the hypothesis that a paucity of compatible EM fungi on Isla Victoria, Argentina, limits invasion by several species of exotic Pinaceae.

METHODS

Study area

Isla Victoria in Nahuel Huapi National Park, Argentina (40°57' S, 71°33' W), is 20 km long and 4 km across at its widest point. Annual mean precipitation in the area is 1300 mm and mean annual temperature is 10.1°C. At the beginning of the last century the island began to suffer major damage from logging, cattle ranching, and fires, which affected as much as 60% of its area. With the establishment of the Nahuel Huapi National Park in 1934, such activities substantially decreased, although cattle and a nursery for exotic forestry trees were not removed until ca. 1960 (Basti 1988), and buildings, trails, roads, abandoned pastures, and plantations of exotic trees still remain. In 1925, the Argentine government started a project to grow forestry and fruit trees that might be suitable in the region (Koutchê 1942). Through 1939, at least 73 conifers—including 60 of the world’s known invasive conifer species—and 62 broad-leaved species were planted on the island. Among them were 43 species of Pinaceae, including 18 of the 28 species of Pinaceae that were planted on the island. Among them were 43 species of Pinaceae, including 18 of the 28 species of Pinaceae that Richardson and Rejmánek (2004) found to be invasive. For most species it is impossible to determine the exact number of individuals planted and at what stages, but numbers were often very large (tens of thousands for several of them) and they occupy an extensive area that persists today (Simberloff et al. 2002). Despite the large number of species and individuals introduced, and their fertility (many species have been reported to produce large numbers of viable seeds [Barrett 1952]), only areas adjacent to plantations (50 m from the plantations) are dominated by exotics (Simberloff et al. 2002, Núñez et al. 2008b). Today most of the island is covered by forest dominated by native Nothofagus dombeyi (Nothofagaceae; primarily ectomycorrhizal) and Austrocedrus chilensis (Cupressaceae; arbuscular mycorrhizal). Soils of the study area are volcanic soils (andisols) typified by low levels of available phosphorus (Satti et al. 2007) (see Appendix A).

Greenhouse experiment

We conducted a greenhouse experiment to assess mycorrhizal colonization and growth of exotic Pinaceae (Pseudotsuga menziesii, Pinus contorta, Pinus ponderosa) with the following soil treatments: (a) soils from areas near plantations (<100 m) where individuals of the genera Pinus and Pseudotsuga are established and (b) soils from uninvaded areas far from plantations (>1000 m); and soils (c) from areas close to plantations and (d) from uninvaded areas far from plantations to both of which we added soil from conspecific plantations on the island. We used soil of conspecific plantations as inoculum since sporocarps of EM fungi are ubiquitous during the fruiting season and thick hyphal mats are found in those soils. We collected these inoculum samples (~1 L each) from 15 haphazardly selected spots in each type of plantation, and we sifted them with a 2-mm mesh to exclude large particles. We pooled these samples by plantation type and added the pooled sample within two weeks of collection. As an additional treatment to detect inadvertent EM inoculation in the greenhouse, we used sterilized soil (autoclaved, cf. Wolf and Skipper 1994) from areas far from plantations. We used 10 pots (1 L each) per species per treatment and added 25 cm$^3$ of soil from plantations for inoculum (1/40 of the total volume) to the pots that required inoculum (soil treatments c and d). Therefore, we grew seedlings individually in 150 pots (3 species, 5 treatments, and 10 replicates of each). Water was added ad libitum, and there were no nutrients added to the pots nor was there artificial light. Temperature in the greenhouse ranged from approximately 10°C to 30°C.

Soils were collected from haphazardly selected areas in mixed Austrocedrus–Nothofagus forests and added in situ to the pots to be used in the experiment. We excluded large roots and stones from our samples. To each pot we added five cold-stratified seeds that were previously surface-sterilized (Reddy and Nataraajan 1997), and only the first seedling to emerge was allowed to grow. We used Pseudotsuga menziesii (Douglas-fir), the most abundant (although still rare) nonindigenous tree on the island outside plantations, plus Pinus ponderosa (ponderosa pine) and P. contorta (lodgepole pine), which are invasive elsewhere (Rejmánek and Richardson 1996, Richardson and Rejmánek 2004) but have failed to spread widely on the island, though individual trees have established and flourished. These three species have extensive ranges in their native distribution in temperate regions (Burns and Honkala 1990) and are successfully planted as forestry species in the Isla Victoria region (Schlichter and Laclau 1998).

All the pots produced seedlings, and we harvested seedlings after one growing season (nine months, from early spring to early winter). We measured aboveground dry biomass of seedlings and analyzed their full root systems under dissecting microscopes to determine presence of EM fungi (based on morphological characteristics) and to calculate the proportion of colonized seedlings (number of seedlings with colonized root tips/total number of seedlings). We also catalogued EM species by randomly selecting EM root tips for molecular identification (see EM fungi identification, below, for more detail) (Gardes and Bruns 1996, Horton and Bruns 2001). The use of these molecular techniques allowed us to determine the EM species supporting Pinaceae seedling establishment in the study, and therefore to estimate the composition of the fungal community in the vicinity of different tree species and at...
different distances to plantations. It also contributed to our understanding of the origin of these fungi (i.e., if they were native or exotic fungi).

Field experiment

We set up a field experiment to analyze growth and EM colonization of seedlings that followed as much as possible the procedures of the greenhouse experiment (e.g., we used soils from the same areas and the same inoculum procedure). Surface-sterilized seeds of three different species, *Pseudotsuga menziesii*, *Pinus ponderosa*, and *P. contorta*, were planted close to (∼100 m) and far from (>1000 m) plantations in late austral winter 2005 in mixed *Austrocedrus–Nothofagus* forests. We set up the plots haphazardly with respect to the plantations of the different species. This experiment had one treatment (with EM additions) and two controls (without EM additions). One control consisted of sterilized samples (25 cm³) of the soil used to inoculate seeds, and the second control consisted of additions (25 cm³) of soil from the same area where seeds were planted. We planted five cold-stratified seeds per plot per species in 50 randomly selected locations both close to and far from plantations. Therefore, we followed 900 plots (3 species, 3 treatments, 2 areas, and 50 replicates of each). Each plot was protected against seed predators with a metal-mesh exclosure (cf. Nuñez et al. 2008b). We followed seedlings for emergence and survivorship monthly for one year and collected seedlings after one year. We analyzed full seedling root systems as in the greenhouse experiment and measured their aboveground dry biomass. Because of low emergence and establishment probabilities only 103 plots had seedlings at the end of the experiment. We analyzed only seedlings established in the growing season (from August to January), which comprised 92% of the total at the end of the experiment, because seedlings established later were notably smaller, likely owing to the low rainfall that characterizes summer in this region and to the shorter time they had to grow. The excluded seedlings (eight in total) belong to all the studied species and were found in areas both near plantations and far from them in treatments with and without added inoculum.

EM fungi identification

From the field and the greenhouse experiments, we collected a representative sample of EM root tips from each colonized seedling (five randomly selected EM root tips per seedling). After removal from the seedlings, root tips were stored in 2× CTAB buffer solution (100 mmol/L Tris-HCl [pH 8.0], 1.4 mmol/L NaCl, 10 mmol/L EDTA, 2% CTAB) (Gardes and Bruns 1996). We extracted DNA from EM root tips with unique morphological characteristics (morphotypes) from each seedling. In cases where two or more morphotypes were observed, we extracted DNA from each unique morphotype. In most cases only one morphotype was observed per seedling, and when two morphotypes were analyzed, they usually proved to represent a single genetic entity. DNA was extracted from a total of 300 mycorrhizal root tips. We never observed more than two distinctive morphotypes in the samples of five tips. From these, 218 root tips yielded clean restriction fragment length polymorphism (RFLP) fingerprints, which we grouped into 35 unique types; we obtained ITS (internal transcribed spacer of the nuclear ribosomal DNA) sequences for 28 of these. BLAST (basic local alignment search tool) search results yielded 20 matches to genus or species, and one type simply as an Ascomycete (Appendix B; some unique RFLP types proved to be the result of intraspecific variation).

DNA extractions, polymerase chain reaction (PCR) amplifications, and RFLP generation procedures followed Gardes and Bruns (1996). The ITS region of the nuclear rDNA was amplified using the primers ITS-1f and ITS-4. RFLPs were generated using *Hinf* I and *Dra*I (New England Biolabs, Ipswich, Massachusetts, USA). We compared ITS-RFLP patterns from ectomycorrhizae to each other and to those from voucher specimens of sporocarps collected on Isla Victoria. We also sequenced the nuclear ITS region of one example of each RFLP type. We were unable to obtain ITS sequences from some samples because of mixed extracts or low quality PCR products.

Resulting sequences were subjected to a BLAST search in Genbank. We identified specimens to genus if the majority of the ITS1 and ITS2 regions overlapped with a sequence in Genbank and had a similarity of 97% or greater. Sequences with similarities below 97% were identified to family level or lower based on a consensus of the Genbank output. Sequences generated for this study are deposited in Genbank (Appendix B).

Data analyses

We analyzed the greenhouse experiments by ANOVA. We tested for differences of seedling biomass in the different treatments with species and treatments as factors with a two-way ANOVA, and we used a Tukey test to analyze differences among treatments (at α = 0.05). We log-transformed data owing to normality issues. We also used two-way ANOVA to test for differences in the numbers of seedlings that emerged among treatments, with species and treatments as factors. ANOVAs were performed using PROC GLM in SAS (all analyses were conducted using SAS 9.13; SAS Institute 2003).

Field experiments were analyzed by ANOVA and logistic regression. To analyze aboveground biomass we used a two-way ANOVA with treatments and locations as factors. We used logistic regression to analyze establishment probabilities using PROC LOGISTIC (SAS Institute 2003) with species, treatment, and distances as factors.

RESULTS

In the greenhouse experiment we found that addition of ectomycorrhizal (EM) soil inoculum increased the
size of seedlings by a factor of 3 in soil from uninvaded areas, demonstrating a deficit of EM fungi in uninvaded areas (Fig. 1). Seedlings in sterile soil, for which visual inspection of their roots showed they were not colonized by EM fungi, grew at a rate similar to that of seedlings planted in soil from uninvaded areas, without EM inoculum. The biomass of seedlings differed significantly by species ($F_{2,152} = 69.21, P < 0.001$) and treatment ($F_{4,152} = 38.15, P < 0.001$), but there was no interaction ($F_{8,152} = 1.32, P = 0.24$). A post hoc Tukey test grouped the treatments with soil from areas near plantations and the treatment with soil far from plantations but with inoculum added together, and treatments of soil far from plantations without inoculum and sterile soil together. Seed germination was high (~85%), and there were no significant differences among treatments ($F_{4,150} = 1.90, P = 0.12$).

Field experiments yielded results in accord with those of the greenhouse study (Fig. 2). We found an effect of location (area: $F_{1,81} = 4.84, P = 0.031$) but not of EM addition (treatment: $F_{2,81} = 0.99, P = 0.376$) for the aboveground biomass for the three species. Seedlings growing far from plantations tended to be bigger if they had EM inoculum, but not significantly so. For establishment (measured as the proportion of plots with seedlings established at the end of the experiment), using logistic regression we found a significant difference in treatment effect (treatment $\chi^2 = 10.31, df = 2, P < 0.006$), with plots with the EM addition treatment having more seedlings than the other two treatments. There were also differences in effect on establishment probabilities for species (species $\chi^2 = 32.8, df = 2, P < 0.001$) and location (higher near plantations, area $\chi^2 = 4.9, df = 1, P = 0.026$).

We collected root tips from 151 colonized seedlings for molecular analyses. Of these, 62 seedlings were from treatments without inoculum additions. Most of them were from areas near plantations. We obtained 10 colonized seedlings grown in soils from treatments from areas far from plantations without inoculum addition. Fungal richness and inoculation probabilities were notably lower in soils from areas far from plantations in both the greenhouse and field studies (Table 1, Appendix C). Inoculation tended to increase both the number of species and probability of successful fungal colonization. There were significant differences among treatments without EM soil inoculum addition in terms of proportion of individuals colonized between soils from areas near plantations and those soils far from them (field, $\chi^2 = 17.28, df = 1, P < 0.0001$; greenhouse, $\chi^2 = 12.60, df = 1, P < 0.0004$). After sequencing, some species (eight ITS types) did not closely match any species in the Genbank database at the genus or species level. We are confident that these are unique genotypes because they are not chimeric (the same results were obtained if ITS 1 and ITS 2 were BLAST-searched individually).

**DISCUSSION**

Understanding which factors determine the success or failure of invasive species is a challenge for basic and applied ecology (Mack et al. 2000). Data from our field and greenhouse experiments suggest that lack of belowground mutualisms may be contributing to hinder the tree invasion on Isla Victoria, Argentina. Growth and survival of seedlings diminishes notably from areas with high levels of inoculum to areas with low levels of inoculum, in support of our hypothesis that suggested that the lack of tree invasion was related to the scarcity of proper mycorrhizal fungi for the introduced Pinaceae. These results are in accord with literature on forestry that have shown that to establish successful plantations of exotic Pinaceae, in areas where Pinaceae are not native, the introduction of mycorrhizal inoculum is needed (Mikola 1970, Read 1998). However, they are in partial disagreement with some other reports that
suggested that fungi introduced to a region may not limit the spread of trees for long owing to the high dispersal potential of fungi (Richardson et al. 1994, Reinhart and Callaway 2006).

We found more fungal species near to plantations than far from them. These differences in fungal richness may be due to different samples sizes, since we found many more colonized seedlings near plantations. However, Wilcoxina mikolae and Rhizopogon sp. dominated on seedlings from soil near plantations and were not found far from plantations. Wilcoxina rarely produces fruiting bodies, limiting spore production so spread

![Fig. 2. Aboveground biomass and establishment from the field experiments; data are means ± SE. (A) Aboveground biomass for the three tree species seedlings. (B) Proportion of plots with seedlings established at the end of the experiment.](image)

<table>
<thead>
<tr>
<th>Species and parameters</th>
<th>Near plantations (&lt;100 m)</th>
<th>Far from plantations (&gt;1000 m)</th>
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<tr>
<td></td>
<td>With inoculum addition</td>
<td>Without inoculum addition</td>
</tr>
<tr>
<td>Pinus contorta</td>
<td>64 (8)</td>
<td>75 (4)</td>
</tr>
<tr>
<td>Pseudotsuga menziesii</td>
<td>86 (5)</td>
<td>88 (4)</td>
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<tr>
<td>Pinus ponderosa</td>
<td>75 (7)</td>
<td>56 (6)</td>
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<td>Average colonization for all tree species</td>
<td>75.0</td>
<td>73.0</td>
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<td>Species richness for all tree species</td>
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<td>11</td>
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**Greenhouse**

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<th>Species and parameters</th>
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<th>Far from plantations (&gt;1000 m)</th>
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<tr>
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<td>With inoculum addition</td>
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<tr>
<td>Pinus contorta</td>
<td>90 (4)</td>
<td>100 (6)</td>
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<tr>
<td>Pseudotsuga menziesii</td>
<td>80 (5)</td>
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<tr>
<td>Pinus ponderosa</td>
<td>90 (4)</td>
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<tr>
<td>Average colonization for all tree species</td>
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<td>93.3</td>
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<td>Species richness for all tree species</td>
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**Notes:** The data are from field and greenhouse studies. Number of fungal species is based on RFLP (restriction fragment length polymorphism) and DNA sequencing analyses.
occurs mostly by vegetative growth and the production of chlamydospores below ground (Trevor et al. 2001). *Rhizopogon*, a hypogeous fungus, needs mammal vectors (to dig up the sporocarps for long-distance dispersal of spores in fecal pellets) that may be absent in the area (e.g., there are no native squirrels in the region). These two fungi were frequently observed on seedlings when soils from near plantations were used as inoculum. Chlamydospores of *Wilcoxina* and basidiospores of *Rhizopogon* are both resistant propagules that function as effective inoculum when soils are used to inoculate Pinaceae seedlings (Baar et al. 1999, Ashkannejhad and Horton 2006, Izzo et al. 2006).

Far from plantations *Suillus luteus* was the most abundant species. *Suillus luteus*, a *Pinus*-specific species, produces sporocarps prolifically, and its fruiting bodies are ubiquitous in the understory of plantations on Isla Victoria, as well as in other plantations in the region. In Ecuadorian *Pinus radiata* plantations, this species can produce hundreds of kilograms of dry mass per hectare, a production level that is an order of magnitude more than all species combined in the highly productive stands of the Pacific Northwest, USA (Chapela et al. 2001). The fact that this species produces large numbers of fruiting bodies aboveground (epigeous), and therefore many wind-dispersed spores, could explain its presence far from plantations. A related species in our study, *Suillus lakei*, is specific to *Pseudotsuga menziesii*. This species produces far fewer fruiting bodies, and this may help explain why it was not frequently observed on *Pseudotsuga menziesii* samples in our study. Indeed, it appears there is very little suitable inoculum for *Pseudotsuga menziesii* far from the plantations, and only seedlings inoculated with soils from near plantations became mycorrhizal. The apparently low abundance of EM propagules in areas far from plantations diminished chances for Pinaceae to find proper EM inoculum. Note that establishment was very poor for all tree species far from plantations without inoculum addition, and this was especially so for *Pinus contorta* and *Pseudotsuga menziesii* (Fig. 2).

Soil properties affect colonization by EM fungi and trees, but we found no soil differences between sites near and far from plantations other than the inoculum potential (see Appendix A). It is possible that factors such as soil properties that we did not measure, or other groups of mutualistic fungi (such as arbuscular mycorrhizal fungi) or bacteria (such as plant-growth-promoting bacteria), can also be contributing to or be driving the observed results. Other mechanisms may also be contributing to the observed lack of invasion, including insect pests, fungal pathogens, herbivory of exotic trees (Nuñez et al. 2008a), seed predation (Nuñez et al. 2008b), or interactions with other components of the biota (Janzen 1970, Connell 1978, Clay and Holah 1999). However, given the similarity in habitat characteristics near to and far from plantations, it appears that a lack of inoculum levels in the soils is an important contributing factor in inhibiting Pinaceae establishment away from the plantations. Our results are supported by studies showing the importance of mycorrhizal inoculum for seedling establishment in native settings (Horton et al. 1999, Dickie and Reich 2005, Nara 2006) as well as when Pinaceae are introduced to novel locations (Hacskaylo 1972, Mikola 1990, Read 1998).

Our results are limited to the early establishment of Pinaceae trees. It is possible that seedlings not colonized the first year could be colonized at a later stage. However, it is likely that the negative effect observed in the first year of life would severely limit the probability that such seedlings would survive or be competitive in later years, even if they were ultimately colonized. Further, the first year of establishment is critical for invasive pines (Grotkopp et al. 2002).

Our results indicate that lack of adequate mycorrhizal fungi has contributed to limiting invasion by the tree species studied in this system. These results support the contention that positive interactions are important in biological invasions. This study could help elucidate the lag time sometimes observed in invasion by different tree species elsewhere. Dispersal by EM fungi may require more time than that by plants because these fungi spread by mycelial growth or may need to build up a sufficient spore bank. Further, long-distance dispersal of spores from epigeous sporocarps is highly diffuse, while that from hypogeous sporocarps requires animal vectors (Allen 1987, Allen et al. 1992).

This study supports the contention that, while we are correct to focus on the global spread of plant invaders, microbial invasions may feed back on the invasiveness of plants, and the spread of mycorrhizal inoculum continues largely unchecked (Schwartz et al. 2006). Therefore, it is important for managers to consider practices, such as soil translocations, that are potentially problematic from the standpoints of both plant invasion and fungal invasion. To control conifer invasion in areas historically dominated by non-ectomycorrhizal species, Thiet and Boerner (2007) suggest it may be more effective to employ a series of management techniques to limit the ectomycorrhizal component of the invasion, for example by trenching, rather than the tree component. Similar techniques may be useful for managers in the southern hemisphere or in areas where Pinaceae are not native.

Lack of proper aboveground mutualists, such as pollinators or vectors for dispersal, has been shown to be a potential barrier for invasions. We show that mutualisms can also be important belowground, owing to plant interactions with fungi—one of the least understood groups of invasive organisms. We suggest that facilitation may be a more general mechanism than is recognized and may help explain patterns of exotic plant invasion, as well as general plant distribution patterns, especially when plants are introduced into areas lacking suitable mutualists.
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LITERATURE CITED


APPENDIX A

Methods of and results from soil analysis (Ecological Archives E090-165-A1).

APPENDIX B

Species, Genbank accession numbers, and BLAST results for sequences from this study (Ecological Archives E090-165-A2).

APPENDIX C

Percentages of mycorrhizal species colonizing roots of seedlings from the field and greenhouse experiments (Ecological Archives E090-165-A3).