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Co-invading ectomycorrhizal fungal succession in pine-invaded mountain grasslands

Tomás Milani [a,*], Jason D. Hoeksema [b], Esteban G. Jobbágy [a], J. Alejandro Rojas [c], Rytas Vilgalys [d], François P. Teste [a,e,1]

[a] Instituto de Matemática Aplicada San Luis (IMASL - UNSL - CONICET), Av. Ejército de los Andes 950, 5700, San Luis, Argentina
[b] Department of Biology, University of Mississippi, University, MS, 38677, USA
[c] Department of Entomology and Plant Pathology, University of Arkansas, Fayetteville, AR, 72701, USA
[d] Biology Department, Duke University, 130 Science Drive, Durham, NC, 27708, USA
[e] School of Biological Sciences, The University of Western Australia, 35 Stirling Highway, Crawley (Perth), WA, 6009, Australia

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ABSTRACT

Ectomycorrhizal (EM) fungal communities that associate with invading pines (*Pinus* spp.) are expected to be poor in species diversity. However, long-term successional trajectories and the persistence of dispersal limitations of EM fungi in the exotic range are not well understood. We sampled the roots and surrounding soil of *Pinus elliottii* and *P. taeda* trees invading mountain grasslands of Argentina. We also sampled the EM fungal spore bank in grassland soil near (~150 m) and far (~850 m) from the original pine plantations. We found 86 different co-invasive EM fungal OTUs. Differential dispersal capacities among EM fungi were detected in the spore bank of grassland soil, but not under mature pines. After thirty years of invasion, the age, but not the degree of spatial isolation of pine individuals affected the EM fungal composition. We showed how EM fungal succession occurs during pine invasions, which may have clear consequences for ecosystem functioning of co-invaded sites.

1. Introduction

During an invasion, plants interact with fungi, including both pathogens and mutualists (Keane and Crawley, 2002; Policelli et al., 2020). Important advances in our understanding of plant-fungal invasions have been achieved during the last decade, yet long-term dynamics, biogeographical comparisons and ecosystem-level impacts remain as major topics for future research (Dickie et al., 2017). The reliance on mutualisms has been recognized as a barrier for different biological invasions (Richardson et al., 2000); and although the strict dependence on mycorrhizal fungi can limit the invasion of some tree species into new sites (Nuñez et al., 2009), it also appears to drive plant invasiveness (Menzel et al., 2017; Moyano et al., 2021). The benefits conferred to the plant by mycorrhizal fungi include increased nutrient acquisition as well as pathogen protection (Smith and Read, 2008), which are crucial to colonize new sites and outcompete the native vegetation of the invaded range (Nuñez and Dickie, 2014; Menzel et al., 2017). However, how these complex mutualistic associations develop over time is still poorly understood.

Pines (*Pinus* spp.) are one of the most concerning groups of woody plant invaders that form obligate associations with ectomycorrhizal (EM) fungi (Dickie et al., 2010; Nuñez and Dickie, 2014). The relatively high specificity of the pine-EM fungal association usually results in a co-invasion into habitats where other pine species are naturally absent, like most of the terrestrial ecosystems of the Southern Hemisphere (Vlk et al., 2020). The multiple 'filters' that operate during the introduction (i.e., plantation) phases and throughout later invasion phases often result in co-invading EM fungal communities that are poor in species when compared to its native range (Hayward et al., 2015b; Gundale et al., 2016; Hoeksema et al., 2020). However, pines can form important novel associations when invading ecosystems that have other native EM hosts, highlighting the context-dependency of this phenomenon.

In absence of alternative EM hosts, the composition and abundance of co-invading EM fungal communities is thought to be primarily controlled by differences in dispersal capacity among the fungal species, resulting in spatially structured invasion fronts, with most of the EM

* Corresponding author.
E-mail address: milanitomas@gmail.com (T. Milani).
[1] Current address: Swift Current Research and Development Centre, Agriculture and Agri-Food Canada, 1 Airport Road, Swift Current, SK, S9H 3X2, Canada.

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fungal species constrained to the proximity of original pine plantations (Hayward et al., 2015a). Dispersal of EM fungi can be defined as a function of how many fungal spores are produced, how far they disperse, the time interval considered and the spore's capability to remain viable (and hence accumulate) in soil over time (Nara, 2009; Nguyen et al., 2012). This last trait is essential during the colonization of novel sites, since some EM fungal spores, unlike extraradical hyphae, can survive unfavorable soil conditions until seedlings establish (Horton, 2017). Hence, the ability to form a resistant spore bank, high production of spores and multiple abiotic and biotic dispersal vectors have allowed good dispersers such as Suilloid fungi (*Suillus* and *Rhizopogon* EM fungal species) to dominate pine invasion fronts worldwide, especially far from the original inoculum source (Nguyen et al., 2012; Policelli et al., 2019).

Although dispersal can be important in structuring the EM fungal community of pines (Ashkannejhad and Horton, 2006; Peay et al., 2007; Glassman et al., 2015), other abiotic and biotic factors have been shown to also play a role. For example, disturbances like fire (Rincón and Pueyo, 2010; Kipfer et al., 2011), drought (Swaty et al., 2004), increased nitrogen deposition (Lilleskov et al., 2002), or clear-cutting (Jones et al., 2003) can influence the structure of EM fungal communities. Another major factor contributing to EM fungal structure in the native range of pines is the stand age. Such changes in EM fungal composition that occur as pine stands get older is also known as EM fungal succession, which was recognized in early studies using sporocarp surveys around *Betula pendula* trees (Deacon et al., 1983), and again later reported using root tips in many other EM woody species including pines (Last et al., 1987; Fastie, 1995; Visser, 1995; Fryar, 2002; Nara et al., 2003a, 2003b; Twieg et al., 2007).

The mechanisms behind compositional changes during EM fungal succession remain unclear, partly because tree age includes both changes in tree ontogeny and time (Jokela and Martin, 2000), yet some propose that these shifts are a consequence of competition-colonization trade-offs (Peay et al., 2007; Teste and Dickie, 2017; Smith et al., 2018). The EM fungi associated with young trees and seedlings typically include good dispersers such as *Suillus*, *Thelephora* and *Rhizopogon*, while those EM fungi that tend to dominate in the roots of adult trees include *Amanita*, *Russula* or *Inocybe* that are thought to be poor dispersers yet better competitors (Nara, 2009). Other studies have suggested that changes in soil nutrient availability and differential extracellular enzyme activities among EM fungi can explain the succession (Kyaschenko et al., 2017). The factors affecting the structure of EM fungal communities are thus diverse and temporally dynamic (Bruns, 1995; Dickie et al., 2013), yet we do not fully understand the trajectories that EM fungal co-invading communities follow in pine invaded ecosystems.

Here, we took advantage of a native mountain grassland ecosystem in central Argentina, which was historically free of EM fungi, but is now invaded by pines, to explore the diversity and structure of the co-invading EM fungal community. We aimed to evaluate compositional changes in the EM fungal community along a distance gradient from the original plantations to assess which EM fungi are dispersal limited. Our specific hypotheses were that: (1) the pine invasion is less species-rich in EM fungi than the pine plantations; (2) richness of the co-invading EM fungi declines with increasing distance from the edge of the plantation where the EM fungal community is dominated by good dispersers; and (3) due to lack of alternative host plants, the EM fungal spore bank in non-pine-invaded grassland soil (hereafter grassland soil) becomes more species-poor and compositionally-simpler with distance from the plantation.

2. Materials and Methods

2.1. Study site

The study was conducted on the east side (31°58′ S, 64°47' W) of *Sierras de Córdoba*, Córdoba province, Argentina. This mountain belt runs North–South across 500 km of rolling hills, elevated plains and deep valleys forming a very heterogeneous landscape. The native vegetation above 1100 m elevation is a mix of C3 and C4 grasses dominated by *Stipa filiculmis* and *Festuca hieronymi* that has been subjected to cattle grazing for the last ~400 y (Cabido et al., 1997; Cingolani et al., 2013). Mean annual precipitation is 850 mm and monsoonal, occurring mainly from October to April (Jobbágy et al., 2013). Fire is an important disturbance of these grasslands, with tight intervals between extensive fires ranging from three to four years (Argañaraz et al., 2015).

Exotic plantations of slash pine (*Pinus elliottii*) and loblolly pine (*Pinus taeda*), both native in the southeastern USA, were established as a result of a tax benefit program by the Argentinian government during the 1970s and 1980s with the aim of promoting the regional forest industry (Izurieta et al., 1993). Neither pruning nor thinning were properly conducted in these exotic pine plantations, resulting in poorly managed pine stands that were partly harvested during the 2000s or suffered severe damage from large wildfires during 2013 (CONAE, 2013). Although some of the original plantations in the region persist, most of these now consist of scattered trees and medium-size aggregates (1–4 ha). In the adjacent grassland, a long-distance and widespread invasion of mixed *P. elliottii* and *P. taeda* has been occurring since the early 1990s (Fig. S1), which has led to an invaded system where pines of different age classes are evenly distributed across the invasion front (Milani et al., 2020). In this study, we refer to the invasion front as the area outside the plantation edges where pines have naturally established (i.e., invaded). Due to the low density of invading pines and widespread nature of this pine invasion, it was not possible to identify a leading-edge of invasion.

2.2. Field sampling of roots, soil and sporocarps

Pine roots, pine soil (i.e., soil below pine-crown projection) and EM fungal sporocarps were sampled across the invasion front and inside the remaining pine plantations. These samples were collected during May 2017 at five sites. Each site was a band 1200 m long and 400 m wide in the direction of the open, yet invaded, grassland from the edge of the original corresponding plantation. Sites were then divided into five distance intervals (0–25, 26–100, 101–300, 301–600, 601–1200 m from the edge of the original plantation). At each distance interval, one mature invading pine tree (i.e., with cones), was randomly sampled (5 sites x 5 distance intervals x 1 invading pine = 25 invading pines) (Fig. 1A). Instead of sampling within the remaining disturbed pine plantations at our sites, the nearest three undisturbed pine plantations were sampled to describe the original EM fungal community. These plantations were full-cover even stands where five pine trees at least 100 m from each other (3 plantations x 5 pines = 15 pines) were selected (Fig. 1D). Around each pine tree sampled, pine roots and pine soil were taken from the mineral layer (0–20 cm below litter layer) in at least three different cardinal points making a composite sample.

To determine the age of the pine trees, two wood cores (4.3 mm diameter) were extracted with an increment borer at 30 cm above ground level and processed using standard dendrochronological methods (see Milani et al. (2020) for details). Furthermore, 27 representative EM fungal sporocarps were collected for molecular identification (see below). Finally, invading pine seedlings (seven invading seedlings) that we encountered around all sampling locations were opportunistically sampled, carefully extracting as much of the root system as possible. Sampled pines both inside plantations and at the invasion front were mainly *P. elliottii*, yet some *P. taeda* individuals were detected across sites. Southern pines can naturally hybridize (Little, 1979; Burns and Honkala, 1990) and we saw some morphological evidence of this in the field. Because of the aforementioned reasons, and also because phylogenetically related pines species tend to share similar EM fungal communities (Ning et al., 2019), we considered them as a pine complex and do not distinguish between the two pine species in the analyses presented here.

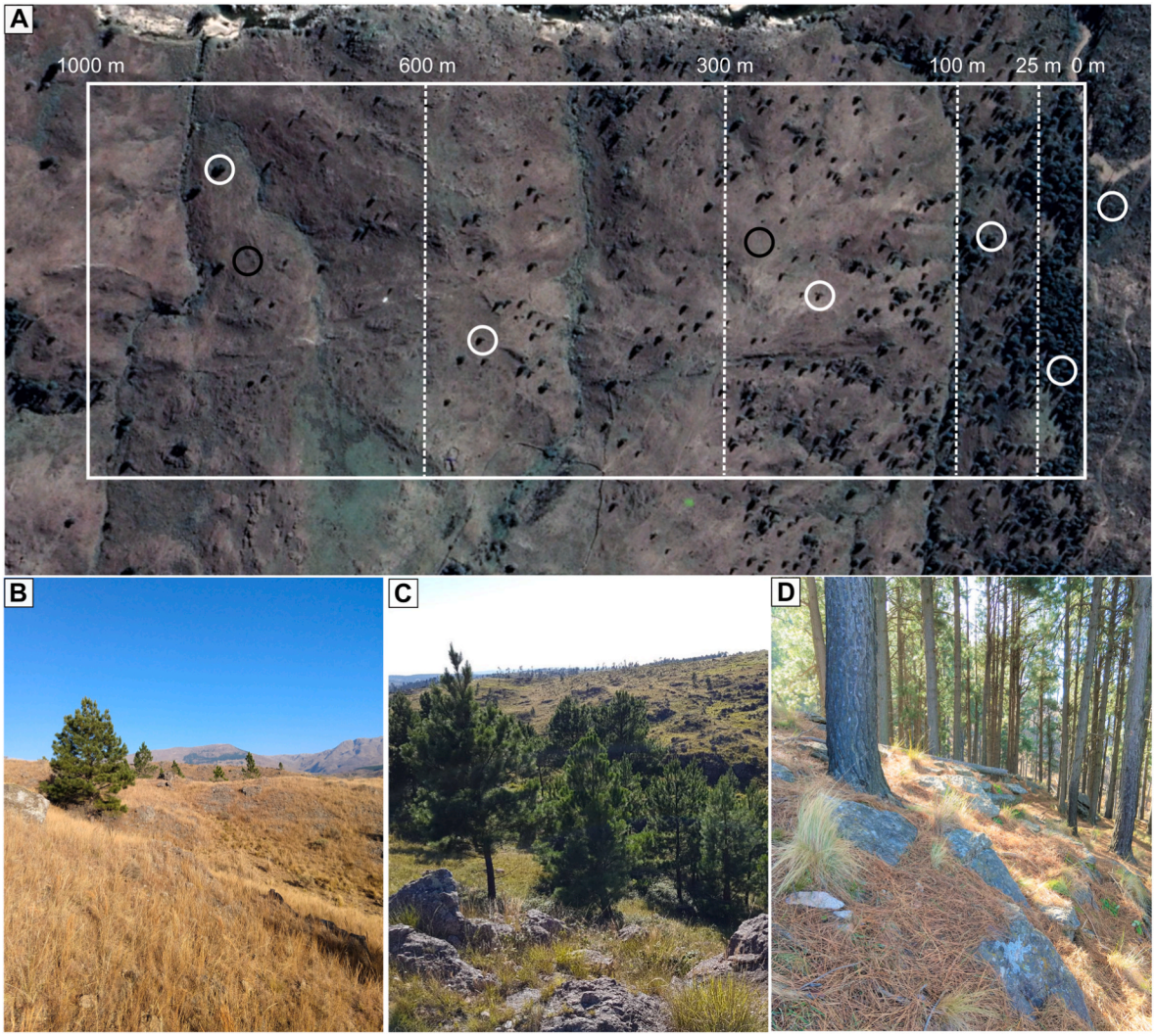

Fig. 1. (**A**) Satellite image taken from Google Earth of one of the field sites in mountain grasslands (Sierras de Córdoba) of Argentina invaded with *P. elliottii* and *P. taeda* along with a scheme of the sampling design to explore its EM fungal community. Black circles represent the locations with native grasslands and no pines, from where soil samples were taken near (~150 m) and far (~850 m) from plantations to explore the EM fungal spore-bank. White circles represent the pine trees (**B**) haphazardly selected within a distance interval from where roots and soil were taken. Old and young isolated pines are found far from plantations while (**C**) density increases close to the plantation edge. (**D**) As plantations associated with the invasion fronts were usually harvested or heavily fire-damaged, nearly undisturbed plantations were sampled instead.

To fully describe the EM fungal community in this pine-invaded ecosystem, the EM fungal spore bank of grassland soil was also sampled on January 2018. Samples were taken near (147 ± 68 m, n = 10) and far (852 ± 197 m, n = 10) from pine plantations, and also far from any host pine tree, sapling, or seedling. All grassland soil samples were collected at a minimum distance of two times the height from all pine individuals (~16 m away), to exclude the effects of pine roots and their extraradical EM fungal mycelium, and to ensure that soil samples were independent and without any confounding factors from nearby host pines. The 'two-times height distance' criterion was determined since the maximum extent of lateral roots in adult pines is around 1.4 its height (Stone and Kalisz, 1991) and their emanating EM mycelium can add another ~40 cm of lateral exploration (Agerer, 2001).

2.3. Sample preparations for molecular analyses

Root fragments of 47 pine individuals (40 adult pines and 7 pine seedlings) were separated from the soil and carefully washed under running water, then cut into 2 cm pieces and homogenized. Subsamples were then haphazardly selected and placed in a Petri plate for morphotype sorting based on shape, color, and emanating hyphae until 200 root tips per sampled pine were scored. During this procedure, the number of root tips belonging to each morphotype, as well as non-mycorrhizal tips, were counted. Regardless if root tips were in clusters or as single tips, we calculated percent mycorrhizal root colonization based on total counts of root tips per sample. A total of 9604 root tips were inspected and one root tip per morphotype per sample (124 root tips) was placed in extraction buffer (Merck KGaA, Darmstadt, Germany) and stored at −20 °C until further processing. Soil samples (pine soil, grassland soil) were kept refrigerated during the field work (5 d) and immediately processed upon arrival at the laboratory. Soil samples were sieved to 2 mm, homogenized, and then 0.5 g of fresh soil was stored in PowerBead tubes (Qiagen, Hilden, Germany) at a temperature between 4 and 6 °C until further processing. Sporocarps collected in the field were stored in wax paper bags and transported to the lab for processing. Small sections of tissue (~0.1 cm^3) were excised with clean forceps and laid down on quadrants of Whatman FTA card (Whatman International Ltd, Maidstone, England) following the protocol from Dentinger et al. (2010). The FTA cards were air-dried and stored at room temperature until processing.

2.4. DNA extractions, amplifications, and sequencing

Fungal DNA was extracted from root tips using 10 μL of Extract-N-Amp Tissue kit (Merck KGaA, Darmstadt, Germany), incubated for 10 min at 65 °C followed by 10 min at 95 °C, after which 30 μL of neutralization solution (Merck KGaA, Darmstadt, Germany) were added. Extracts of DNA were diluted to 20% concentration by adding PCR-grade water. Amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA was done using REDTaq ReadyMix (Merck KGaA, Darmstadt, Germany) along with ITS1-F and ITS4 primers (Gardes and Bruns, 1993) and the parameters described in Table S1. Using gel electrophoresis, PCR products were checked with a 1% agarose gel. Samples with clear bands of around 800 bp were cleaned enzymatically using ExoSAP-IT (USB corporation, Cleveland, USA). Sanger sequencing was performed in both the forward and reverse directions, using primers ITS1-F and ITS4, respectively and BigDye Reaction Premix (Thermo Fisher Scientific, Waltham, Massachusetts, USA) under the following conditions: 1 min at 96 °C followed by 45 cycles (20 s at 95 °C, 20 s at 52 °C, 240 s at 60 °C) and a final extension phase of 4 min at 60 °C. The reactions were dried and sent to the DNA Laboratory at the School of Life Science at Arizona State University where they were purified and read on an Applied Bioscience 3730 capillary genetic analyzer (ThermoFisher, Waltham, MA, USA).

Fungal DNA from soil samples was extracted using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was diluted to 50% by adding PCR-grade water. A fungal amplicon library targeting the ITS region using primers ITS1-F and ITS2 (White et al., 1990) was prepared using a frame-shift tagging approach (Lundberg et al., 2013). This approach reduces the need for Phi X spiking and it consists of three amplification steps: enrichment, tagging and sample-barcoding (Table S2). The 'enrichment' step consisted of 10 PCR cycles with the ITS1-F and ITS2 primers using 2 μL of DNA diluted 1:1 ratio. The 'frame-shift tagging' step also consisted of 10 cycles with the modified ITS1-F and ITS2 primers including an Illumina-bridge adapter (Fig. S2, Tables S3 and S4); the forward primer also included a frame-shift section as described in Lundberg et al. (2013) and 2.5 μL of DNA derived from the first step served as the template. The last step consisted of 10 cycles using sample-specific Illumina adapters, where the reverse primer contained 10 bp sequences derived from Golay primers. The last step used 5 μL of DNA derived from the frame-shift tagging step. The different PCR steps were performed in 25 μL reactions containing 2 mM of $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of forward and reverse primers, 1X of PCR buffer II and 1 U of AccuStart II Taq polymerase (Quantabio, MA, USA) and 0.4 mg mL^{-1} of bovine serum albumin (BSA); water was adjusted based on the volume of DNA template used for every step. For quality control, a mock EM fungal community and negative control were included on every plate. Using gel electrophoresis, PCR products were checked on 1% agarose gels and quantified using Quant-iT HS DNA assay kit (Invitrogen, NY, USA). The PCR products were pooled, normalizing by molarity to ~10 nM, into a single library and the final library was purified twice using Mag-Bind TotalPure NGS (Omega Bio-tek, GA, USA) following manufacturer instructions. Sequencing was conducted on paired-end Illumina MiSeq (MiSeq Reagent Kit v3, Illumina Inc., San Diego, CA, USA) at the Centre for Genomic and Computational Biology, Duke University, USA.

For EM fungal sporocarp identification, discs (6.35 mm diameter) from FTA cards were extracted using a paper hole puncher and placed in 0.2 mL tubes. Fungal DNA was extracted from these discs using 20 μL of Extract-N-Amp Tissue kit and incubating them for 10 min at 95 °C. After the incubation, 20 μL of BSA at 3% were added to the extraction and the solution was used as a template for PCR. Amplification of the ITS region was done using ITS1-F and ITS4 primers and the parameters described in Table S1. The PCR reactions had a final volume of 25 μL containing 2 μL of template DNA, 2 mM of $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of ITS1-F and ITS4 primers, 1X of PCR buffer and 1 U of DreamTaq polymerase (ThermoFisher, Waltham, MA, USA) and 0.2 mg mL^{-1} of BSA. The PCR products were checked in 1% agarose gels and positive samples were cleaned with a mix of exonuclease I (10 units/μL) along with shrimp alkaline phosphatase (1 unit/μL). Six μL of the exonuclease I and the phosphatase mix (M0293S and M0371S, NEB, Ipswich, MA, USA) were added to the PCR products and then were incubated at 37 °C for 30 min, then at 85 °C for 10 min in a thermal cycler. Sanger sequencing was performed in both the forward and reverse directions, using primers ITS1-F and ITS4 at Eurofins Genomics (Eurofins Genomics, NC, USA).

2.5. Bioinformatics

Forward and reverse DNA sequences from pine roots were assembled *de novo* using *GENEIOUS* software (Biomatters Ltd, Auckland, New Zealand). Each sequence was checked and corrected with its complementary strand, so a longer and higher quality sequence was obtained. Only *de novo* sequences longer than 200 bp and with less than 3% of unidentified bases continued in our workflow. Forward and reverse sequences from sporocarps were edited using *Sequencher* (GeneCodes, MI, USA) in the same way. Root and sporocarp sequences were grouped together into operational taxonomic units (OTUs) of 97% similarity using CAP3 software (Huang and Madan, 1999).

Taxonomy assignment of root and sporocarp OTUs was done using parallel BLAST into the UNITE (Kõljalg et al., 2005) + INSD database (https://unite.ut.ee/index.php). The reference sequence with the higher bit score was used for the taxonomy assignment to species level when match was ≥99%, genus for 95–99%, and family for 90–95% (Tedersoo et al., 2010; and see Table S7). Singletons coming from root tips and sporocarp samples were preserved and manually incorporated to OTUs if the match was identical at the species level. The taxonomy assignments from molecular analyses were then extrapolated to the number of root tips with the same morphotype of the corresponding sample. Root morphotypes that were visually confirmed to be ectomycorrhizal (i.e., had fungal mantle and presence of Hartig net) but that were not properly identified by molecular analyses, were named as 'Unidentified EM fungi' and given consecutive numbers (see Table S7, Fig. S3).

Raw sequences of DNA from soil samples (~300 bp) were processed using the FAST pipeline (https://github.com/ZeweiSong/FAST). Illumina adapters and sequences shorter than 50 bp were processed using Cutadapt v1.18 (Martin, 2011) and overlapping sequences were merged using PEAR v0.9.8 (Zhang et al., 2014). Merged sequences were filtered by low quality and expected error using VSEARCH v2.12.0 (Rognes et al., 2016). A total of 2,767,437 sequences were kept and used for de-replication, chimera check and OTU clustering at 97% similarity (singletons were removed at this step) using VSEARCH in the FAST pipeline (all soil DNA processing code available in Data Accessibility section).

A novel database using OTUs of roots and sporocarps with assigned taxonomy along with UNITE + INSD was created prior to soil OTU BLAST. This was done in an attempt to take advantage of long and high-quality sequences (ITS1-F/ITS4, bidirectional sequencing) to go into a deeper taxonomy assignment and confirmation of soil sequences. Those soil OTU samples that matched a root and/or sporocarp OTU received the same taxonomic assignment. Soil OTUs that did not match a root and/or sporocarp continued as unique OTUs with an assigned taxonomy from UNITE + INSD database. Sequence data from all roots and sporocarps collected are publicly available in the National Centre for Biotechnology Information (NCBI) GenBank database (accessions nos. **ON406880**-**ON406916**). Short reads from Illumina sequencing are also publicly available in the NCBI Short Read Archive (SRA) (accessions nos. **SAMN28115804**-**SAMN28115884**).

2.6. Data analyses

Soil OTUs were first filtered as 'possible' EM fungi using the FUNGuild database (Nguyen et al., 2016). Those soil samples that had less than five EM fungal reads were considered to have no EM fungi in our

statistical analyses. Analyses of EM fungal richness were done at the OTU level. In the case of pine roots and pine soil samples, a joint presence/absence dataset was also created. Comparison of OTU richness between plantations and the invasion front were done by fitting Arrhenius models (Dengler, 2009) to the sample-based rarefaction curves and testing five random fitted values of plantation and invasion at equivalent levels of sampling effort. Differences in OTU richness per tree between distance intervals were tested using one-way analysis of variance (ANOVA), while comparisons of OTU richness and proportion of EM fungal reads out of total reads between grassland soil samples taken near and far from pine plantation were made by *t*-tests. For roots and soil independently, we rarefied the data prior to comparisons of richness per tree, because there were differences in the number of root tips/fungal reads per sample. On the other hand, when richness was compared within the combined dataset, we used the total observed richness. Relationships of pine age with root colonization and proportion of EM fungal reads were tested using regression analyses. In all cases we checked that the data met model assumptions.

The EM fungal community analyses were done at the genus level due to the high variability in OTU composition among samples. We also filtered to include only those EM fungal genera that were present in at least three samples to increase data connectivity. Analyses were done independently for: (a) joint pine dataset (pine roots + pine soil), (b) pine soil, (c) pine roots and (d) grassland soil. All analyses were also done considering plantation and invasion together or only invading pines. The relationships between community structure and the evaluated variables (stand origin, site, pine age, distance) were tested with Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations using the adonis2 function from the vegan package in R (Anderson, 2001; Oksanen et al., 2019). Jaccard dissimilarity index was used in the case of the joint pine dataset while Bray-Curtis dissimilarity index was used for individual datasets that were previously transformed to relative frequency. Community structure was then visualized by ordination using Non-metric Multi-Dimensional Scaling (NMDS). All analyses were done using R version 4.0.2 (R Core Team, 2020).

3. Results

A total of 113 EM fungal OTUs and 26 EM fungal genera were detected in our study. Furthermore, 86 EM fungal OTUs and 24 EM fungal genera were observed at the invasion front while 43 EM fungal OTUs and 15 EM fungal genera were observed inside plantations (Fig. S3). Of the total EM fungal OTUs, 35 corresponded to unidentified EM fungi that were found on pine roots (see Materials and Methods section for details) and accounted for 29% of the total root tips inspected. From the root tips selected for molecular analyses, 96% were properly amplified. Out of these, 74.8% yielded sequences assigned to EM fungi, while <1% were pathogenic fungi and 24.3% were low-quality sequences. Of the soil samples taken under pines, 80% (32 out of 40 pine soil samples) had EM fungi, while in the case of soil samples from grassland soil this value dropped to 62.5% (25 out of 40 grassland samples). All samples of EM fungal sporocarps were properly amplified (Fig. 2, Figs. S1B–G).

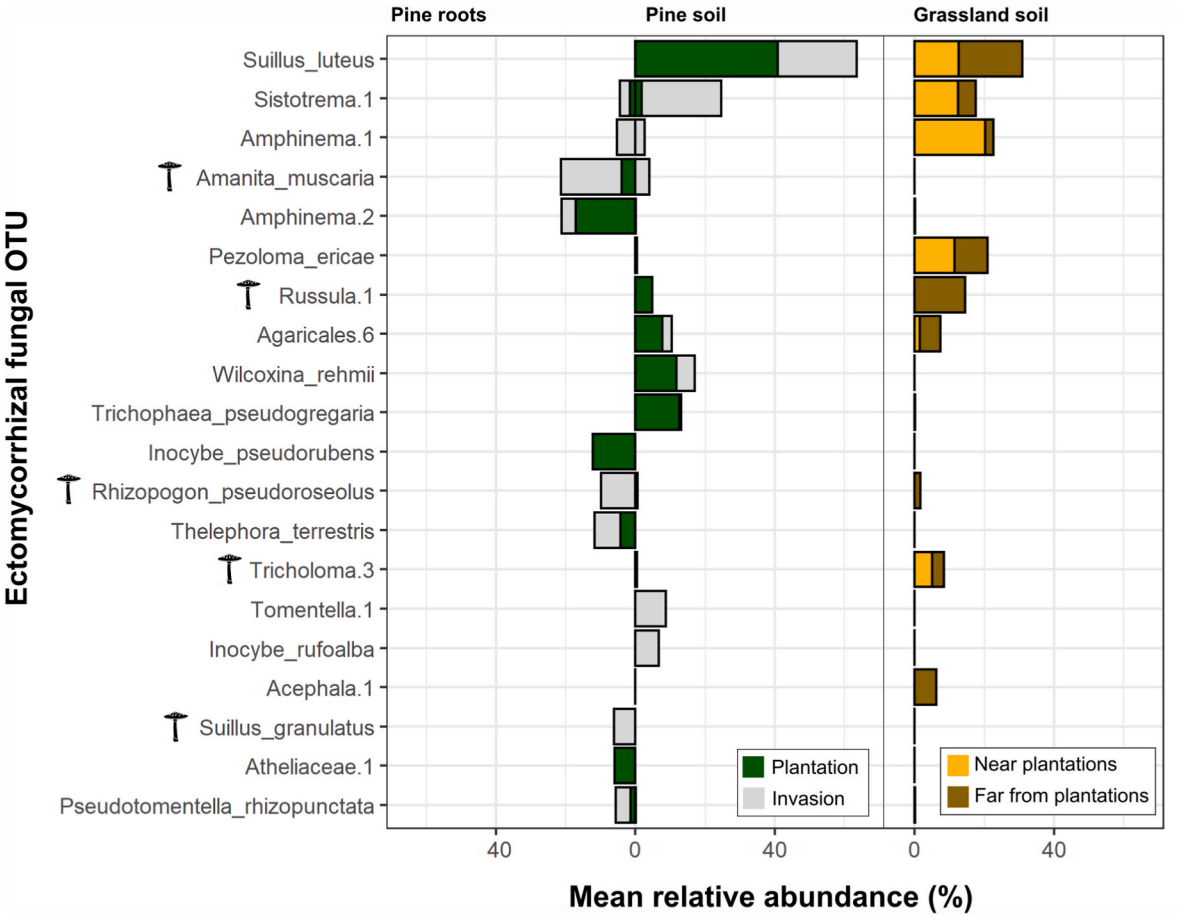

Fig. 2. Ectomycorrhizal (EM) fungal community found in pine-invaded mountain grasslands (*Sierras de Córdoba*) of Argentina. On the left panel, EM fungal operational taxonomic units (OTUs) from pine roots and pine soil taken from plantations and from the invasion front. On the right panel, EM fungal OTUs from the spore bank found in grassland soil. Taxa marked with illustrations of mushrooms are the OTUs that were also identified from sporocarps. Only the top 20 EM fungal OTUs with the highest mean relative abundance shown. A full version of this figure shown in Fig. S3.

The EM fungal OTU with the highest mean relative abundance in pine soil samples was *Suillus luteus*, followed by *Sistotrema1*, *Wilcoxina rehmii,* and *Trichophaea pseudogregaria* (Fig. 2). Within these pine soil samples, *Sistotrema1* had a greater mean relative abundance than *Suillus luteus* at the invasion front while the opposite occurred inside plantations. *Trichophaea pseudogregaria* was relatively more abundant in pine soil inside plantations than outside them. In the case of grassland soil samples, *Suillus luteus* and *Sistotrema1* were also important components of the community, along with *Amphinema1*, *Pezoloma ericae, and Russula1*. These fungal OTUs, highly represented in soil samples, were scarce on pine roots, where *Amphinema2*, *Amanita muscaria*, *Inocybe pseudorubens, Thelephora terrestris* and *Rhizopogon pseudoroseolus* were found instead. *Amphinema2* was an important component of the EM fungal community on the roots of planted pines whereas *Amanita muscaria* had higher mean relative abundance on the roots of invading pines. The sporocarps collected in the field corresponded to seven different EM fungal OTUs (Fig. S3). Five of them were also detected on pine roots while only four were detected in soil samples. All *Suillus* sporocarps collected corresponded to the OTU *S. granulatus* instead of *S. luteus* which was abundant in the soil.

There were no statistical differences in the number of EM fungal OTUs (pine soil and roots) between plantations and the invasion front when comparing at equivalent levels of sampling effort (Table 1, Fig. 3). This was not the case when analyzing pine roots separately, where plantations were found to have 28.5% more EM fungal OTUs than the invasion front, yet the opposite pattern occurred for pine soil samples separately (Table 1). There were similar values of EM fungal root colonization and percent of EM fungal reads in pine soil samples between plantations and the invasion front (Table 1). Mean OTU richness per sample was affected by pine age in the roots and pine soil although showing opposite patterns (Fig. S4). Neither percent root colonization nor percent of EM fungal reads were affected by pine age (Fig. S4). The EM fungal spore bank in the grassland soil had similar richness near or far from plantations (Table 1).

Community composition was affected by distance in grassland soil (Table 2, Fig. 4B). In these samples, *Suillus*, *Sistotrema*, *Trichophaea*, *Tomentella* and *Rhizopogon* were frequently found both near and far from plantations while *Cortinarius*, *Amphinema*, *Pezoloma,* and *Pseudotomentella* were more frequent near the plantation edge. The EM fungal community associated with pines was affected by pine stand origin (plantation or invasion), site and pine age, but no statistically significant effect of distance was detected when analyzing only the invasion front or both invasion and plantation together (Table 2, Fig. 5). Site effect was stronger in pine soil samples while differences in EM composition

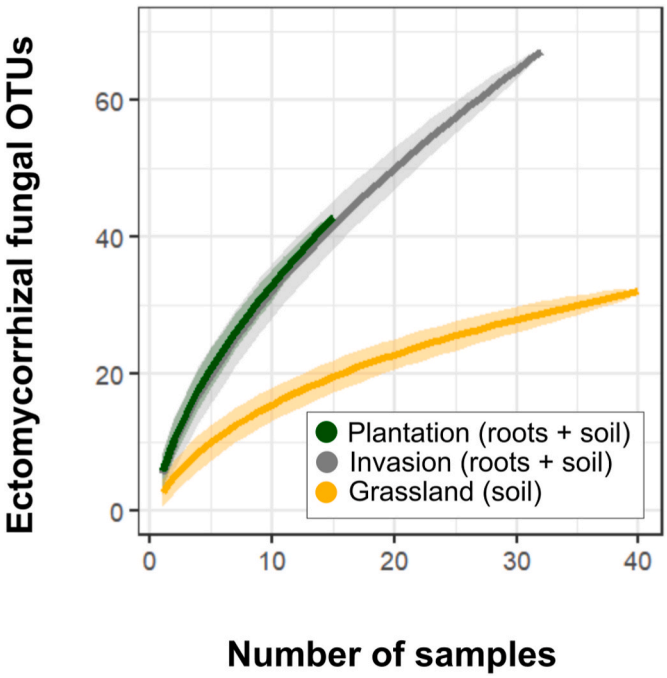

Fig. 3. Sampled-based accumulation curves for ectomycorrhiza fungal OTUs found in plantations (dark green), associated with invading pines (grey), and in grassland soil (yellow) in *Sierras de Córdoba,* Argentina. The joint dataset (pine root + soil) was used to construct plantation and invasion curves.

Table 1
Richness of ectomycorrhizal (EM) fungal operational taxonomic units (OTUs) founded inside of plantations and at the invasion front in mountain grasslands (*Sierras de Córdoba*) of Argentina. Pine stand origin refers to planted or invasive pines while distance refers to sampling distance intervals.

Sampling	Variable	Plantation	Invasion front					p-value	
		0 m	0–25 m	26–100 m	101–300 m	301–600 m	601–1200 m	Pine stand Origin	Distance
Pine roots + Pine soil	Rarefied richness (n = 15)	**42.23** (40.51–43.55)	**40.99** (38.52–42.13)					0.17	–
	Mean observed richness per tree	**5.60** (2–10)	**5.37** (1–11)					0.81	–
			7.37 (2–11)	**5.00** (2–10)	**4.50** (1–9)	**5.50** (2–9)	**4.43** (1–8)	0.82	0.36
Pine roots	Rarefied richness (n = 15)	**29.12** (27.70–30.05)	**22.66** (19.64–25.10)					**< 0.01***	–
	Mean rarefied richness per tree	**2.98** (2.00–4.78)	**2.12** (1–3.59)					**< 0.01***	–
			2.15 (1.00–2.98)	**2.28** (1.00–3.00)	**1.75** (1–2.52)	**2.00** (1.00–3.00)	**2.35** (1.00–3.59)	**< 0.01***	0.76
	Root-tip EM colonization (%)	**97.00** (79.30–100)	**97.20** (92.20–100)	**98.30** (96.70–100)	**96.70** (94.40–100)	**98.30** (95.80–100)	**98.50** (94.40–100)	0.46	0.85
Pine soil	Rarefied richness (n = 10)	**14.57** (13.80–15.70)	**21.35** (17.99–23.35)					**< 0.01***	–
	Mean rarefied richness per tree	**1.63** (1.08–2.59)	**2.14** (1–3.59)					**0.04***	–
			2.63 (1.01–3.46)	**2.34** (1.00–3.59)	**2.38** (2.06–2.86)	**1.64** (1.00–2.78)	**1.77** (1.26–2.48)	0.07	0.20
	EM fungal reads/ total fungal reads	**0.16** (0.01–0.86)	**0.19** (0.01–0.73)					0.81	–
			0.23 (0.07–0.37)	**0.05** (0.01–0.12)	**0.06** (0.01–0.09)	**0.28** (0.01–0.59)	**0.28** (0.01–0.73)	0.79	0.38
Grassland soil	Total observed richness (n = 20)	–	**22**			**20**		–	–
	Mean rarefied richness per sample	–	**1.85** (1.00–2.90)			**1.63** (1.02–2.09)		–	0.31
	EM fungal reads/ total fungal reads	–	**0.14** (0.01–0.50)			**0.43** (0.02–0.76)		–	**< 0.01***

Table 2
Results of Permutational Analysis of Variance (PERMANOVA) analyses of the co-invading ectomycorrhizal (EM) fungal community composition at the genus level in mountain grasslands (*Sierras de Córdoba*) of Argentina. Factors considered were: pine stand origin (plantation or invasion), site, pine age (as a factor of 5 years intervals), and distance from plantations (distance intervals). For joint dataset (pine roots + pine soil, presence/absence data), the Jaccard dissimilarity index was used (Faith et al., 1987). For pine roots, pine soil, and non-pine-invaded-grassland soil datasets, we performed PERMANOVAs using relative frequency (abundance data) with the Bray-Curtis dissimilarity index.

Sampling	Subsetting	Statistical values																	
		Pine stand origin					Site				Pine age				Distance				
		Df [a]	*SumSqs* [b]	*R2* [c]	*F* [d]	*P* [e]	*df*	*SumSqs*	*R2*	*F*	*P*	*df*	*SumSqs*	*R2*	*F*	*P*	*df*	*SumSqs*	*R2*	*F*	*P*
Pine roots + Pine soil	Invasion + plantation	1	0.85	0.05	2.93	**0.01***	6	2.72	0.17	1.57	**0.01***	3	1.83	0.11	2.11	**0.01***	4	1.57	0.10	1.36	0.09
	Invasion	–	–	–	–	–	4	1.53	0.15	1.30	0.15	3	1.83	0.17	2.07	**0.01***	4	1.57	0.15	1.34	0.10
Pine roots	Invasion + plantation	1	1.50	0.09	4.18	**0.01***	6	2.02	0.12	0.94	0.59	3	2.11	0.12	1.96	**0.01***	4	1.78	0.10	1.24	0.20
	Invasion	–	.	–	–	–	4	1.39	0.13	0.94	0.58	3	2.14	0.20	1.93	**0.01***	4	1.70	0.16	1.15	0.30
Pine soil	Invasion + plantation	1	1.00	0.09	3.22	**0.01***	6	3.04	0.28	1.64	**0.01***	2	0.86	0.08	1.38	0.17	4	0.88	0.08	0.71	0.85
	Invasion	–	–	–	–	–	4	2.43	0.35	2.18	**0.01***	2	0.86	0.12	1.54	0.14	4	0.88	0.13	0.79	0.75
Non-native grassland soil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1.02	0.11	2.81	**0.01***

[a] Degrees of freedom.
[b] Sum of squares.
[c] R-Squared.
[d] F-value.
[e] *P-value*.

related to age were stronger in pine roots (Fig. 5D). *Suillus*, *Rhizopogon,* and *Trichophaea* were more associated with the roots of young pines whereas *Inocybe* and *Pseudotomentella* were associated with old pines inside plantations. The genera *Amphinema*, *Amanita*, *Sistotrema,* and *Thelephora* were abundant EM fungal genera associated with intermediate pine age classes at the invasion front (Fig. 5C).

4. Discussion

Our results show that pine invasions can involve higher EM fungal richness, including fungi of differing life history strategies, than usually reported in other studies. Contrasting dispersal capacities among these fungi were evident in the composition of the EM fungal spore-bank in non-pine-invaded grasslands near and far from plantations. However, the EM fungal community of established pines did not vary consistently with distance; instead, we found that tree age was a more important factor that structured the co-invading EM fungal community. These results imply that although dispersal ability among EM fungal genera may be important for the occupancy of non-invaded sites, this factor may be overcome in a relatively short time at the invasion front, allowing fungal succession to continue. In contrast with previous studies which mostly focused on the importance of single species of EM fungi (e.g, Hayward et al., 2015b; Nuñez and Dickie, 2014; Policelli et al., 2019), our study is the first to report EM fungal succession by a more diverse EM fungal community co-invading highland grasslands.

4.1. Diversity of exotic ectomycorrhizal fungi

The diversity of co-invasive exotic EM fungi that we found in these mountain grasslands was surprisingly high for an ecosystem historically (i.e., naturally) free of EM fungi (Moeller et al., 2015) (Table S5). In the Southern Hemisphere, exotic pines mostly rely on co-introduced EM fungi to successfully establish and invade (Dickie et al., 2017; Vlk et al., 2020). However, not all EM fungi are able to widely disperse out of plantations (Hayward et al., 2015a), typically resulting in impoverished co-invading fungal assemblages (Hoeksema et al., 2020). For instance, Gundale et al. (2016) showed that *P. contorta* hosted 88 EM fungal OTUs in its native range (northwestern North America) while 25 EM fungal OTUs were found inside commercial plantations of Chile and New Zealand and only 19 EM fungal OTUs outside of them. In line with this, it has been shown that these EM fungal communities are even simpler when invading non-EM systems such as grasslands (Hayward et al., 2015b; Moeller et al., 2015). The high EM fungal diversity that we detected in central Argentina can be partly explained by the favorable conditions for growth and dispersal of EM fungi, including strong winds (Urcelay et al., 2017) and multiple animal vectors, both native and exotic, that are able to disperse the exotic EM fungi (Aguirre et al., 2021). Furthermore, the comprehensive combination of field sampling techniques (sporocarp survey, pine-root and pine-soil sampling, grassland soil sampling) and the molecular tools used (Sanger and Illumina DNA sequencing) allowed us to detect high EM fungal diversity in these pine-invaded mountain grasslands, hence caution must be taken when comparing with other studies (Table S5). Although our results do not support our hypothesis that EM fungal richness is higher inside plantations than at the invasion front, there are some insights that call for caution. Our sample-based accumulation curves do not reach a *plateau,* hence comparisons between plantation and invasion richness were made at the step phase of the curve. Moreover, many EM fungal OTUs found at the invasion front were not detected inside plantations, yet our study site did not have alternative EM hosts beside pines. Consequently, more information is needed to explore the filters that are expected to operate during pine invasions (Hoeksema et al., 2020).

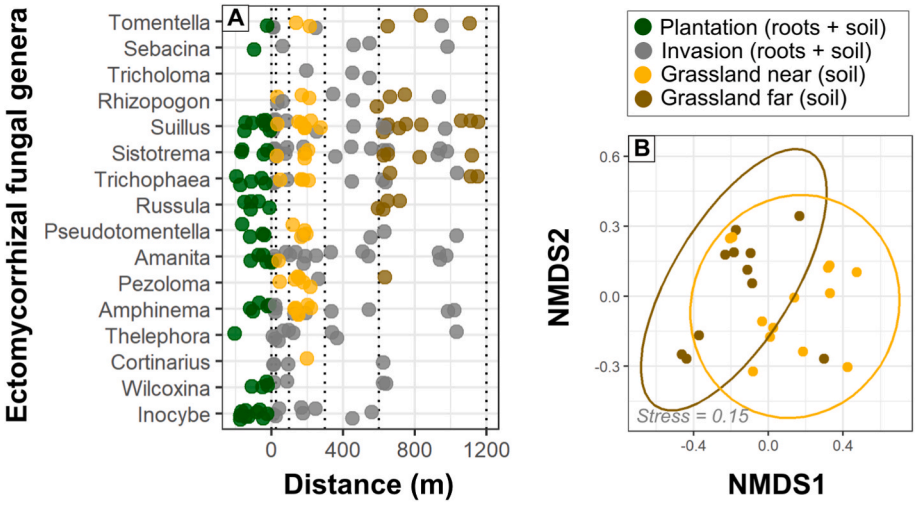

Fig. 4. (**A**) Ectomycorrhizal (EM) fungal genera found in pine roots, pine soil and grassland soil across distance from plantations. Dots indicate the presence of a particular genus at a given distance. Genera are ordinated based on pine roots and pine soil mean across distance. (**B**) Differences in the EM spore bank in grassland soil samples shown in the Non-metric Multi-Dimensional Scaling (NMDS) using relative frequency and Bray-Curtis distance. Each dot in the NMDS represents a sampling point. No differences were found in the EM fungal composition of pine roots and pine soil across distance from plantations (Table 2).

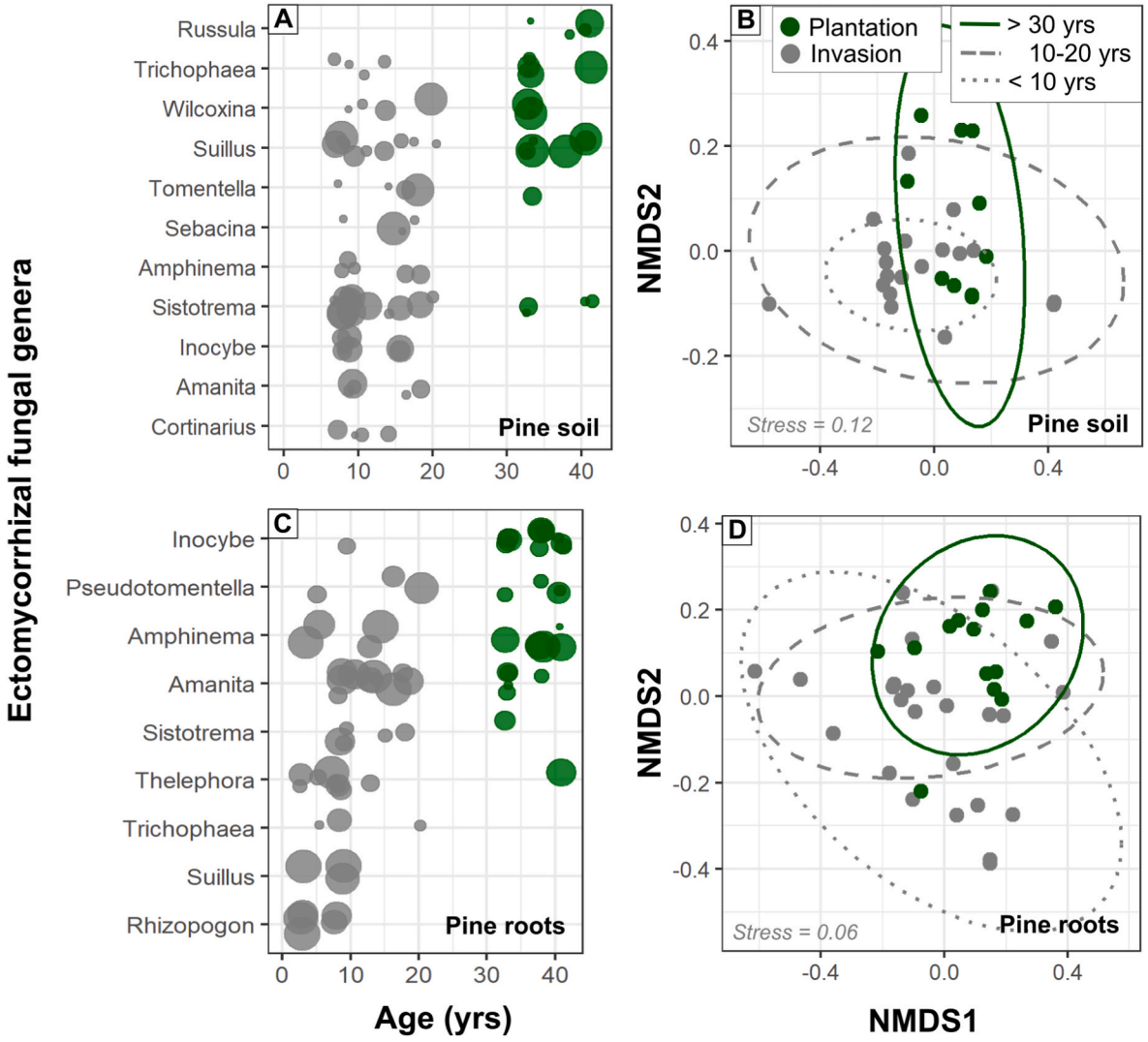

Fig. 5. Ectomycorrhizal (EM) fungal genera found in (**A**) pine soil and (**C**) pine roots across the pine age gradient. Genera are ordinated based on their means across pine age. Dot size represents the relative abundance of an EM fungal genus on a given sample. Differences in the EM fungal community shown in the Non-metric Multi-Dimensional Scaling (NMDS) for (**B**) pine soil and (**D**) pine roots using relative frequency and Bray-Curtis distance. Each dot on the NMDS represents a sampling point. There was an effect of pine age in the community composition of pine roots but a weaker effect was found for pine soil (Table 2).

4.2. Effects of distance on the composition and abundance of co-invading ectomycorrhizal fungi

The grassland soil was compositionally different near and far from plantations, supporting our hypothesis that isolation has an effect on the structure of the exotic EM fungal spore bank. Most of our knowledge on the exotic EM fungal spore bank in pine invasions has been achieved by means of bioassays, where seedlings are used as bait plants (Davis and Smaill, 2009; Nuñez et al., 2009; Wood et al., 2015). Although these experiments can give valuable information on the initial composition of the EM fungal community at the start of the invasion, most of the EM fungal species would remain undetected. This detection bias would result since the majority of the EM fungal spores do not readily germinate because they: (i) can remain dormant for considerable time, (ii) senesce before colonizing pine roots, or (iii) may not survive transport/bioassay conditions (Nara, 2009; Moeller et al., 2015). The molecular detection tools that we used in our study allowed us to detect differences between the spore bank near and far from plantations that are likely a result of different dispersal abilities and life history strategies of EM fungi (Ishida et al., 2008).

Contrary to what we observed in the spore-banks of grassland soil samples, the EM fungal community associated with invasive pines (i.e., pine roots and pine soil) was not structured by distance from the original plantations. This finding does not support our hypothesis that distance from plantations has an effect on the EM fungal richness associated with co-invasive pines, along with a dominance of good dispersers at great distances from plantations. This result also contrasts the major finding of previous studies such as Hayward et al. (2015a), that EM fungal diversity was low at further distances from plantations, where the EM fungal community was dominated by Suilloid fungal species. In other pine-invaded systems, the co-invading EM fungal communities showed compositional differences across distance gradients too, and these shifts were attributed to differential dispersal capacities across fungal species (Nuñez et al., 2009; Hynson et al., 2013). In fact, some studies in this region have already explored the dispersal limitations of EM fungi across altitudinal gradients and at the scale of several kilometers away from plantations (Urcelay et al., 2017), yet we analyzed the EM fungal community in much higher spatial detail (i.e., at the scale of the invasion front) and looked for the persistence of dispersal limitations in advanced stages of pine invasion beyond the seedling stage. However, our approach of focusing on adult pines may also have included a survival bias, since non-mycorrhizal pine seedlings that died were not included in our study design. As a consequence, we cannot confirm with certainty that pine seedlings have EM fungal inoculum available in the early stages of invasion. In fact, we found a decoupling between the EM fungal composition of the spore bank in grassland soil and the roots and soil of invading pines suggesting that dispersal limitation would be more important in non-invaded sites but less critical for advanced stages of invasion.

4.3. Changes in the ectomycorrhizal community associated with pine age

A more important factor than distance in determining the structure of the EM fungal community was the age of the invading pine trees. Changes in the EM fungal community associated with host age (i.e., EM fungal succession) have been shown to be important in the native range, yet are rarely accounted for in invasion ecology (Dickie et al., 2010; Hynson et al., 2013; Gundale et al., 2016). Commonly, the distant end of the invasion fronts is dominated by young pines, making it challenging to decouple the effects of distance and host age on the EM fungal community (Hayward et al., 2015a) (Fig. 6A). Although some studies have tried to control this through the use of seedlings across distance gradients, and hence keeping age constant (e.g., Nuñez et al., 2009; Peay et al., 2011), the EM fungal species that commonly associate with seedlings (i.e., 'early stage') represent a small fraction of the whole EM fungal community (Nara et al., 2003b). Moreover, fungal dispersal

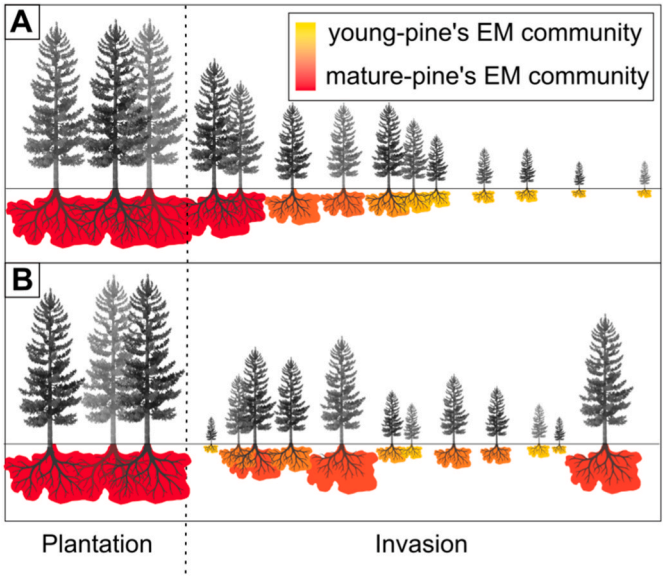

Fig. 6. Conceptualized belowground co-invading ectomycorrhizal (EM) fungal succession occurring in pine-invaded grasslands, based on Fig. 4 and Table 2 and adapted from Milani et al. (2020). (**A**) In invasion fronts where pine age and distance co-vary, EM fungi that tend to dominate on young pine trees, like *Suillus* and *Rhizopogon*, also dominates far from plantations. (**B**) When pine age and distance do not co-vary, the EM fungal community is not spatially structured and pine age becomes a more important factor. Note that we highlight that the shift from young to mature pine's EM fungal community is a continuum.

constraints are expected to be stronger for the EM fungi that tend to associate with old trees, which are misrepresented in such seedling experiments due to the aforementioned reasons (Kennedy et al., 2011). Our study system has mature pines established across the invasion front, which allowed us to detect clear changes in EM fungal community composition as pines got older at different distances from plantations.

Classic examples of EM fungi that dominate on pine seedlings include prolific dispersers like *Suillus* and *Rhizopogon* (Ashkannejhad and Horton, 2006; LeDuc et al., 2013), which were only abundant in young pines up to ten years-old in our sites but an important component of the spore-bank in non-invaded grasslands. Interestingly, *S. luteus* was the most abundant EM fungal OTU of soil samples (pine soil or grassland soil), yet it was undetected in pine roots or sporocarps, where *S. granulatus* was found in low abundance instead. This could be the result of methodological biases or fine-scale seasonal changes in the EM fungal community that should be further explored in future studies. On the other hand, *Amanita* or *Cortinarius* are typically reported in association with adult trees (Nara, 2009) and were abundant in pines older than eight years-old. The co-invading EM fungal succession pattern that we observed coincides with proposed trade-offs between competition and dispersal capacity for EM fungal species (Peay et al., 2007; Smith et al., 2018; Thoen et al., 2019).

Despite dispersal capacity, there might be some other key mechanism that can explain our fungal succession. We found that certain EM fungal genera were frequent in the spore bank and only associated with older pines, while other EM fungal genera were abundant on young pines but were undetected in the spore bank. For instance, *Tomentella* spores were abundant and dispersed over great distances, yet frequently associated with older pines. Other EM fungi typically associated with adult trees, such as *Russula* spp., were also detected in non-pine-invaded grassland soil at distances >700 m from the edge of the original plantations but only associated with the roots of old pines inside plantations. In line with this, Ning et al. (2020) showed how *Pinus elliottii* seedlings prefer to associate with *Rhizopogon* instead of *Russula* or *Tomentella* EM fungal species because the former had higher exoenzymatic activity to

acquire N and P. It is well known that tree age involves both the passage of time as well as changes in tree ontogeny and soil environment (Jokela and Martin, 2000), and experimentally decoupling these factors was beyond the scope of our study, yet further research is needed to better understand the underlying controls of EM succession.

4.4. Implications for pine-ectomycorrhizal invasion research

Invasion fronts are usually characterized by a gradual movement of pines over the landscape that result in a dominance of younger trees far from plantations and older trees close to them (Peña et al., 2008; Langdon et al., 2010). As EM fungi that commonly associate with young trees tend to be also good dispersers (Nara et al., 2003b; Peay et al., 2012), spatial factors (e.g., distance) that affect the structure of the EM fungal community result both from isolation and the age of the invading trees at a given distance (Fig. 6A and B). This is a key finding since it implies that working with invasions as natural experiments (Hoeksema et al., 2020) should not assume distance from plantations to be a proxy of EM fungal richness or community composition without previously testing for that. Moreover, we showed here that fungal dispersal at the invasion front should be considered as a spatial and temporal process, since the passing of time (i.e., age of trees) also increases the opportunity for EM fungal dispersal. This was evident in the negligible differences in EM fungal composition between adult pines located at contrasting distances from plantation. Finally, as these adult pines are able to support the production of EM sporocarps and consequently acting as an inoculum source, EM fungal communities of pine invasions with an aboveground age structure like that described here should be considered within a metapopulation model (i.e., patches of fungal inoculum source) instead of an island-mainland one (i.e., distance from plantation or a defined invasion edge).

Although our data supports the role of Suilloid fungi as an important EM fungal group in pine invasions, particularly for seedling establishment (Policelli et al., 2019), other genera also showed high invasive potential. The EM fungal genus *Sistotrema* was abundant in the spore bank of non-pine-invaded grassland soil both near and far from plantations, and it was also associated with pine seedlings and saplings (i.e., 0 to ~ 5-y-old pines) across the invasion front. In fact, *Sistotrema* had been previously reported at these invaded mountain grasslands (Urcelay et al., 2017), as well as in other pine invasions of the Southern Hemisphere (Gundale et al., 2016). Our study focused on the EM fungal community of mature pines, and further research is needed in this ecosystem to better understand the succession of the early EM fungal community on invading pine seedlings (but see Urcelay et al., 2017). Nevertheless, we provide evidence that a 'no Suilloids' scenario (e.g., commercial EM fungal inoculum for plantations without Suilloids as proposed by Hayward et al. (2015a)) would not be sufficient to avoid or even reduce pine invasions, since highly diverse EM fungal communities may still have other genera like *Sistotrema*, *Amanita*, *Amphinema*, and *Trichophaea* that could potentially replace the functional role of *Suillus* or *Rhizopogon* during co-invading EM fungal succession (Shah et al., 2016).

5. Conclusions

Here we show how an ongoing pine invasion in central Argentina can host a highly diverse co-invading EM fungal community, thus challenging the idea that EM fungal communities at the invasion front are necessarily depauperate. Although some differences in the dispersal ability of EM fungal species were seen in the spore bank of non-pine-invaded grasslands, they disappeared for invading pines established more than twenty years ago and up to 1200 m away from original plantations. With the dispersal of EM fungi assured during the life span of invading pines, EM fungal communities seem to be predominantly structured by host tree age, and biotic-driven EM fungal succession becomes more important than distance from EM fungal sources. Our results not only improve the general understanding of EM fungal dispersal and succession, but also show how important biotic filters can be overcome, allowing the rapid and widespread expansion of exotic pine populations.

Author contributions

T.M. and F.P.T. conceived the study. T.M. collected and processed the data with help from F.P.T., E.G.J., J.D.H., R.V. and A.R. T.M. wrote the first manuscript draft, while T.M., F.P.T., J.D.H., E.G.J., A.R. and R. V. participated in data interpretation and manuscript revisions.

Data Accessibility and benefit-sharing statement

Processing of soil data available on https://github.com/alejorojas2/TomasAR/blob/master/Tomas_preprocessing.md.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2022.101176.

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