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REGULAR ARTICLE

Native and non-native trees can find compatible mycorrhizal partners in each other's dominated areas

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Abstract

Aims Biological invasions have historically been addressed mostly from an aboveground perspective, so little is known about the impacts of belowground invasions. We studied the impact of belowground invasions on growth of native tree species and test the possibility of novel interactions between native and non-native hosts and native and non-native belowground symbionts.

Methods We combined field and growth chamber studies. With a growth chamber bioassay we compared growth and root colonization percentage of native *Nothofagus* and non-native invasive pine species, both highly dependent on ectomycorrhizal fungi (EMF), growing in pine invaded and non-invaded soils from native *Nothofagus* forest. We evaluated the identity of EMF species associated with both hosts in the different soil sources from the bioassay and we performed an *in situ* root sampling in the field.

Results We found that both hosts grew equally well in both soil sources in terms of biomass, with high percent of root colonization, and no cross-host colonization of symbiotic EMF except for one species of *Sistotrema* found on both hosts.

Conclusions Soil where invasive hosts are absent is already conditioned by the presence of non-native invasive EMF. Native trees may be able to remain in the invaded area due to the presence of native EMF. The presence of native hosts is not hindering the invasion of non-native hosts and the presence of native belowground fungal mutualists seems not to hinder the spread of their non-native counterparts.

Keywords Ectomycorrhizal fungi · Patagonia · Pinaceae · Plant invasion · Plant- soil feedbacks

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Introduction

Biological invasions are a global problem that historically has been mainly addressed from an aboveground perspective (Bohlen 2006; Rundel et al. 2014; Wardle and Peltzer 2017). Invasion of animals or plants have received a great deal of attention as they are conspicuous and have visible impacts (Brussaard 1997, Vilà et al. 2011). However, belowground invasions also occur and are at least as widespread (Callaway et al. 2004; Simberloff et al. 2013; Rodríguez-Echeverría and Traveset 2015). Earthworms, insects, and fungi together with other soil microorganisms are readily transported by humans, usually inadvertently, being able to establish in novel habitats, reproduce, and spread without any further human assistance (Lockwood et al. 2007; Blackburn et al. 2011). Belowground invaders can determine aboveground communities' assembly and impact ecosystem processes (Wardle et al. 2004; Bohlen 2006; van der Putten et al. 2007; Suding et al. 2013; Wardle and Peltzer 2017; Peay 2018). Due to their cryptic nature and the methodological difficulties in studying them, belowground invasions and their impact are scarcely reported in the literature, with a few exceptions as the case of pathogenic microbes (Reinhart and Callaway 2006, Inderjit and van der Putten 2010). Although our understanding of the interactions between the aboveground and the belowground components of the invasion is increasing, the effects of non-native belowground biota on the aboveground community are poorly understood.

Pine invasions in the Southern Hemisphere are an ideal system to study how the belowground effects of the invasion can affect the native community (Simberloff et al. 2010; Gundale et al. 2014). Pinaceae is among the most invasive plant families in the world, causing alterations on the disturbance regime, nutrient availability, and ecosystem processes, provoking in turn severe economic loss (Richardson and Rejmánek 2004; Nuñez et al. 2017). Pines are obligate partners with ectomycorrhizal fungi (EMF) and without them they are unable to invade (Nuñez et al. 2009). Pines co-invade with their EMF symbionts and in the invaded range they both disperse independently (Pringle et al. 2009; Dickie et al. 2010; Hayward et al. 2015a, b). Paired with the aboveground changes produced by pine invasions, there are many new species of EMF introduced belowground, most of them with novel physiological traits that may confer an advantage compared to native EMF. Some of these non-native EMF are even capable of forming novel associations with native hosts (Dunk et al. 2012; Wolfe and Pringle 2012; Nuñez and Dickie 2014; Truong et al. 2017). Native woody species might be negatively affected in terms of their growth by this change in soil biota provoked by the invasion, but this is still unclear. In Patagonia, many pine-EMF coinvasions occur in native forests, where the dominant woody vegetation, the genus *Nothofagus*, is associated with native EMF. This constitutes a unique scenario to test how non-native invasive EMF co-invading with non-native invasive trees may affect native trees and their native EMF.

We aim to determine how a non-native invasive pine species grow in soil from non-invaded stands dominated by native ectomycorrhizal *Nothofagus*, and how *Nothofagus* grow in soil from stands highly invaded by non-native ectomycorrhizal pine hosts. We expect that native trees will perform better in soil from native stands compared with soil from already invaded stands due to the presence of native EMF, and that non-native trees will grow better in soil from invaded stands compared to native stands due to the presence of non-native EMF. We also aim to address the possibility of novel-interactions: non-native fungi with native trees and native fungi with non-native trees (native EMF associated with *Nothofagus* that are capable of associating with non-native pines). To test these two aspects we used both experimental and observational approaches, pairing a growth chamber bioassay with *in situ* root sampling. We expect to provide new experimental evidence on how EMF invasion may impact native aboveground biota and how EMF native community responds to non-native EMF invasion. This will contribute to the understanding of which factors determine the success or failure of invasive species while also considering their effects on native communities.

Materials and methods

Study site

Soil sampling and root tip collection was performed in the Malalcahuello National Reserve, La Araucania region of Northern Patagonia, Andes Mountains, Chile (38°25'28''S 71°32'35''W; 1420 m above sea level). The Reserve is one of the few in South America that protects endangered monkey puzzle tree (*Araucaria*

araucana, IUCN Red List of Threatened Species, 2018) forest mixed with dense patches dominated by the southern beech *Nothofagus antarctica.* Together with *Nothofagus antarctica*, other *Nothofagus* species present in surrounding forest are the only native species capable of forming symbiosis with EMF in this ecosystem (Palfner et al. 2008; Nouhra et al. 2013). The understory is dominated by the bamboo *Chusquea quila* and the grass *Festuca scabriuscula*. Mean annual precipitation is 3038 mm and mean annual temperature is 8.5 °C. The topography of the area is heavily influenced by glaciation and volcanic activity (Peña et al. 2008), and the study site is located on the southern slope of the Lonquimay volcano. Predominant winds are from the northwest, going down from the volcano hillside. The area is undergoing an increasing invasion of pine species, mainly *Pinus contorta*. This tree species is native to the Northern Hemisphere in Western US forests. In early 1970s *P. contorta* was planted in a small set of trials plots mostly for erosion control (Peña et al. 2008). This species, considered one of the most aggressively invasive tree species (Ledgard 2001; Richardson and Rejmánek 2011; Richardson et al. 2014), now covers more than 100 ha in an invasion gradient that goes from areas highly invaded by pines near plantations to areas with no pines, dominated by *N. antarctica* and *A. araucana* trees. In the highly pine invaded areas there are still some isolated *N. antarctica* trees. This pine invasion threatens native communities above and belowground (Cóbar-Carranza et al. 2014, García et al. 2018).

Soil sampling

Soil was collected from two different areas in Malalcahuello: one area with the highest *P. contorta* invasion density (hereafter "dense pine invaded area") with ca. 5,000 pines ha^{-1} (pine trees taller than 1 m) and the other dominated by *N. antarctica* and with no *P. contorta* present (hereafter "non-invaded area"). In order to characterize the level of *P. contorta* invasion in each of these areas four plots of 10 × 10 m were randomly established and the height and diameter of each individual pine tree was measured. The two areas were 600 m apart. Despite the absence of invasive pines nearby, non-invaded areas were selected close enough to match habitat conditions with invaded areas (Hejda et al. 2009). During the austral spring (November) of 2015, we randomly distributed six sampling plots (10 × 10 m) in the dense invaded area and in the non-invaded area. In each sampling plot we extracted a total of six soil samples of 900 cm^3 (10 cm diameter x 12 cm deep) each using ethanol-sterilized PVC pots trying to disturb the soil inside and outside of the PVC pot as little as possible. After collecting the sample, each PVC pot was covered with two ethanol-sterilized PVC caps and wrapped in plastic film. To avoid cross-contamination between sites, we used sterilized gloves and we washed all the instruments used with ethanol after collecting each sample and during all the manipulation. At the end of each sampling day we stored the PVC pots at 4 °C until we set up the experiment. Additionally, we collected 36 soil samples (18 from invaded and 18 from non-invaded areas) that were left as separate and sterilized individually, to be used as control soil to detect inadvertent EMF inoculation during the experiment. The total of 108 pots (six for each sampling plot x six sampling plots x two areas –dense invaded, non-invaded- plus 36 sterile control pots) were transported to a growth chamber. For sterile control soils, we pre-incubated the samples to be sterilized for three days at room temperature to stimulate microbial growth. We then autoclaved the soil twice at 0.10 MPa and 121 ºC for 1 h each time and with an incubation of two days at room temperature in-between. To assure the heat reached evenly throughout the sample we spread the soil to a depth less than 2.5 cm for incubations and sterilizations (Wolf and Skipper 1994). We kept samples individually sterilized without mixing soil during all the sterilization process.

To compare soil conditions at invaded and non-invaded sites, we measured soil variables (pH, phosphorus, nitrate, ammonium, carbon nitrogen ratio, and organic matter) from samples collected at invaded and non-invaded stands.

Growth chamber bioassay

To determine the effect of soil biota from dense invaded and non-invaded areas in the growth of native and non-native tree species, and determine the possibility of crossed- colonization (i.e. non-native EMF associated with native trees and/or native EMF associated with non-native invasive trees) we conducted a growth chamber bioassay at the University of Concepción, Chile. For the experiment, we used three tree species commonly found in the study area. Two are ectomycorrhizal: the non-native invasive *Pinus contorta*, and the native

Nothofagus antarctica. We also used one non-mycorrhizal native species found in the area as a control for possible abiotic soil changes between treatments (Kahiluoto et al. 2000; Koide and Li 1989; Teste et al. 2014, 2015): *Embothrium coccineum* (Proteaceae). We collected seeds of the three species from the study site. Seeds were previously stratified (Arana 2011) and surface- sterilized (Sudhakara Reddy and Natarajan 1997) to avoid possible contamination due to fungal propagules present on the seed surface. Once germinated under sterile conditions, we planted three seedlings of the same species in each pot collected in the field. We used 12 pots per species per treatment. Therefore, we grew seedlings in 108 pots (three species, 12 pots with plants of each species in each of two soil sources: invaded or non-invaded, plus 12 pots with plants of each species in sterile soil − 6 pots from invaded and 6 pots from non-invaded sources). After one month, only the tallest seedling was allowed to continue growing, the rest were cut at the soil level, to avoid intraspecific competition effects on seedling growth. During the experiment, water was added *ad libitum*, and there were no nutrients added to the pots. Temperature in the growth chamber ranged from 18 to 20 °C. Light conditions were 200 μmol $m^{-2}s^{-1}$ with a light-dark cycle of 16:8 h. After eight months of growth, we harvested the plants. We carefully rinsed the seedling root of adhering soil, separated them at the soil line into a root and shoot fractions, and placed the shoot and root fractions into an envelope to be dried in an oven at 65 °C for two days. We measured the biomass of the dried shoot and root fractions using an electronic balance with accuracy to 0.1 mg. We carefully examined the root system of each *P. contorta* and *N. antarctica* seedling under a dissecting microscope (Nikon SMZ645) and compound microscopes (Nikon E600 DIC and Nikon E200) to address the extent of ectomycorrhizal colonization (based on morphological characteristics). We placed roots on a petri dish, and we recorded the number of fine root tips colonized and not colonized by ectomycorrhizal fungi.

In situ root sampling

We identified the EMF species associated with the roots of pine trees and *N. antarctica* trees present in the densely invaded area (same area with the highest *P. contorta* invasion density from where soil samples were collected) and in the pine invasion front (native *N. antarctica* forest with the lowest pine invasion density, where sparse and solitaire pine trees are still present). During austral autumn of 2016 (May), just after the peak of mushroom fruiting season, we carefully extracted fine roots of 24 randomly chosen juveniles of *P. contorta* and 24 *N. antarctica* (where present). Each tree was at least 20 m apart from the nearest sampled tree. We placed EMF colonized root fragments of each individual tree and their adjacent soil in coin envelopes and then into gallon bags and stored them at 4 °C to be processed the same night after collecting. We carefully rinsed soil from roots under tap water and we randomly chose five EMF colonized root tips under a dissecting microscope, separated them according to their morphology, and preserved them in 2% CTAB buffer for later DNA analysis (100 mmol/l Tris-HCl [pH 8.0], 1.4 mmol/l NaCl, 10 mmol/l EDTA, 2% CTAB; Gardes and Bruns 1993). We performed the *in situ* root sampling in an extra site to test the consistency of our observations and to analyze possible effects of the study system on the EMF composition associated with the hosts' roots. We also collected root tips in an ecosystem with different climate conditions and vegetation characteristics but with *N. antarctica* patches and a *P. contorta* invasion gradient, following the same experimental design. This site was a Patagonian steppe near the city of Coyhaique in the Aysen region, southern Chile (45°30'2" S, 71°42'15" W); for more details about the site and a comparison with the Malalcahuello site, see Langdon et al. (2010), Hayward et al. (2015b), and Franzese et al. (2017).

Identification of ectomycorrhizal fungi

From the growth chamber bioassay and the field, we collected a representative sample of each EM morphological type (morphotype) on root tips from each colonized seedling. Immediately after being removed from the seedlings, root tips were stored in 2% CTAB buffer solution. We extracted DNA from each unique morphotype present in each seedling (Agerer 1987). In cases where two or more morphotypes were observed, root tips were analyzed separately to check their identity based on restriction fragment length polymorphism (RFLP) and sequencing of the ITS region. We sorted by morphotypes only within samples, without lumping between samples. We extracted DNA from a total of 381 mycorrhizal root tips following the protocol of Gardes and Bruns (1993). We amplified the internal transcribed spacer (ITS) region of fungi in DNA extracts

using the forward primer ITS1f (Gardes and Bruns 1993) and the reverse primer ITS4 (White et al. 1990). PCR conditions followed Gardes and Bruns 1993: 3 min at 94 °C, followed by 35 cycles of 35 s at 94 °C, 55 s at 53 °C, and 45 s at 72 °C, adding 2 s per cycle to the extension time, with a final extension period of 10 min at 72 °C. We generated RFLP fingerprints from PCR products using the restriction enzymes *Hinf* I and *Dpn* II (New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocols. We visualized restriction fragment patterns on 3% agarose gels following Gardes and Bruns (1993). We sequenced the nuclear ribosomal ITS region from at least three exemplars of each RFLP type, except when fewer than three exemplars were present, in which case all exemplars were sequenced. We used the same two primers for the initial PCR product and ITS1f for the sequencing reactions. Sequencing was performed on an ABI3750XL sequencer at the laboratories of Operon, inc. (Eurofins MWG Operon, Huntsville, AL, USA) using standard chemistry. 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 Basic local alignment search tool (BLAST) search in Genbank. We named operational taxonomic unit (OTUs) based on BLAST comparisons to GenBank: we considered a sequence conspecific with named GenBank sequences at 97% similarity if at least 60% of the ITS region was alignable.

Data analyses

To analyze how aboveground dry biomass of the different plant species changed with soil source we assumed normal distribution of the response variable "biomass", and we used linear mixed-effects model fit by residual maximum likelihood (REML) (nlme package, lme function) (Pinheiro et al. 2007). We analyzed each tree species separately. We included "soil source" as a fixed factor with three levels (soil from dense pine invaded area, soil from non-invaded area, and control sterile soil) and "plot" as a random factor in the model. We validated the model using Shapiro–Wilk as a test of normality and we also tested for homoscedasticity. To analyze pine and *Nothofagus* EMF root colonization in the different soil sources, we assumed a binomial distribution for the response variable "root colonization" calculated as number of colonized root tips/total number of root tips, and used Generalized Linear Mixed Models (GLMM) based on Laplace approximation and a logit link function (lme4 package, glmer function) (Bates et al. 2018). If overdispersion was present, as in the case of *Nothofagus* root colonization, we included an observation-level random effect for modelling the overdispersion (Harrison 2014). We used a Tukey test to analyze differences among treatments (at $\alpha = 0.05$). All analyses were performed with R 3.4.0 statistical software (R Core Team 2018).

For molecular data, assignment to species level was made with ITS dissimilarity < 3% and no obvious conflicting assignments in the top 25 BLAST hits (a few cases of incorrect data in Genbank were ignored, Bidartondo 2008); assignment to genus was made with ITS dissimilarity between 3% and 10% and no obvious conflicting assignments in the top 25 BLAST hits; assignment to family was made with ITS dissimilarity between 10% and 20% and no obvious conflicting assignments in the top 25 BLAST hits. In cases in which we did not obtain a clear sequence, we identified the RFLP unique fingerprint with a number and treated it as a different OTU. We then compared the frequency of colonized hosts by each of the OTU's found between pine and *Nothofagus* hosts.

Results

There were no differences in growth between pine seedlings grown in soil from dense pine invaded areas or soil from non-invaded areas, but they grew less in sterile soils (Fig. 1a, linear mixed model parameters in Online resource 1). Similarly, native *N. antarctica* had higher aboveground biomass in soils from invaded and non-invaded areas compared to sterile soil (Fig. 1b). There were no differences in growth of native non-mycorrhizal *Embothrium coccineum* grown in the three soil sources (Fig. 1c). We found the same pattern for belowground biomass (Online resource 2). Non-native pines nearly doubled the aboveground biomass reached by native *Nothofagus* growing under the same period of time and conditions (Fig. 1a, b).

Both non-native pines (Fig. 2a) and native *Nothofagus* (Fig. 2b), had a high proportion of root tips colonized by EMF (higher than 0.8 for pines and higher than 0.6 for *Nothofagus*, respectively) with no differences between soil from invaded and non-invaded areas for either plant species. For pine trees, the suilloid dichotomously branched morphotype (Agerer 1987)

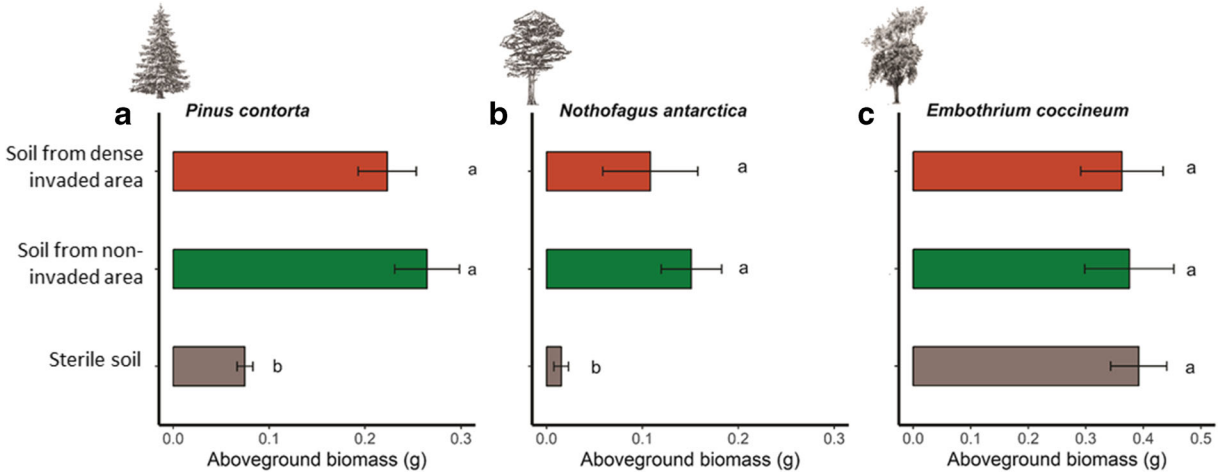

Fig. 1 Mean aboveground dry biomass for (**a**) non-native invasive *Pinus contorta* seedlings, (**b**) native *Nothofagus antarctica* seedlings, and (**c**) native non-mycorrhizal *Embothrium coccineum* seedlings, growing for eight months in three different soil sources in a growth chamber bioassay: soil from pine invaded areas (red bars), soil from native stands of *Nothofagus*, non-invaded by pines (green bars), and sterile soil used as control (grey bars), data from sterile soil is presented together as we did not find differences between control soil from invaded and non-invaded areas for each plant species. Data was analyzed using a linear mixed-effects model fit by residual maximum likelihood (REML). Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). Error bars show standard error

was dominant in roots from both soil sources compared to other morphotypes. For *Nothofagus* we did not find a dominant morphotype. The roots of the seedlings growing in sterile soils were not colonized by EMF, showing there was no inadvertent EMF inoculation in the growth chamber (Fig. 2, GLMM parameters in Online resource 3).

More than half of the root tips analyzed (210 out of 368; 57%) yielded ITS amplicons. Root tips not yielding amplicons showed signs of being senescent or dead. Although senescent root tips might yield amplicons, we did not pursue these data after 2 attempts at PCR amplification. These samples were scattered throughout the pool of samples, without any clear patterns. A total of 5 different molecularly identified taxa were observed on *P. contorta* in both field and growth chamber samples (Fig. 3). For field samples, *Suillus luteus, Hebeloma* sp., *Thelephora terrestris*, and *Sistotrema* sp. were found associated with pine roots in the pine invasion front, while *Amanita muscaria, Suillus luteus*, and *Sistotrema* sp. were present on pine hosts in the densely invaded area. For growth chamber samples, *Suillus luteus, Hebeloma* sp., and *Thelephora terrestris* were observed on pine seedlings growing in soil from non-invaded areas, while *Suillus luteus*, *Amanita muscaria*, and *Sistotrema* sp. were observed on pine seedlings growing in soil from invaded areas. *Suillus luteus* was present on pine roots from both soil sources in the field and the growth chamber samples (Fig. 3, Online resource 4, 5). While *Suillus luteus* was the most dominant taxon for the field samples and appeared on nearly 75% of the pine hosts analyzed, it was the second most dominant species in the growth chamber samples, where *Amanita muscaria* was the most dominant (observed on more than half the pine trees analyzed, Fig. 3). Other taxa were observed on less than 10% of the pine seedlings in both field and growth chamber samples (Fig. 3).

A total of 8 different taxa were observed on *N. antarctica* (Fig. 3, Online resource 4, 5). For field samples, *Sistotrema* sp., *Clavulina* sp., *Tomentella* sp., *Tricholoma* sp., *Porpoloma terreum*, and *Rickenella minuta* were found associated with *N. antarctica* trees growing in the dense pine invaded area. *Inocybe* sp. was found associated with *N. antarctica* trees from the pine invasion front. *Cortinarius* sp. was found associated with *N. antarctica* trees from both the dense pine invaded area and the invasion front. For growth chamber samples, *Inocybe* sp., *Tricholoma* sp. and *Corinarius* sp. were found associated to *N. antarctica* seedlings in soil from non-invaded areas and *Tricholoma* sp. was also found on *N. antarctica* seedlings in soil from dense pine invaded area. While *Sistotrema* sp. was the most common taxon in both field and growth chamber *Nothofagus* samples, *Cortinarius* sp. was the second

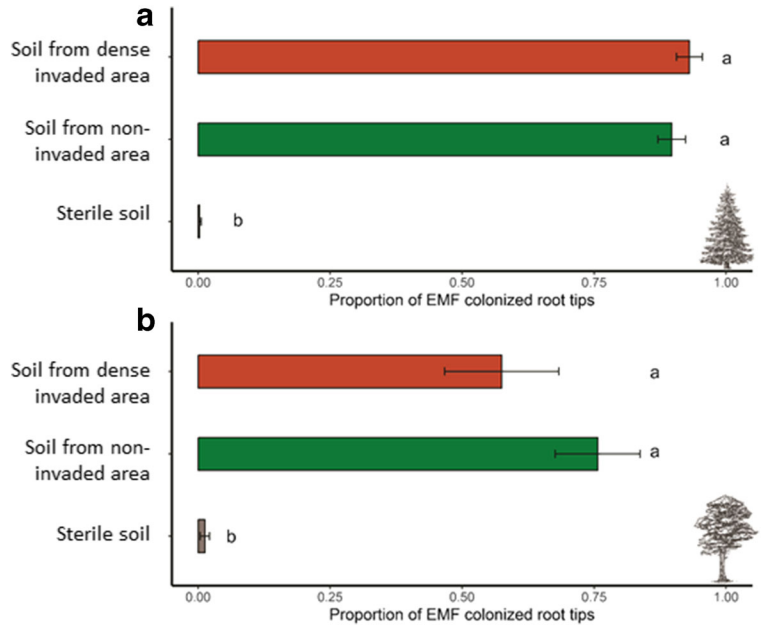

Fig. 2 Mean proportion of EMF colonized root tips (colonized root tips/total root tips) for (**a**) non-native invasive *Pinus contorta*, and (**b**) native *Nothofagus antarctica*, growing for eight months in three different soil sources in the growth chamber bioassay: soil from pine invaded areas (red bars), soil from native stands of *Nothofagus*, non-invaded by pines (green bars), and sterile soil used as control (grey bars), data from sterile soil is presented together as we did not find differences between control soil from invaded and non-invaded areas for each plant species. Data were analyzed using Generalized Linear Mixed Models (GLMM) based on Laplace approximation and a logit link function. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). Error bars show standard error

most common taxon in field samples, and *Tricholoma* sp. was the second most common taxon on *Nothofagus* in the growth chamber bioassay (Fig. 3, Online resource 4, 5). *Sistotrema* sp. was the only taxon that associated with both *P. contorta* and *N. antarctica* (Fig. 3). Field samples from Malacahuello and Coyhaique showed very similar patterns of EMF associations with a greater predominance of *S. luteus* associated with *P. contorta* in the case of root tip samples from Coyhaique (Online resource 6) compared to Malalcahuello.

We did not find significant differences in most soil variables measured (phosphorus, nitrate, ammonium, carbon nitrogen ratio, and organic matter) between invaded and non-invaded areas (Online resource 7). Soil from invaded sites had slightly lower pH compared to non-invaded sites (5.2 ± 0.2 and 5.6 ± 0.2 respectively, Online resource 7).

Discussion

Our results show that due to the presence of non-native EMF inocula, non-native invasive trees are able to establish and grow in still non-invaded places where native tree species dominate. Non-native invasive pines grow equally well and have high EMF colonization percentage both in soil from highly invaded areas and in soil for non-invaded stands. We also found that even in highly invaded sites there is still enough native EMF inoculum for native trees to establish and grow. *Nothofagus* aboveground biomass and percentage of EMF colonization did not vary between soils from dense pine invaded and non-invaded areas, which supports the idea that there is no negative interference between native and non-native EMF species for the set of fungi observed, at least for resistant inoculum as evidenced from soil bioassays in the growth chamber. These results suggest that at our sites the availability of ectomycorrhizal fungi is not a limiting factor for the establishment and growth of native and nonnative trees in areas dominated by the other tree species. We do not discard effects on the plants' growth of other soil-borne organisms (e.g. pathogens) in our results. However, given the higher growth rates of pines, and their lack of ectomycorrhizal symbionts limitations, it is

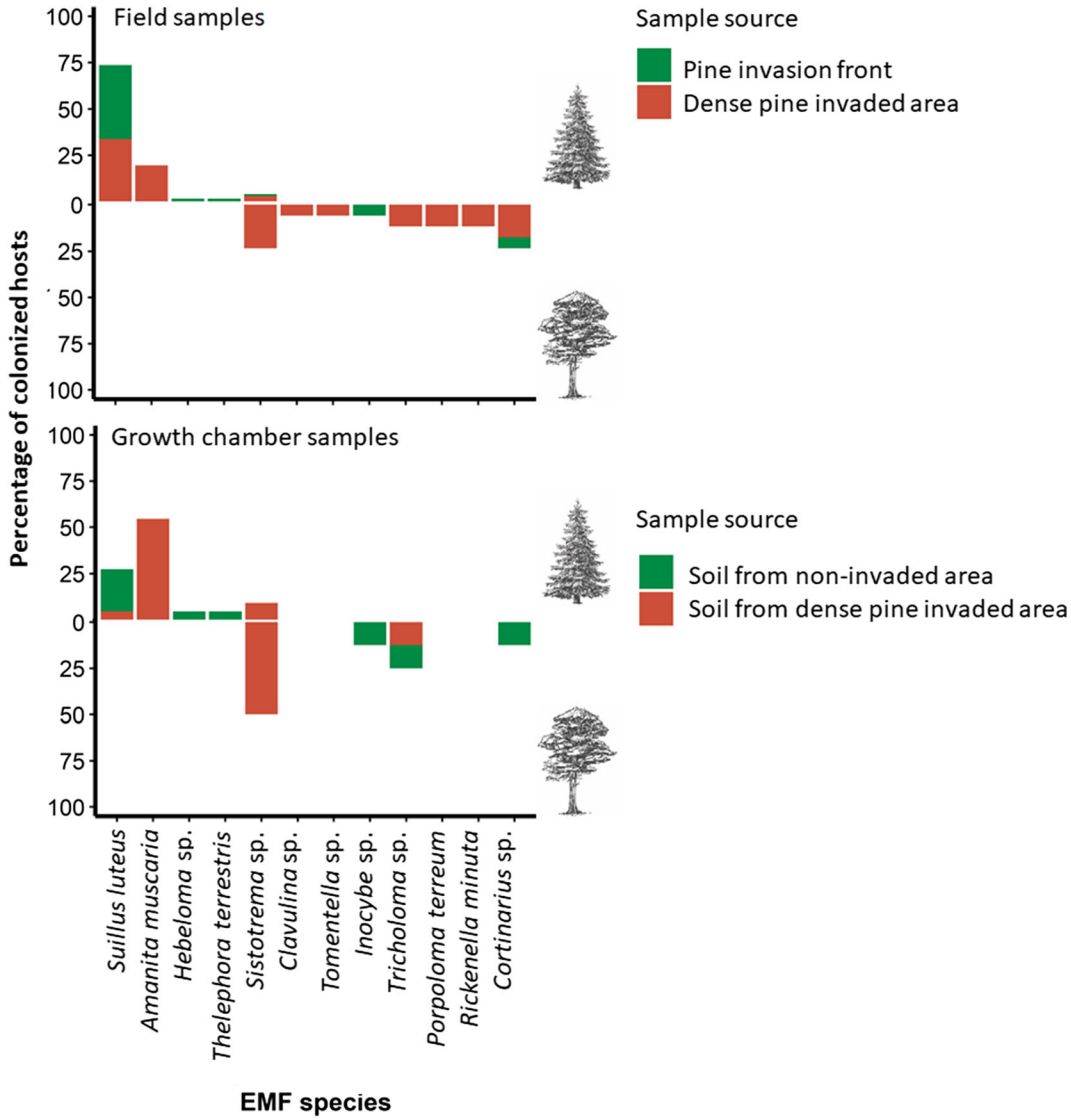

Fig. 3 Percentage of each host colonized by different EMF species obtained from the molecular analyses of root tips. Upper panel: root samples collected from the field, for both adult hosts of *Pinus contorta* (above, n = 24) and *Nothofagus antarctica* (below, n = 19), in two different areas, the pine invasion front (green bars) and the dense pine invaded area (red bars) in Malalcahuello, Chile. Lower panel: root samples collected from the growth chamber bioassay, for both hosts *Pinus contorta* seedlings (above, n = 24) and *Nothofagus antarctica* seedlings (below, n = 20) growing in soil from dense pine invaded areas (red bars), and soil from native stands of *Nothofagus*, non-invaded by pines (green bars) in Malacahuello, Chile. In both panels the bars show the percent of hosts colonized by each EMF species relative to the total hosts analyzed for each plant species

expected that pines will continue to increase their range and invade native forests.

A restricted set of co-invasive EMF allow pines to establish and survive once they reach places far from the inoculant source. In corroboration with other authors (Nuñez et al. 2009; Hayward et al. 2015b; Urcelay et al. 2017), we found *Suillus luteus* as the main non-native invasive EM fungus colonizing pine seedlings in field samples and soil bioassay from the growth chamber, even where pines were not yet present locally. Most of the previous works found this pattern for non-forest settings (Hayward et al. 2015b), as we did in the steppe ecosystem in Coyhaique, but our data support this pattern also for a native forest ecosystem (Ashkannejhad and Horton 2006). Co-invasive suilloid fungi, and *S. luteus* in particular, have been described as global

drivers of pine invasions due to their spore resistance, long distance dispersal and reactivity of the spores (Horton 2017; Policelli et al. 2019). Together with *S. luteus* we found *Hebeloma* sp. and *Thelephora terrestris* which have also been reported associated with *P. contorta* in the invasion front (Nuñez et al. 2009; Hayward et al. 2015a). Due to the presence of this set of invasive EMF that are waiting for their hosts to arrive and may persist in the soil for many years (Nara 2008; Bruns et al. 2009; Nguyen et al. 2012; Policelli et al. 2019), native stands where pines are still not present should be recognized as potentially vulnerable to invasion.

We provide evidence that native EMF colonize native hosts in invaded stands. We found that *Cortinarius* sp. is capable of colonizing *Nothofagus* hosts both in soil coming from highly pine invaded stands and in soil from native stands without pines. Particularly for EMF that do not disperse well through spores and appear to colonize mostly by hyphal spread (Agerer 2006; Peay et al. 2011), the presence of viable mycelia in the soil might be key to allow colonization of native seedlings. Our results are limited at distinguishing between different species of *Cortinarius*, a genus of great diversity and highly complex taxonomy (Garnica et al. 2016). Different species might have shown the same or even similar RFLP patterns that our analysis did not separate. We did find other species of native EMF that are coincident to what is reported for native *Nothofagus* stands (Nouhra et al. 2013, Fernandez et al. 2015). However, rarefaction curves showing the diversity of EMF associated with *Nothofagus* suggest we did not sample enough to fully characterize the species richness of the *Nothofagus* EMF (online resource 4, Chao et al. 2014; Hsieh et al. 2016).

The availability of native inocula in previously invaded soil, may be strongly dependent on two different traits of native EMF: their dispersal capacity and the formation of long-lived spore banks (Grove et al. 2017). Dispersal capacity is directly related to distance from native EMF sources of inocula. Lack of ectomycorrhizal inoculum has been found to limit establishment of *Nothofagus* even at short distances from the forest edge in grassland systems (Dickie et al. 2012). As fungal species might not be able to disperse at long distances, dispersal limitation strongly impacts spore availability (Galante et al. 2011; Peay and Bruns 2014; Horton 2017) so the presence of native inocula nearby seems a necessary condition to support colonization of native hosts. We were not able to distinguish if the native EMF inocula present in the invaded stands was in the form of active mycelia, a spore bank in the soil, or if it continuously arrives through dispersal. Future bioassays could address if native EMF spores actually disperse to the site and how long they remain in soils as resistant propagules in the absence of a suitable host. Other aspects of native fungal colonization, such as access to mycorrhizal networks available near native hosts (Horton et al. 1999; Nara and Hogetsu 2004; Nara 2006; Grove et al. 2019), might be relevant to achieve successful establishment of native woody plants in the field and worth further research.

We found *Sistotrema* sp. colonizing both the native *Nothofagus* and non-native *P. contorta*. *Sistotrema* spp. have been previously reported and characterized as forming ectomycorrhiza with *Pinus* and other genera in Pinaceae, as well as with Fagaceae (Kim et al. 2005; Nilsson et al. 2006; Dunham et al. 2007; Di Marino et al. 2008; Münzenberger et al. 2012). This species might have been co-introduced with pine trees and was able to associate with native *Nothofagus* roots. Generalist EMF species, such as *Amanita muscaria*, have been reported to cross-colonize Nothofagaceae and Pinaceae species (Dunk et al. 2012; Nuñez and Dickie 2014; Truong et al. 2017). Our results might be limited in terms of the number of EMF species found both from the growth chamber bioassay and the field samples, and we cannot completely rule out the possibility of cross colonization of other EMF species. Particularly we cannot discard that non-native EMF may associate with native hosts or native EMF with non-native hosts (Bahram et al. 2013), which may be more likely for phylogenetically close host species (Hoeksema et al. 2018). In our study, we did not find cross-colonization of the EMF species except for *Sistotrema* sp. Plant-soil feedbacks may depend on whether or not plants share soil mutualists (Crawford et al. 2019).

Invasive plants are able to modify existing interactions between native plants and native soil biota, or establish novel mutualistic interactions with native soil biota to thrive and invade in their new range (Reinhart and Callaway 2006; Nuñez and Dickie 2014; Wardle and Peltzer 2017). Even for native EMF mutualists, high abundance of non-native hosts increases the frequency with which they encounter non-native species rather than native species, increasing in turn the probability of forming a novel interaction (Aslan et al. 2015). For pines invading *Nothofagus* forests in the Southern

Hemisphere, the absence of a phylogenetically close group of hosts may prevent them from associating with native EMF yet still be highly successful (Reinhart et al. 2012; Hoeksema et al. 2018). Co- invasion is the main mechanism to allow introduced plants to maintain their symbionts, especially if the recipient community has no closely related hosts (Tedersoo et al. 2007; Dickie et al. 2010; Nuñez and Dickie 2014; Hayward et al. 2015a, b). The presence of novel interactions in the other way (non-native EMF associated with native trees) have been scarcely reported although it has been previously evaluated (Sulzbacher et al. 2018).

Differences between the EMF species found associated to field samples and growth chamber samples were expected, as generally growth chamber bioassays tend to select for EMF species with resistant spores that survive the disturbance caused by the collection of the sample (Taylor and Bruns 1999; Baar et al. 1999; Izzo et al. 2006). Also the EMF communities associated with new seedlings of pine trees and Nothofagus is generally different from that of already established, although young, trees in the field (Peay et al. 2011; Twieg et al. 2007). In the growth chamber bioassay, we did find some colonization of EMF species that typically colonize through mycelial networks (i.e. non spore colonizers) such as *Amanita muscaria* for *Pinus contorta*, or *Cortinarius* sp. for *Nothofagus antarctica*, where readily active hyphae present in the soil cores might have been the primary inoculum source. The role of remnant *Nothofagus* trees present in the dense pine invaded area might be crucial as inoculum source to new native seedlings, particularly for those native EMF that do not actively colonize through spores (Teste et al. 2009).

Availability of native soil mutualists would not be a limiting factor for restoration of sites previously invaded by pine trees. Re-establishment of disrupted associations between mycorrhizal fungi and their hosts can be necessary for successful restoration of target species (Kardol and Wardle 2010). In pine invaded habitats, however, native communities of EMF may remain in the soil, allowing native trees to successfully establish and grow. Applying soil collected from reference sites or even spores of certain EMF species is increasingly used as a way of ensure mycorrhizal colonization and improve seedling establishment (Maltz and Treseder 2015; Patterson 2018, but see Grove et al. 2019). In some cases, however, it might not be necessary as viable inoculum might still be present in the soil, which may reduce costs involved in restoration efforts.

Generally in plant communities, non-native species negatively affect native species more than they affect other non-natives, and natives have neutral or even negative effects on native neighbors (Kuebbing and Nuñez 2016). In our study both native and non-native hosts grew equally well in native and non-native soil due to the presence of their own specific fungal partners. Soil where invasive hosts are absent is already conditioned by the presence of non-native invasive EMF, and native trees may be able to remain in the invaded area due to the presence of native EMF. The presence of native hosts is not hindering the invasion of non-native hosts and the presence of native belowground fungal mutualists seems not to hinder the spread of their non-native counterparts.

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