Symbiosis
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Low specificity and high variability of ectomycorrhizal association in *Salix humboldtiana* along its southern latitudinal distribution

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Abstract
The preference in the ectomycorrhizal (ECM) - plant association exhibits a wide degree of variation and depends on the identity of the plant but also of the fungal species. Evaluating the degree of specificity of the plant species toward ECM symbionts is an important clue for understanding the functioning of the ECM symbiosis itself. In this work we set out to investigate the patterns of association and specificity of *Salix humboldtiana* in a wide range of distribution. To do this, we evaluated in a greenhouse experiment if this species establishes symbiosis with ECM fungi belonging to its own rhizosphere (using soil from three provenances of *S. humboldtiana* distribution in: north, center and south of Argentina) and the rhizosphere of *Alnus acuminata* and Nothofagaceae species, with which share distribution in the north and the south of Argentina, respectively. Trees of *S. humboldtiana* associate with ECM fungi belonging to its rhizosphere in the north, center and south of Argentina and from the *A. acuminata* rhizosphere, but not with those belonging to the Nothofagaceae rhizosphere. Furthermore, PERMANOVA showed that the composition of the associated ECM fungi differed between inoculum provenances. Our results suggest that *S. humboldtiana* shows low specificity on ECM symbiosis, associating with a small group of fungi that differ in abundance and composition throughout their distribution in Argentina.

Keywords Fungi · *Alnus acuminata* · Nothofagaceae · Mycorrhizal inoculum · Greenhouse

1 Introduction

The association patterns of plant and mycorrhizal fungi are influenced by numerous factors (Taylor 2008; Tedersoo et al. 2012), among them, the presence and dominance of specific host plants (Haskins and Gehring 2005; Bahram et al. 2012; Tedersoo et al. 2012, 2013, 2014; Roy et al. 2013; Urbanová et al. 2015) has been postulated as an important driver of the fungal community.

Within the diversity of ectomycorrhizal (ECM) plant lineages, a degree of preference and specificity for the associated ECM fungi is observed (Smith and Read 2008; Molina and Horton 2015). In this way, some tree genera are highly specific and associate to a reduced group or set of specific fungi throughout its distribution, as it is among *Alnus* species (Kennedy et al. 2011; Roy et al. 2013; Põlme et al. 2013). On the other hand, other tree species show low specificity, and sometimes an ample array of fungi associated throughout their distribution, as for example *Pseudotsuga sinensis* (Wen et al. 2015).

Salix humboldtiana Willd. (Salicaceae), *Alnus acuminata* Kunth (Betulaceae) and Nothofagaceae Kuprian. trees are known to be associated to ECM fungi (Becerra et al. 2005a; Becerra et al. 2005a; Becerra et al. 2005a; 2009a; 2009b; Fracchia et al. 2009; Pritsch et al. 2010; Nouhra et al. 2012a, b, 2013; Fernández et al. 2015; Wicaksono et al. 2017) in Argentina. These tree species have widespread distributions, spanning in the case of *S. humboldtiana*, from northern Mexico to the Patagonia region in the south of Argentina, occurring principally in riparian zones in the Yungas, Andean valleys, and periphery of the Amazonia towards the Andes (Gallo et al. 2021). On the other hand, *A. acuminata* is distributed from southern Mexico to north-west Argentina through Central American mountains and along the Andes mountains (Weng et al. 2004), while Nothofagaceae species are present in sub-Antarctic forests along the southern Andes in Argentina and Chile below 30º

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S latitude (Nouhra et al. 2019). In Argentina, these tree species show different distributions along the Andes mountains in the latitudinal gradient. In the northwest, *A. acuminata* occurs in the subtropical Yungas region reaching its southernmost distribution in the Catamarca province (Becerra et al. 2005c), overlapping with *S. humboldtiana* distribution (Ragonese et al. 1987) along the lower altitudinal vegetation belt of the Yungas. However, *S. humboldtiana* extends further south through the flat central Pampa and Chaco regions into northern Patagonia. In the latter area, isolated populations of *S. humboldtiana* thrive in lower sections of watersheds that are occupied upstream in the Andean highlands by Nothofagaceae species such as *Nothofagus alpina* (Poepp. & Endl.) Oerst., *N. obliqua* (Mirb.) Oerst., *N. antarctica* (G. Forst.) Oerst., *N. dombeyi* (Mirb.) Oerst. and *N. pumilio* (Poepp. & Endl.) Krasser (Moreira-Muñoz 2011), some of them reaching Cape Horn at 55 degrees south.

These woody ECM species occurring in Argentina seem to present a wide degree of specificity on the associated ECM fungi. *A. acuminata* shows relatively few but a highly specific set of ECM fungal taxa in the roots with relative no changes of composition throughout its latitudinal distribution (Becerra et al. 2005a, c; Kennedy et al. 2011, 2015; Geml et al. 2014; Nouhra et al. 2015). Furthermore, *A. acuminata* is highly dependent on fungi in order to supply the high phosphorus (P) consumption demanded by the root actinorrhizal bacteria *Frankia* for the N fixation process (Russo 1989; Benson and Clawson 2000; Becerra et al. 2009b; Kennedy et al. 2015). On the other hand, Nothofagaceae species seem to show a less specific association to ECM fungi than *A. acuminata*, and tree species in this family are considered to have a wide range of receptivity to various fungal lineages as recently shown (Nouhra et al. 2013; Fernández et al. 2015; Truong et al. 2017, 2019). Similar to *A. acuminata*, they are highly dependent on the ECM symbionts for survival which seems to be related to the type of soils of the Andes in Patagonia (Andisols) that have a high natural retention of phosphorus (a limiting nutrient for plants), and whose availability is favored by the ECM fungal growth (Diehl et al. 2003, 2008; Mazzarino and Gobbi 2005). So far it is known that *S. humboldtiana* is associated with a few ECM fungal species of which only three were identified within the genera *Inocybe*, *Tomentella* and *Tuber* in the linage puberulum (*Tuber*/puberulum) and additional five unidentified ECM morphotypes (Becerra et al. 2009a; Lugo et al. 2012; Bonito et al. 2013). Interestingly, a matching sequence of *Tuber* in the mentioned puberulum lineage was also found associated with *Nothofagus alpina* in Patagonia (UDB007212), raising questions on the shared ECM partners between these ECM hosts trees (Bonito et al. 2013; Nouhra et al. 2013). In addition, it is known that *S. humboldtiana* is associated with arbuscular mycorrhiza and dark septate fungi (Becerra et al. 2009a; Fracchia et al. 2009).

Salix species are considered in general terms to be facultative to the ECM symbiosis according to reports of some individuals that are not colonized in the North hemisphere (Meyer 1973; Cázares et al. 2005; Brundrett and Tedersoo 2020). We refer by facultative to tree species capable of associating with ECM fungi but also able to thrive without them (Meyer 1973; Molina et al. 1992; Cázares et al. 2005; Smith and Read 2008). Also, it is known that many *Salix* species establishing opportunistic and generalist association with widely distributed and generalist ECM fungi that are compatible with a large number of plants taxa (Nara and Hogetsu 2004; Nara 2006; Tedersoo et al. 2010, 2013; Ryberg et al. 2011; Arraiano-Castilho et al. 2020, 2021).

In Argentina, little is known about the *S. humboldtiana* ECM associated fungal community and the level of specificity that supports the symbiosis. This species belongs to the subgenus *Salix* which is characterized mostly by tree habit, unlike other subgenera (*Chamaetia* and *Vetrix*) that are characterized by dwarf and multi-stemmed shrubs (Newsholme 1992). *S. humboldtiana* is distributed along the tropical and subtropical regions of Mexico, Central America and South America (Lauron-Moreau et al. 2015), and usually occurs on wetlands and riparian zones (Ragonese et al. 1987; Camacho-Rico et al. 2006; Isla et al. 2010). *S. humboldtiana* relationship with the environmental variables can be quite different from those Northern hemisphere *Salix* shrubby species from boreal forests and arctic habitats, which in general do not thrive in wetlands and flooded environments (Nara 2006; Schmidt et al. 2010; Ryberg et al. 2011).

Our aims were to identify the ECM fungi associated with *S. humboldtiana* and determine the specificity and association patterns of this tree with fungi from different inoculum sources: (i) its own rhizosphere along the latitudinal gradient; (ii) the rhizosphere of neighboring *A. acuminata* populations in the north, (iii) the rhizosphere of Nothofagaceae in the south. Based on the literature, and due to the wide and sometimes overlapped distribution of *S. humboldtiana* with other ECM woody species in Argentina, as mentioned above, we hypothesized that *S. humboldtiana* will show low specificity ECM associations with a reduced set of widely distributed ECM fungal species, including some taxa belonging to *A. acuminata* rhizosphere in its northern distribution, and some belonging to Nothofagaceae rhizosphere in the south. In order to address this hypothesis, we evaluated in a greenhouse experiment if *S. humboldtiana* plant roots will indistinctly establish symbiosis with ECM fungi belonging to its own rhizosphere and the rhizosphere of *A. acuminata* and Nothofagaceae species.

2 Materials and methods

2.1 Obtaining plants

S. humboldtiana plants were obtained from stem cuttings of two adult individuals located in La Calera locality in central Argentina (31°21'20.0"S; 64°21'42.1"W) in July 2015. The stem cuttings were 30–40 cm long and 1 cm in diameter approximately. In the greenhouse, rooting hormone (gibberellic acid) was added to the base of the cuttings and then placed in pots filled with autoclaved sand on a heated table for 30 days until roots emerged. The pots were irrigated by automatic sprinklers three times per day (08:00 h., 13:00 h. and 18:00 h.). Growing conditions for cuttings were provided by a specialist of INTA (The National Agricultural Technology Institute), EEA Bariloche.

2.2 Soil selection

A. acuminata and Nothofagaceae species soil samples were collected from northern Argentina (Yungas) and southern Argentina (Patagonia) respectively, while *S. humboldtiana* soils were collected in the north, central and south of Argentina. Specifically, *A. acuminata* soil was collected from three sites, in Tucumán province localities: Lamadrid, Las Lenguas and Tafí del Valle. Nothofagaceae species (*N. dombeyii*, *N. antartica*, *N. pumilio*, *N. alpina* and *N. oblicua*) soil was collected from eight sites, three in Lanín National Park: Hua hum; Laguna Verde and Lahuencó and five in Nahuel Huapi National Park: Road to Siete Lagos, Brazo la Última Esperanza, Pampa Linda, Lago Mascardi y Cerro Tronador. Finally, *S. humboldtiana* soil samples were collected from the following locations: Lamadrid and Rio Chico in Tucumán province in northern Argentina; Piedra Parada, in Chubut province in southern Argentina and 2 sites along Río Chico de Nono in Córdoba province in central Argentina (Fig. 1).

At each site, soil samples were collected with a spade under the trees upon removal of the litter layer. Each soil sample consisted of a mixture of four or five sub-samples of approx. 2 kg randomly taken from the top 30 cm depth of soil around the site and separated by about two meters from each other. Then, soil sub-samples from each site and host plant were mixed to form the soil inoculums and stored at 4 °C for a period of two weeks until the cuttings were ready for the greenhouse experiment.

2.3 Experimental design

For the greenhouse experiment, natural soil from *A. acuminata*, Nothofagaceae species and *S. humboldtiana* from each region were maintained separated totalizing five soil inoculums (Fig. 1): (1) Soil from *S. humboldtiana* from the Yungas forests in the north (hereafter SN), (2) Soil from *S. humboldtiana* from Chaco forest in the central region (hereafter SC), (3) Soil from *S. humboldtiana* from Patagonia, in the south (hereafter SS), (4) Soil from *A. acuminata* from the Yungas, in the north (hereafter A) and, (5) Soil from Nothofagaceae species from Patagonia, in the south (hereafter N).

The greenhouse experiment involved the rooted cuttings and the five inoculum treatments described above, with 12 replicates (plants) for each inoculum and 12 replicate controls per treatment. Rooted cuttings were placed individually in plastic containers (capacity of 650 ml). The substrate was prepared by thoroughly mixing each inoculum soil and autoclaved sand (in proportions 1:1). For controls, the inoculum soil was autoclaved, stabilized for two weeks and then mixed in equal proportions with sterilized sand. Autoclaved soil for controls and sand was at 120° C and 1.5 atm during 1 h. To prevent cross-contamination between pots, a thin layer of autoclaved coarse sand was added at the top of each plastic container. The temperature in the greenhouse was between 20 and 30 °C. Plants were irrigated in the same way as we did for the cuttings, and fertilizer was not added. After seven and nine months, half of the plants per treatment (n = 6) were uprooted respectively, and shoots and roots were separated. Shoots were kept in paper bags and dried 72 h at 70 ºC to measure biomass. Roots were gently washed with water to remove adhering particles and taken to the laboratory to observe the root tips under a stereomicroscope.

2.4 Measurements and ECM morphotype identification

Shoot biomass was obtained from weighing the dry shoot material subtracting the weight of the cutting to consider only the biomass produced during the experiment.

The percentage of total ECM colonization for each seedling was calculated as the number of ECM root tips divided by the total number of root tips and multiplying by 100 (Gehring and Whitham 1994) in a uniformed sample of roots displayed on a Petri dish and covering the whole surface (63.62 cm^2). The ECM root tips were carefully extracted from the roots samples and sorted into morphotypes according to their morphological and anatomical features, by using a Wild M5A stereomicroscope at x10-40 magnification. Criteria for sorting ECM morphotypes included mantle coloration, mantle layers, branching pattern, emanating hyphae and presence of rhizomorphs and cystidia, following Agerer's methodology (Agerer 1991, 1995). The percentage of colonization by each ECM morphotype was calculated by dividing the number of root tips colonized by each morphotype by the total number of root tips colonized in the

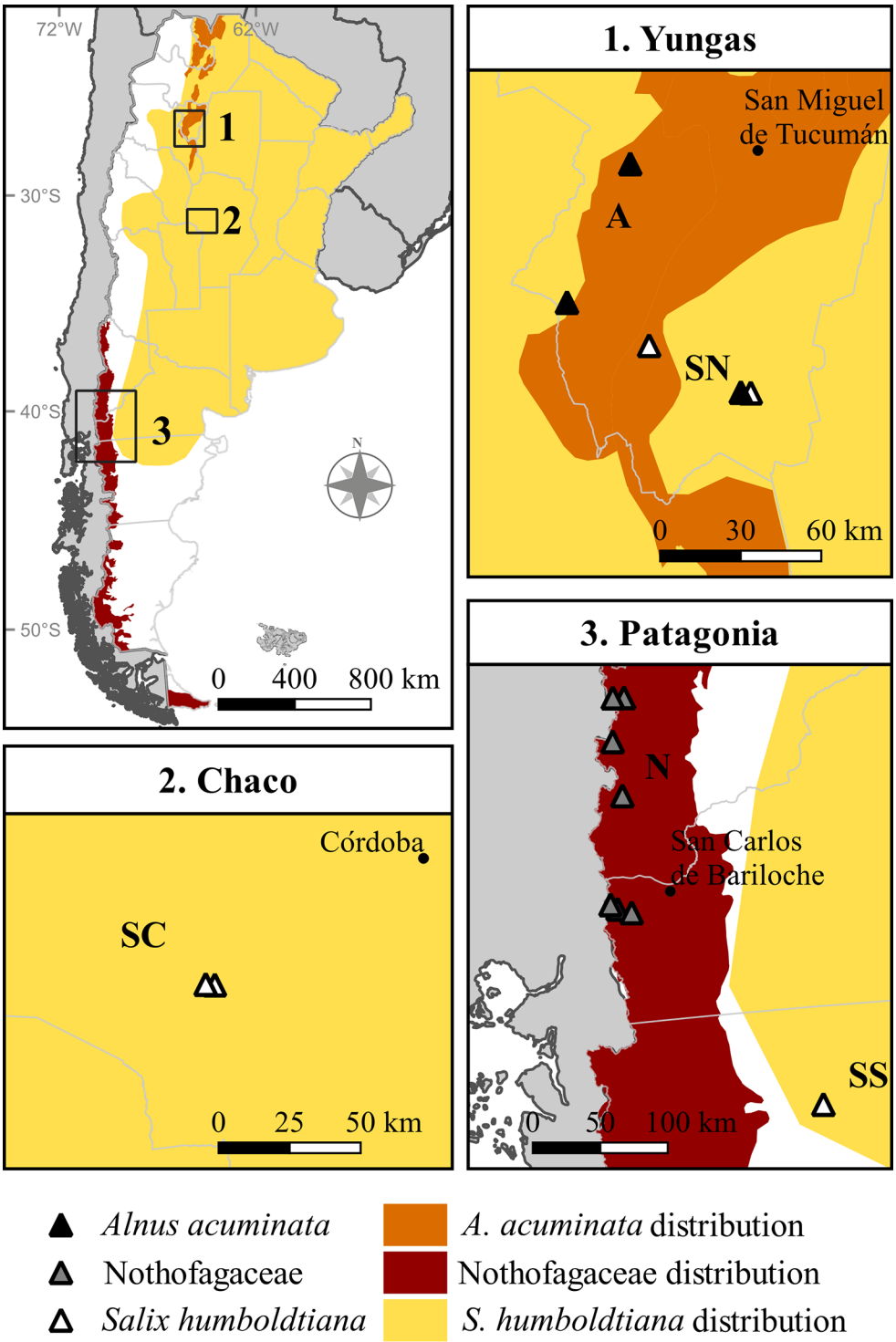

Fig. 1 Sampled sites and distribution of *Salix humboldtiana*, *Alnus acuminata* and Nothofagaceae species in Argentina. Important city of each region near the sample sites is represented with a black circle. A: soil from *Alnus acuminata*; SN: soil from *Salix humboldtiana* from the Yungas forests in the north; SC: soil from *S. humboldtiana* from Chaco forest in the central region; SS: soil from *S. humboldtiana* from Patagonia in the south; N: soil from Nothofagaceae species from Patagonia, in the south

sample and multiplying by 100 (Helm et al. 1999 modified). Morphotype richness was calculated as the total number of different ECM morphotypes encountered in each treatment.

2.5 Molecular identification of ECM root samples

ECM root tips belonging to individual morphotypes were placed into 1.5 ml Eppendorf tubes containing 500 µl 2% CTAB DNA extraction buffer (2% cetyltrimethylammonium bromide, 100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, and 20 mM EDTA) and were stored at -20 °C. Five to eight root

tips from one to five samples from each morphotype were subjected to DNA extraction by CTAB chloroform method (Rogers and Bendich 1994). Fifty (50) µL of extracted DNA was suspended in TE buffer. All the samples of extracted DNA were amplified. The full ITS rDNA repeat, including the 5.8 S region, was amplified via PCR with ITS1F and ITS4 as well as ITS1F and ITS4B primer pairs (Gardes and Bruns 1993; White et al. 1990). One to four amplified products per morphotype were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing using the BigDye™ terminator kit and run on ABI 3730XL. ITS sequence chromatograms of ECM root sequences were visually revised and manually corrected where necessary using BioEdit 7.0.5.3 (Hall 1999). The sequences generated for this study from the root tips DNA have been deposited into GenBank. ECM fungal taxon names were based upon the taxonomic level supported by online BLAST (Basic Local Alignment Selection Tool) results (Table 1). Sequences were generally assigned to species based on ITS sequence similarity threshold (≥97%) and to ECM fungal lineages according to Tedersoo et al. (2010). Identification was provided by running BLAST searches on the curated GenBank and UNITE + INSD fungal ITS sequence databases containing identified fungal sequences (Altschul et al. 1997; Nilsson et al. 2018). We also obtained from UNITE, Species Hypotheses which were assigned for the taxa delimited in clustering on different similarity thresholds (97–99%) (Kõljalg et al. 2013).

2.6 Data analysis

We fitted a one-way ANOVA to analyze the variation of shoot dry biomass in plants treated with inoculum using colonization as the factor (levels of the factor: colonized/not colonized).

We fitted linear models in order to analyze the variation of ECM fungal colonization percentage in relation to fixed factors: "Inoculum type" and "harvest time". Tukey's HSD tests were used to detect significant differences between the levels of explanatory factors with the HSD.test function from R package agricolae (de Mendiburu 2021). Variation in ECM fungal community composition, both qualitative (presence-absence) and quantitative (relative abundance) data was analyzed with permutational multivariate anova (PERMANOVA) with 999 permutations using the function adonis to test whether 'Inoculum type' affected ECM fungal community. Multilevel pairwise comparison of PERMANOVA was used to show differences between factor levels using the function pairwise.adonis from the pairwiseAdonis package (Martinez Arbizu 2017). To visualize dissimilarities (Bray-Curtis and Sorensen) in ECM composition we used non-metric multi-dimensional scaling (NMDS) from the vegan package (Oksanen et al. 2020). We reported the stress value that is an index to indicate how faithfully the high-dimensional relationships among samples are represented in a two-dimensional ordination plot. NMDS with stress values ≤ 0.1 are considered to be an acceptable representation (Clarke 1993). We assessed possible variations in multivariate dispersion (analogous to homoscedasticity) between groups with the betadisper function from the vegan package (Oksanen et al. 2020). All the graphs were performed with package ggplot2 (Wickham 2016; Wickham et al. 2021). The analyses were conducted in R (R Core Team 2021).

3 Results

3.1 Colonization

Ectomycorrhizal colonization occurred in *S. humboldtiana* individuals planted in A, SN, SC and SS, while it was not present in those planted in N. There were significant differences in total ECM colonization percentages between inocula ($P = 0.027$, $F = 3.43$; Fig. 2a). SS with a mean colonization of 38.23% was higher than SC with 23.92%, while A and SN showed no differences and were colonized by 26.63% and 26.92% respectively (Fig. 2a). There were no

Table 1 Ectomycorrhizal fungal sequences from *Salix humboldtiana* roots and additional information of the Best BLASTn identified ITS matches from GenBank and Species Hypothesis from Unite

Taxon	GenBank accession number	GenBank information		Unite information
		Best BLASTn identified ITS match		Unite SH
		Specimen	Identity (%)	
Geopora sp. 1	MN381163	*Geopora* sp. KU991189	97.42	SH1268267.09FU
Serendipita sp. [a]	MN381164	*Sebacina* sp. MH794948	99.82	SH1218835.09FU
Tomentella sp. 1	MN381166	*Tomentella* sp. MH795004	99.66	SH1177728.09FU
Tomentella sp. 2	MN381168	*Tomentella* sp. HG426008	98.72	SH1177728.09FU
Tuber sp.	MN381171	*Tuber* sp. JQ925631	99.64	SH0916819.09FU
Peziza sp.	MN381172	*Peziza* sp. HM105556	95.45	SH1067858.09FU
Geopora sp. 2	MN381173	*Geopora sp.* KP745606	98.17	SH1268216.09FU

[a] Assigned as *Serendipita* sp. instead of *Sebacina* sp. based in the Species Hypothesis name in Unite

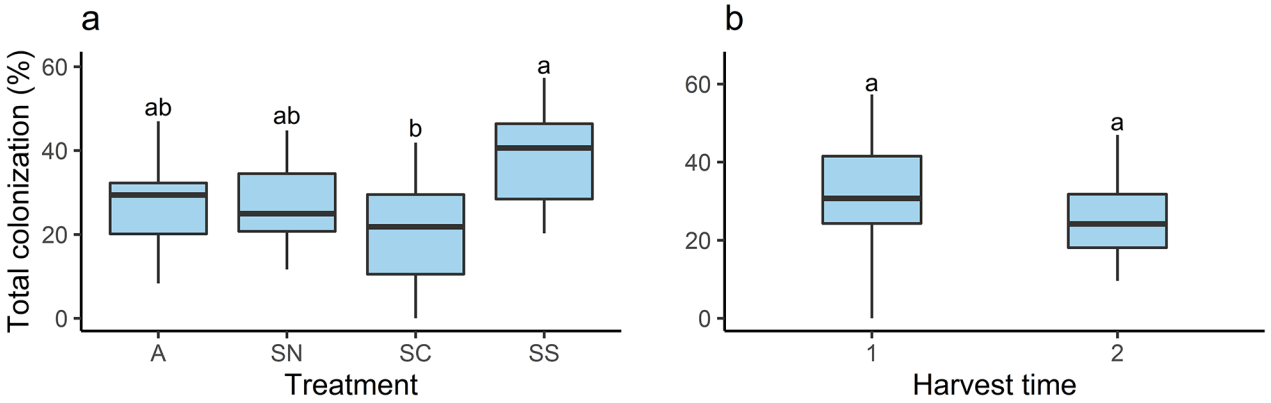

Fig. 2 Total colonization percentage of ECM fungi in *Salix humboldtiana* plants with different soil inoculum and harvest times. Abbreviations of the treatment names are the same as in Fig. 1. Boxplots represent the median, the first and the third quartile. Different letters in boxes indicate significant differences ($P < 0.05$). (a) Colonization in plants with A (n = 12), SN (n = 11), SC (n = 9) and SS (n = 11) inoculum. (b) Colonization in first (1, n = 22) and second (2, n = 21) harvest time

Table 2 Percentages of colonization of each ECM fungi taxon in seedlings of *Salix humboldtiana* treated with A, SN, SC and SS inoculum. Abbreviations of the treatment names are the same as in Fig. 1. The values are mean ± sd

ECM taxon	Inoculum soil			
	A	SN	SC	SS
Geopora sp. 1	-	21.6 ± 20.20	24.46 ± 24.96	-
Serendipita sp.	-	-	25.21 ± 23.51	-
Tomentella sp. 1	-	58.24 ± 24.08	6.97 ± 7.23	-
Tomentella sp. 2	-	3.55 ± 8.28	-	-
Tuber sp.	-	-	0.20 ± 0.61	-
Geopora sp. 2	99.21 ± 2.12	-	-	-
Peziza sp.	-	3.38 ± 7.51	41.97 ± 29.35	100.00 ± 0.00
ECM sp. 1	-	-	0.17 ± 0.52	-
ECM sp. 2	-	5.19 ± 6.71	-	-
ECM sp. 3	-	-	1.01 ± 3.03	-
ECM sp. 4	0.79 ± 2.12	8.04 ± 9.93	-	-

differences in total ECM colonization between first and second harvest time ($P = 0.055$, $F = 3.92$; Fig. 2b). All replicates of these treatments were colonized by ECM fungi except one planted in SC in the first harvest time, which was not included in the statistical analysis. As was expected, replicates from control treatments were not colonized, except for some replicates (two in autoclaved SS, four in autoclaved A and eight in autoclaved N) that were weakly colonized by *Tomentella* sp. 1 in some cases and by *Serendipita* sp. in others in values minors to 5%.

There were in total five plant replicates that died during the experiment, one of them inoculated with SN, one with SC, one with SS and two with autoclaved SS soil (control). None of the individuals planted in N and only one individual planted in SC was not colonized by ECM fungi, however, they grew well during the experiment. There were no significant differences in dry shoot biomass between colonized and not colonized plants treated with inoculum soil (ESM, Table S1).

Eleven ECM morphotypes were identified in the samples and later seven of them determined through molecular analysis in the following taxa: *Geopora* sp. 1 (MN381163), *Geopora* sp. 2 (MN381173), *Serendipita* sp. (MN381164), *Tomentella* sp. 1 (MN381165; MN381166; MN381167; MN381169), *Tomentella* sp. 2 (MN381168), *Peziza* sp. (MN381172), and a *Tuber* sp. (MN381170; MN381171) (Table 1), belonging to the lineages /geopora, /serendipita, /tomentella-thelephora and /puberulum (Tedersoo et al. 2010; Bonito et al. 2013). *Peziza* sp. sequence was not assigned to a specific lineage due it can not be assigned unambiguously to a well defined lineage based on the references (Tedersoo et al. 2010). In addition in a more recent study, Tedersoo and Smith (2013) stated: "Peziza sensu lato is still treated as a large, paraphyletic group even though it includes several well defined lineages that include sequestrate and epigeous species as well as both EcM and non-EcM taxa". Four of the determined taxa belong to Ascomycota (*Geopora* sp. 1, *Geopora* sp. 2, *Peziza* sp. and a *Tuber* sp.) and three of them belong to Basidiomycota division (*Serendipita* sp.,

Tomentella sp. 1, *Tomentella* sp. 2). It was not possible to precisely determine the other four morphotypes due to the poor quality of the sequences, and these were named as ECM sp. 1, ECM sp. 2, ECM sp. 3, and ECM sp. 4.

3.2 ECM fungi composition

S. humboldtiana individuals planted in SN and SC were colonized by six and seven taxa, respectively, while a lower number of ECM taxa were found in those planted in A and SS that were colonized only by two and one ECM taxa, respectively (Table 2). The total ECM richness found in the whole experiment was 11 species. Four ECM taxa were observed in more than one treatment. In this way, *Geopora* sp. 1 and *Tomentella* sp. 1 were present in individuals planted in SN and SC, *Peziza* sp. was present in those planted in SN, SC and SS (all the 3 inoculum soils from *S. humboldtiana* rhizosphere), and ECM sp. 4 was present in individuals planted in A and SN (Table 2).

The identity of the most abundant ECM varied throughout the inoculum treatments. In individuals planted in A, we found *Geopora* sp. 2 in 99.21% of colonized roots. Instead, in individuals planted in SN, *Tomentella* sp. 1 occupied 58.24% of the colonized roots, while *Peziza* sp. 41.97% of colonized roots in individuals planted in SC and 100% in those planted in SS (Table 2).

Many of the ECM taxa were rare in some treatments being present in low percentages (Table 2) due to being present only in a few plants (ECM sp. 4 in three individuals planted in A, *Tomentella* sp. 2 in three individuals planted in SN, *Peziza* sp. in two individuals planted in SN, ECM sp. 1 in an individual planted in SC, *Tuber* sp. in an individual planted in SC, ECM sp. 3 in an individual planted in SC).

PERMANOVA test indicated that the composition (qualitative and quantitative) of ECM fungal species in *S. humboldtiana* seedlings differed between inoculum treatments (PERMANOVA Sorensen, $P = 0.001$; PERMANOVA Bray-Curtis, $P = 0.001$). The differences in composition between inoculum treatments appeared to be attributable to both differences in multivariate location and differences in multivariate dispersion, due to a significant beta-dispersion ($P < 0.05$). Communities from A and SS had significantly lower dispersion than communities from SN and SC. The pairwise PERMANOVA contrast indicated that the composition of ECM taxa associated with all inoculum treatments differed significantly from one another ($P = 0.001$ for all the pairwise comparisons). NMDS ordination showed variation in ECM fungi composition for the inoculum treatments forming four different groups (Fig. 3a, b).

4 Discussion

In this study, we provide evidence showing that *S. humboldtiana* seedlings establish low-specificity symbiosis with a set of ectomycorrhizal fungi that varies throughout their distribution in Argentina. Specifically, the results of the greenhouse experiment indicate that the *S. humboldtiana* plants were colonized early by 11 ECM fungi from four of the five inocula tested, including those fungi from their own rhizosphere from three regions along the entire latitudinal range of distribution in Argentina, and fungi from the *A. acuminata* rhizosphere in the north (one region).

More specifically, we found two fungi taxa from the *A. acuminata* rhizosphere colonizing *S. humboldtiana* roots (*Geopora* sp. 2 almost in all the colonized roots in this treatment and ECM sp. 4 in low percentage). Species of *Salix* from the northern hemisphere showed a similar pattern of association, sharing some ECM fungi with coexisting trees. For example, in a field experiment with seedlings carried out in Mt. Fuji, Japan, it has been shown that *Salix reinii* was associated with five ECM fungi, five of which are shared with *Betula ermanii* and three with *Larix kaempferi* (Nara and Hogetsu 2004); increasing up to 15 and 13, respectively, in naturally established woods where *S. reinii* is associated to a set of 21 taxa fungi in total (Nara 2006). Besides, Obase

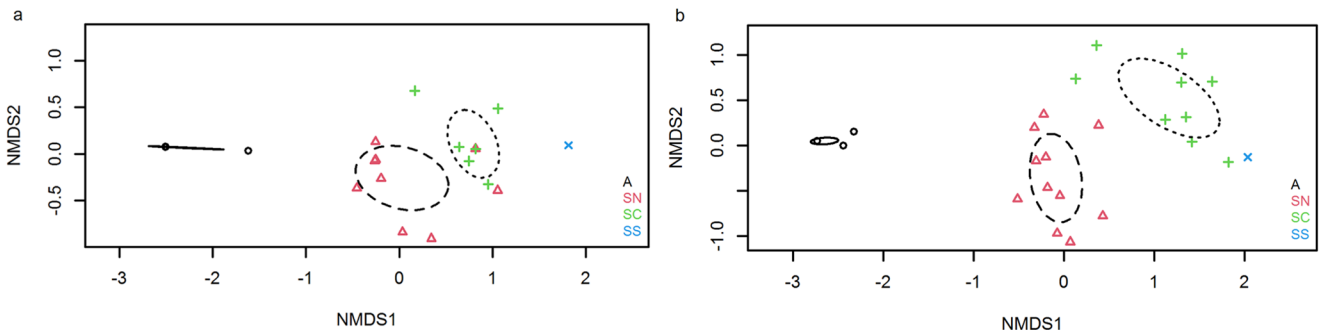

Fig. 3 Non-metric multi-dimensional scaling (NMDS) plots of variation in ECM fungi composition in plants of *Salix humboldtiana* planted in A, SN, SC and SS. Abbreviations of the treatment names are the same as in Fig. 1. (a) Qualitative (presence–absence based) NMDS; $P = 0.001$; Stress: 0.022 and (b) quantitative (relative abundance based) NMDS; $P = 0.001$; Stress: 0.029

et al. (2007) carried out a field study in Mt. Usu, Japan and found that *S. integra*, *S. udensis* and *S. caprea* share various ECM fungal species with neighboring *Populus suaveolens* f. suaveolens, *B. pendula* subsp. mandshurica and *Quercus mongolica* var. crispula. On the other hand, it was found that *S. reticulata* and *Dryas octopetala* in Sweden, shared 18 out 59 ECM fungi species (Ryberg et al. 2009), meanwhile *S. fragilis* and *A. glutinosa* shared eight out 25 in New Zealand (Bogar et al. 2015). Similarly, it has been observed that different *Salix* species can share ECM fungi symbionts when growing side by side, suggesting that is the abiotic niche of fungi that determines the fungal species available to associate with host plants within an habitat (Erlandson et al. 2016; Arraiano-Castilho et al. 2020).

The results partially support our hypothesis as no ECM colonization occurred in the individuals planted in soil from Nothofagaceae species. Bogar et al. (2015) found similar results in a study with *S. fragilis* and indigenous fungal symbionts of Nothofagaceae in New Zealand. In general, Nothofagaceae species do not show high specificity and are considered to have a wide range of receptivity to various ECM fungal lineages (Nouhra et al. 2013; Fernández et al. 2015; Tedersoo et al. 2008a, 2009a; Truong et al. 2017, 2019). Therefore, we would expect that a number of the Nothofaceae associated ECM fungi to be compatible with *S. humboldtiana*. In contrast, our results shown that *S. humboldtiana* is not able to associate with ECM fungi from the Nothofagaceae rhizosphere under the provided greenhouse conditions, suggesting that *S. humboldtiana* would not share ECM symbionts with Nothofagaceae species. This could be due to diverse historic biogeographic patterns in *Salix* and Nothofagaceae, e.g. the latter are associated with various ECM fungal lineages that are mostly of Gondwanic origin (Nouhra et al. 2013, 2019; Truong et al. 2017), while those associated with *S. humboldtiana* and *A. acuminata*, appear to have migrated from the northern hemisphere (Kennedy et al. 2011; Lauron-Moreau et al. 2015; Nouhra et al. 2019). Moreover, *S. humboldtiana* and Nothofagaceae take place in extremely different habitats, *S. humboldtiana* inhabits subtropical floodable riparian zones and in general, Nothofaceae is found in dense temperate forests on cool and drained mountain slopes (Hauenstein et al. 2005). We consider that these profoundly different environmental conditions did not favor the sharing of ECM fungal species in our experiment. However, as previously mentioned, data on Tuberaceae biogeography, highlights the existence of sequences in the /puberulum lineage obtained from ectomycorrhizas sampled in natural stands of *N. alpina* from Patagonia and *S. humboldtiana* from central Argentina (Bonito et al. 2013; Nouhra et al. 2013). Previous data indicate that species in this fungi group are well adapted for long-distance dispersal, however, the origin and or migration pattern of this mycobiont of *S. humboldtiana* and *N. alpina* is not known (Bonito et al. 2013; Nouhra et al. 2013). In any case, it is clear that additional studies would be necessary to clarify these perspectives in our research, in particular focusing on knowing both the taxonomic diversity and the qualities of the ECM inocula used.

By and large, ECM associations are a prerequisite for many tree species to grow and survive (Smith and Read 2008). However, our results suggest that *S. humboldtiana* is a host tree with low dependence on ECM fungi to survive, at least in the provided conditions during the experiment, considering that some of the inoculated plants (planted in N and a replica planted in SC) showed no colonization but they grew just like the colonized (ESM, Table S1).

We are aware that morphotyping followed by DNA sequencing of a limited number of samples could underestimate the diversity of ECM fungi in a system (García-Guzmán et al. 2017). However previous studies in the field also reported low diversity (Becerra et al. 2009a) and incidence of ECM on root samples of *S. humboldtiana* subjected by seasonal flooding in central Argentina, or no colonization at all (Fracchia et al. 2009; Lugo et al. 2012). This is in agreement with previous studies that reported no ECM colonization on various species of *Salix* in the field (Meyer 1973; Cázares et al. 2005; Brundrett and Tedersoo 2020). This attribute may be advantageous for *S. humboldtiana* occurring in riparian flooded soils, where the ECM inoculum may be scarce and the percentage of gravimetric water in the soil is high (Lugo et al. 2012).

In soil treatments from central Argentina we found ECM taxa that coincide with some described (*Tuber* sp. and *Tomentella* sp.) by Bonito et al. (2013) and Becerra et al. (2009a) in root samples obtained in the field in the same region. On the contrary, it was not so for *Inocybe* sp., which were registered by Becerra et al. (2009a). *Tomentella*, *Geopora*, *Peziza* and *Tuber* are ECM taxa commonly found associated to *Salix* species around the world (Püttsepp et al. 2004; Parádi and Baar 2006; Ryberg et al. 2009; Ishida et al. 2009; Hrynkiewicz et al. 2009, 2012, 2015; Erlandson et al. 2016; Arraiano-Castilho et al. 2020). The results of our experiment are consistent with that, having registered high colonization percentages of *Tomentella*, *Geopora* and *Peziza* in one or more of the treatments. These taxa are globally distributed and associated with many species of *Pinus*, *Quercus*, *Picea* and *Betula* (Smith et al. 2007; Tedersoo et al. 2008b; Leonardi et al. 2013; Taniguchi et al. 2021). On the other hand, *Tuber* sp. was rare and only present in one individual planted in SC, however, taxa from the /puberulum clade would be adapted to dispersal over long distances compared to other *Tuber* clades, which explains why it is the only group present in our region (Bonito et al. 2013). Unfortunately, we have not found additional data on ECM

fungi associated with *S. humboldtiana* from other South American regions.

Based on our results we could say that *S. humboldtiana* also associates with widely distributed and generalist ECM fungi. Furthermore, some of the ITS sequences obtained in our study have a high percentage of identity match (greater than 97%) with sequences from ECM fungi from other *Salix* and *Quercus* tree species from distant regions of the world (data from GenBank database). This suggests that *S. humboldtiana* might associate with unspecific ECM fungi, which is consistent with other Salicaceae species associated with ECM fungi compatible with multiple hosts, over a large geographic scale (Tedersoo et al. 2013).

Interestingly we found high colonization rates of *Geopora* sp. 2 which was the dominant species from the *A. acuminata* inoculum capable of colonizing *S. humboldtiana* under greenhouse conditions, and at very low rates the presence of ECM sp. 4. Consequently, several OTUs corresponding with *Geopora* spp. were recorded in the *A. acuminata* rhizosphere in the Yungas (Pastor N. PhD thesis unpublished). The absence of other ECM fungi from the *Alnus* soil inoculum on the *Salix* roots suggests that these host trees would share few ECM symbionts. This observation is consistent with the pattern of reciprocal specificity between *Alnus* and its associated set of ECM fungi (Tedersoo et al. 2009b; Kennedy et al. 2011, 2015; Nouhra et al. 2019). The presence of *Geopora* sp. 2 and ECM sp. 4 in *S. humboldtiana* plants inoculated with soil from the rhizosphere of *A. acuminata* suggest that *S. humboldtiana* is capable of associating with very few ECM fungi that would not be exclusive to *A. acuminata*.

Last, the identity of the most abundant species was different among the inoculum sources. In other words, *S. humboldtiana* appears to be associated with a reduced pool of ECM fungi that differ in their composition depending on the origin of the inoculum along its latitudinal distribution in Argentina. These results indicate that it is most likely a tree species of low specificity associated with a non-conserved pool of fungi.

Although the results of this work are preliminary, they are relevant since they address aspects of the specificity of *S. humboldtiana* ECM associations not previously studied. To our understanding, experimental studies of this type are necessary to understand the association patterns of ectomycorrhizal plants. Nevertheless, we consider that these results from a greenhouse experiment should be cautiously extrapolated to natural habitats. To fully understand the specificity patterns of *S. humboldtiana* ECM association, we desire to complement these results with further field research, such as studying ectomycorrhizae from roots samples from trees in natural populations along the analyzed gradient.

5 Conclusions

This study provides novel information about the diversity and community composition of ectomycorrhizal root symbionts in *S. humboldtiana* in Argentina.

Our results suggest that *S. humboldtiana* seedlings could have low specificity in their ECM associations, establishing symbiotic relationships with a small pool of fungi that differ in abundance and composition according to different soils from sites sampling along its latitudinal distribution in Argentina. We showed that at greenhouse experiment conditions, *S. humboldtiana* seedlings associate with ECM fungi belonging to its own native soils from the northern, central and southern sections of Argentina and from the *A. acuminata* soils from northern Argentina where their distributions overlap. On the other hand, *S. humboldtiana* did not associate with ECM fungi belonging to the Nothofagaceae soils in our experiment, although additional studies would be necessary to reveal if *Salix humboldtiana* and Nothofagaceae share other symbionts in the field.

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Code Availability The code used in the current study is available from the corresponding author on reasonable request.

Statements and Declarations

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