Mycoscience (2009) 50:343–352
DOI 10.1007/s10267-009-0490-4

FULL PAPER

Alejandra G. Becerra · Eduardo R. Nouhra
Mariana P. Silva · Donaraye McKay

Ectomycorrhizae, arbuscular mycorrhizae, and dark-septate fungi on *Salix humboldtiana* in two riparian populations from central Argentina

Received: September 5, 2007 / Accepted: February 10, 2009

Abstract Colonization of *Salix humboldtiana* (Salicaceae) by ectomycorrhizae (ECM), arbuscular mycorrhizae (AM), and dark-septate endophytic (DSE) fungi was studied throughout autumn on two riparian populations in central Argentina. AM and DSE infection on roots ranged from 0% to 17% and from 2% to 20% respectively, whereas ECM colonization was higher, varying between 33% and 99% for both sites. Seven ECM morphotypes were found on *S. humboldtiana* roots. The nuclear rDNA internal transcriber spacer (ITS) region from the ectomycorrhizal root tips was amplified using ITS-1F and ITS-4 primers. Two of the seven ECM types were identified by searching GenBank blasts: one attributed to the genus *Tomentella* (Thelephoraceae) and the second most closely matched to *Inocybe* sp. (Cortinariaceae). The ECM colonization varied among sampling dates and sites, whereas AM and DSE colonization varied only among sampling dates. Diversity values for the ECM morphotype were not significantly different for autumn months or among the two sites. Positive correlations were found between *Inocybe* sp. and sites and between *Inocybe* sp., *Tomentella* sp., morphotypes III, IV, and VI, and sampling dates. This article provides the first documented evidence of co-occurrence of ECM, AM, and DSE in *S. humboldtiana*.

Key words ITS sequence analysis · Morphological characterization · Mycorrhizae · *Salix*

A.G. Becerra (✉) · E.R. Nouhra
Instituto Multidisciplinario de Biología Vegetal (CONICET–Universidad Nacional de Córdoba), CC 495, X5000JJC Córdoba, Argentina
Tel. +54-351-433-1056; Fax +54-351-433-1056
e-mail: beceale@gmail.com

M.P. Silva
FAUBA, Estación de Biología Sierras, Huerta Grande, Córdoba, Argentina

D. McKay
USDA, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, Corvallis, OR, USA

Introduction

Mycorrhizae are mutualistic associations between fungi and plant roots. The two most widespread types of mycorrhizal associations are ectomycorrhizae (ECM) and arbuscular mycorrhizae (AM). Some species of *Salix* (Lodge 1989), *Populus* (Lodge 1989), *Eucalyptus* (Lapeyrie and Chilvers 1985; Jones et al. 1998), and *Alnus* (Molina et al. 1994; Becerra et al. 2005a) form both ECM and AM. Field observations have shown that dominance of either type of mycorrhizae could occur in *Salix* and *Populus* species (Lodge 1989).

In addition to ECM and AM fungi, roots of many plant species are often colonized by fungi collectively called dark-septate endophytes (DSE). DSE are characterized by melanized, thick-walled, septate hyphae (Jumpponen and Trappe 1998; Jumpponen 2001; Yu et al. 2001; Cázares et al. 2005). The function and taxonomic affinities of these fungi are poorly understood, but they are ascomycetes (Mandyam and Jumpponen 2005).

Different species of *Salix* are grown all over the world for fodder, fuel, construction material, and environmental preservation because of their wide adaptability. They can grow in poor and exposed soils (Roberston 1984). In Argentina, *Salix humboldtiana* Willd. (Sauce criollo in Spanish) is the only native species of *Salix*. Their populations are distributed along streams, rivers, and lakeshores, and it occurs in habitats in which soils are heavily influenced by water-level fluctuations (Ragonese 1987). This tree is harvested for firewood, pulp, and timber (Dimitri et al. 1998).

Previous studies have focused on the occurrence of ectomycorrhizae, arbuscular mycorrhizae, and dark-septate endophytes of *Salix* species from North America, Europe, and Australia (Antibus et al. 1980; Marshall and Pattullo 1981; Harley and Harley 1987; Lodge 1989; Read 1989; Cázares 1992; Khan 1993; Dhillion 1994; Graf and Brunner 1996; van der Heijden et al. 1999; van der Heijden and Vosatka 1999; Jumpponen et al. 1999; Sasaki et al. 2001; van der Heijden 2001; Baum et al. 2002; Trowbridge and Jumpponen 2004). In addition, *Salix* spp. roots were most

commonly colonized by ECM fungi and less by AM fungi (Helm et al. 1996; van der Heijden and Vosatka 1999; Trowbridge and Jumpponen 2004). In contrast, Read and Haselwandter (1981) and Graf and Brunner (1996) found only ECM colonization in *S. herbaceae* L.

Considering the lack of studies on the mycorrhizal status of *Salix humboldtiana* in Argentina, the aims of this study were (a) to determine the type and abundance of mycorrhizal symbioses present in *S. humboldtiana* on two different riparian populations in central Argentina during the autumn season; (b) to determine the fungal sporocarps, diversity, and morphology of the ECM; and (c) to identify the ECM-associated taxa throughout the GenBank blast searches of mycorrhizal fungal DNA sequences.

Materials and methods

Sampling sites

The field sites are located in Córdoba Province (central Argentina): (1) Hayke, Punilla Department, elevation 839 m, 31°29′54.1″ S, 64°35′40″ W, average precipitation 720 mm, soil classified as Lythic Udorthent; and (2) La Calera, Colón Department, 492 m elevation, 31°21′10.3″ S, 64°21′26.6″ W, average precipitation 400–500 mm, soil classified as Paralitic Ustorthent. Soil physicochemical characteristics are presented in Table 1. Mean annual temperatures range from 8° to 24°C for both locations. The plant community is dominated by trees such as *Fagara coco* (Gill.) Engler (Rutaceae), *Acacia caven* (Molina) Molina, *Prosopis alba* Griseb., and *Geoffroea decorticans* (H. & Arn.) Burk. (Fabaceae), and shrubs such as *Celtis chichape* (Wedd.) Miquel (Celtidaceae), *Schinus praecox* (Griseb.) Speg. (Anacardiaceae), and *Colletia spinosissima* Gmel. (Rhamnaceae) (Luti et al. 1979).

Salix humboldtiana populations (height, 6–15 m; age, 20–30 years) in the Hayke site are located along the riverbanks of San Pablo Creek and in the La Calera site are located along the Suquía River. In both sites, the areas remained water saturated during the rainy seasons (summer–autumn), the root systems of trees being partially submerged during this time.

Sampling of fine roots, soil, and sporocarps

Five square plots (10 m × 10 m) were established randomly within a homogeneous area (100 × 50 m) in each site during autumn, sampling monthly from April to June of 2003. A mature tree (i.e., an individual producing female and male flowers) with a trunk diameter of 65–100 cm was sampled inside each plot, and soil samples of 15 × 15 cm and 25 cm depth were excavated at 15 cm to 1 m from the trunk of each tree. The majority of *S. humboldtiana* roots occurred in the top 20 cm of the soil at both sites. Two soil samples per each individual tree were taken each sampling time. The samples were placed in plastic bags and stored at 4°C during transport to the laboratory. Sporocarps occurring in each site during autumn were sampled and identified using various identification manuals (Wright et al. 1973; Largent 1986; Largent et al. 1986; Moreno et al. 1986; Singer 1986; Wright and Albertó 2002).

Analysis of ectomycorrhizal root samples

Salix humboldtiana roots (the only ectomycorrhizal tree in the area) were easily distinguished by their morphological appearance, although herbaceous plant roots were often present in the samples. Every root sample was checked for ECM roots. The ECM were carefully extracted and sorted into morphotypes according to their morphological features. Criteria for sorting included color, mantle layers,

Table 1. Soil properties of the two sites Hayke (Punilla Department) and La Calera (Colón Department) as analyzed from soil profiles taken during fieldwork

Parameter[a]	Hayke	La Calera
Soil type	Lythic Udorthent	Paralitic Ustorthent
pH 1:2.5	7.36 ± 0.25	7.43 ± 0.25
Electrical conductivity (dS m^{-1})	0.23 ± 0.05	0.50 ± 0.09
Available phosphorus (mg kg^{-1})	4.04 ± 0.86	14.89 ± 3.02
Total nitrogen (%)	0.12 ± 0.04	0.20 ± 0.06
Organic matter (%)	2.16 ± 0.65	3.8 ± 1.14
Cation-exchange capacity (cmol kg^{-1})	11.56 ± 1.31	17 ± 1.93
Ca^{+} (cmol kg^{-1})	7.56 ± 2.09	11.23 ± 3.10
Mg^{+} (cmol kg^{-1})	2.05 ± 0.95	2.35 ± 1.09
Na^{+} (cmol kg^{-1})	0.54 ± 0.46	0.34 ± 0.29
K^{+} (cmol kg^{-1})	0.89 ± 0.59	1.12 ± 0.74
Cu (ppm)	0.55 ± 0.12	0.89 ± 0.20
Fe (ppm)	40.89 ± 11.87	20.13 ± 5.85
Zn (ppm)	0.44 ± 0.09	0.65 ± 0.14
Mn (ppm)	4.42 ± 2.09	1.62 ± 0.77
Texture	Sandy loam	Loam sandy

Data are mean ± standard error values of 15 soil samples

[a]The analyses were Bray and Kurtz I method for available phosphorus; micro-Kjeldhal method for total N; Nelson and Sommers (1982) for organic matter; electric conductivity and pH in 1:2.5 suspension of soil in water; cations after equilibrium of soil in 0.1 N NH_4Cl

branching pattern, emanating hyphae, characteristics of rhizomorphs, and cystidia, following Agerer's (1991, 1999) methodology. Roots were analyzed under a Zeiss stereo microscope at 10–40× magnification and photographed with a Zeiss Axiophot light microscope at 200–1000×. Presence of Hartig net was confirmed in all morphotypes.

The percentage of root tips colonized by ECM fungi was determined as described by Gehring and Whitham (1994). ECM roots were distinguished from nonmycorrhizal roots by the occurrence of a fungal mantle or Hartig net or both. The roots in each sample were divided into three subsamples because of the large number of root tips (200–400). The subsamples were randomly distributed on a plastic plate grid of 9 × 6 compartments each measuring 2.5 × 2.5 cm. The percentage of ECM colonization was calculated as the number of ECM root tips divided by the total number of root tips (Gehring and Whitham 1994). The percentage of colonization for each ECM morphotype was calculated for each sampled tree by dividing the number of root tips of each ECM type by the total number of root tips and multiplying by 100 (Helm et al. 1999).

Diversity of mycorrhizal morphotypes was calculated by the reciprocal of Simpson's dominance index (SR) (Simpson 1949) using the mean relative percentage of each morphotype associated with each tree. Relative colonization of morphotype t on a root system was calculated by dividing the percentage of morphotype t by the total percentage:

$$\mathrm{SR} = \left(\sum_{t=1}^{m} p_t^2 \right)^{-1}$$

where p_t is the relative colonization of ECM morphotype t and m is the number of ECM morphotypes. Simpson's diversity index tends to be less sensitive to sample size and minor species compared with other diversity indexes (Helm et al. 1996).

Molecular identification of ECM

Three to five mycorrhizal tips of each of the seven morphotypes from both sites were prepared for DNA extraction (Gardes and Bruns 1993). When available, two replicates from each morphotype were extracted. The internal transcribed spacer (ITS) region for the nuclear rDNA repeat was amplified with the polymerase chain reaction (PCR) using ITS-1F/ITS-4 primers (White et al. 1990; Gardes and Bruns 1993). This primer pair preferentially amplifies the fungal ITS region. We used the reagents, protocols, and cycling parameters described previously by Gardes and Bruns (1996). DNA products were purified with a QIAquick Purification Kit, following the manufacturer's protocol, and then sequenced with the primers ITS-1F and ITS-4 on an ABI Model 373A (Perkin-Elmer) automated DNA sequencer at the Center of Gene Research and Biotechnology at Oregon State University. DNA sequencing Analysis (version 2.01) and Sequence Navigator software were used to process the raw data. The resulting edited sequences were queried in GenBank databases at the National Center for Biotechnology Information (NCBI), using the BLAST algorithm (Altschul et al. 1997). ECM taxon names were based upon the taxonomic level supported from the BLAST results.

Analysis of arbuscular mycorrhizae and dark-septate endophytes

Nonectomycorrhizal roots were randomly sampled for AM and DSE observation and stored in 50% alcohol at 4°C until processed. Samples were cleared in 5 ml 20% KOH solution. The beakers were maintained at room temperature for 24 h. After clearing, the roots were washed and acidified in 5 ml 2% HCl for 4 min. Roots were then stained in 5 ml 5% aniline blue at room temperature for 24 h (Grace and Stribley 1991). After staining, the roots were stored in 50% glycerin. AM and DSE colonization was observed and quantified with stereo- and compound microscopy. Multiple root samples (between 15 and 20 roots, 1 cm long) from each plant were mounted on slides and viewed under a compound microscope at 40× (McGonigle et al. 1990). The presence of AM or DSE fungal structures was scored for 100 intersections of root and reticle line per plant. An intersection was considered colonized if the reticle intersected vesicles, arbuscules, nonseptate hyphae, DSE-melanized septate hyphae, and microsclerotia. The colonization percentages are expressed as colonized intersects/total number of intersects × 100.

Voucher mycorrhizae of all types were deposited in the "Museo Botánico de Córdoba" Herbarium (CORD) (Holmgren et al. 1990).

Statistical analysis

Analysis of variance (ANOVA) using the *Infostat* statistical package (Di Rienzo et al. 2002) was used to examine the relationships between the response variables (ECM colonization, AM colonization, and DSE fungi colonization) and sites and sampling dates. Before analysis, ECM colonization and DSE colonization data expressed as percentages were transformed to rank and analyzed statistically by ANOVA, the equivalent to the nonparametric analyses (Zar 1999). AM colonization was arcsine square root transformed. All differences among means were evaluated by the Tukey test.

Kruskall–Wallis ANOVA test for ranks and χ^2 median tests were used to test for the ectomycorrhizal diversity and for the differences in the percentage of each morphotype as influenced by sites and sampling dates, as most data did not follow the assumptions of ANOVA even after various transformations.

Results

Both soils present a neutral soil pH but differed in texture and in nutrient content (Table 1). Soils from La Calera site

(Colón Department) had higher contents of organic matter and total N, a higher electrical conductivity and higher levels in P than soils from the Hayke site (Punilla Department).

Ectomycorrhizae

Seven ECM morphotypes were found to be associated with *Salix humboldtiana*. All of them appeared mostly turgid and active, with thin mantle layers and without rhizomorphs. In a few cases, root tips appeared senescent and mycorrhizae were dark colored, probably indicating their inactive stage. A brief description of their most prominent morphological and anatomical features is given in Table 2.

Molecular identification

Morphotypes I and II matched ECM taxa when blasted to the respective resulting consensus sequence in the NCBI database (Table 3). The fungal symbionts in both matches are basidiomycetes, belonging to the genus *Tomentella* in the Thelephoraceae and *Inocybe* sp. within the Cortinariaceae. Amplification of DNA from the rest of the morphotypes failed. In those cases, PCR protocols were modified to optimize amplification (increase of the amount of purified DNA from 0.5 up to 3 µl and lowering annealing temperature to 50°C). Despite variations on the technique, no suitable amplification was obtained.

Ectomycorrhizal colonization and sporocarps

The ECM colonization of *S. humboldtiana* differed between the two sites ($P < 0.001$) and sampling dates ($P < 0.001$) (Table 4). There was no interaction site × sampling date effect ($P = 0.66$). ECM colonization was 71.13% [standard error (SE) = 24.7] at the Hayke site and 86.31% (SE = 17.2) at the La Calera site, varying from 33% to 99% at both sites. The ECM colonization significantly differed among sampling dates (from April to June), being higher in June and May than April (Table 4).

The morphotypes I–*Inocybe* sp., II–*Tomentella* sp., III, IV, and VI presented a different degree of colonization between sampling dates (Table 5). The morphotype I–*Inocybe* sp., morphotype II–*Tomentella* sp., and morphotype IV were three of the ECM morphotypes regularly occurring in almost all plots. One of the seven morphotypes found on *S. humboldtiana* roots showed significant differences in the percentage of occurrence as related to site. The ECM morphotype I–*Inocybe* sp. was more common in La Calera site (Table 5).

Diversity (Simpson's diversity index) was not significantly different at sampling dates (from April to June) ($P = 0.176$) and the two sites (for April, $P = 0.4974$; for May, $P = 0.07$; for June $P = 0.378$).

No ECM sporocarps were found during the sampling period, although saprophytic species were observed under *S. humboldtiana* only in Hayke site: *Mycena* sp., *Clavulina* sp., *Agrocybe cylindrica*, *Hygrocybe lepida*, and the polipores *Funallia troggi*, *Gloeophyllum striatum*, and *Phellinus* sp.

Arbuscular mycorrhizae colonization

Salix humboldtiana root samples from both sites were colonized by AM, some roots previously selected as nonectomycorrhizal, showed an incomplete thin mantle when they were observed under the microscope. AM fungal colonization was characterized by external branched nonseptate hyphae of 4–9 µm diameter and intracellular branched hyphae of 2–6 µm diameter, some hyphae forming coils (Fig. 1H). Intracellular elliptical vesicles, 25–55 µm in diameter with smooth walls and a subtending hyphae of 2–8 µm diameter, were observed. AM colonization of *S. humboldtiana* differed between sampling dates ($P < 0.001$) (Table 4), showing a significant interaction effect between sites and sampling dates ($P < 0.01$). AM colonization at the Hayke site was 4.05% (SE = 4.49), and 3.12% (SE = 2.98) at the La Calera site; the colonization ranged from 0% to 17% for both sites. AM colonization significantly differed among sampling dates, being higher in April at Hayke and in May at La Calera site (see Table 4).

Dark-septate endophyte colonization

DSE fungi were observed in almost all roots colonized by AM fungi. DSE colonization was externally characterized by light brown to black, loosely arranged hyphae, forming an incomplete mantle-like structure composed of intercellular parallel light brown hyphae, 2–3 µm in diameter, and globose to epidermoid light brown cells, 4–16 µm long and 4–9 µm in diameter. The roots also contained intracellular hyphae 2–3 µm in diameter along the periphery within the epidermal cells. The melanized hyphae formed a mass of densely packed, dark hyphae filling epidermal and exodermis cells. These structures were typical of what has been described as mature microsclerotia (Fig. 1I). DSE colonization differed between sampling dates ($P < 0.001$) (Table 4), and there was interaction site × sampling date effect ($P < 0.001$). DSE colonization at the Hayke site was 9.08% (SE = 5.98), and 7.21 % (SE = 5.00) at the La Calera site, ranging between 2% and 20%. DSE colonization significantly differed among sampling dates, being higher in May for both sites (Table 4).

Discussion

This is the first study describing the mycorrhizal status in *Salix humboldtiana* populations of central Argentina. The roots of *S. humboldtiana* were found to be colonized by ECM, AM, and DSE. As stated by Walker and McNabb (1984) and Dhillion (1994), the presence of different mycorrhizal types is the norm within roots of *Salix* and *Populus* species.

Table 2. Brief description of morphological and anatomical characters of seven morphotypes of *Salix humboldtiana*

Name	Mantle color, type, and thickness[a]	Root morphology	Emanating elements (hyphal diameter)	Hartig net[b]	Differentiating features
Inocybe sp. (Fig. 1A), AB 14 (CORD)	Yellow-brown to dark brown mantle, plect. (35 µm)	Simple ramification to irregular pinnate, straight to tortuous tips	Rhizomorphs lacking, hyaline clamped emanating hyphae, smooth surface (2–5 µm)	Paraep.	Emanating hyphae clamped
Tomentella sp. (Fig. 1B), AB 15 (CORD)	Yellowish- to dark brown mantle, plect. (31 µm)	Simple ramification to monopodial pinnate, straight to tortuous tips	Rhizomorphs lacking, hyaline clamped emanating hyphae, smooth surface, not branched (2–5 µm)	Periep.	Acute tips
Morphotype III (Fig. 1C), AB 16 (CORD)	Light brown to dark brown mantle, plect. (16 µm)	Simple ramification, straight to tortuous tips	Rhizomorphs lacking, emanating hyphae lacking	Paraep.	Cylindrical tips with mantle
Morphotype IV (Fig. 1D), AB 17 (CORD)	Light yellow to dark brown mantle, plect. (16 µm)	Simple to monopodial pinnate ramification, straight to tortuous tips	Rhizomorphs lacking, emanating hyphae lacking	Periep.	Cylindrical tips with mantle
Morphotype V (Fig. 1E), AB 18 (CORD)	Light brown to dark brown mantle, plect. (20 µm)	Simple ramification, straight to tortuous tips	Rhizomorphs lacking, few light brown emanating hyphae, not clamped, smooth surface, branched (2–5 µm)	Paraep.	Acute tips with mantle
Morphotype VI (Fig. 1F), AB 19 (CORD)	Light brown to dark brown mantle, plect. (15 µm)	Simple ramification, bent to tortuous tips	Rhizomorphs lacking, few light brown emanating hyphae, clamped, smooth surface (2–5 µm)	Paraep.	Cylindrical tips with mantle
Morphotype VII (Fig. 1G), AB 20 (CORD)	Light yellow to brown mantle, plect. (30 µm)	Simple to monopodial pinnate ramification, straight to tortuous tips	Rhizomorphs lacking, few hyaline emanating hyphae, not clamped, smooth surface (2–5 µm)	Periep.	Cylindrical, some acute tips with mantle

[a] Plect., plectenchymatous (hyphae of mantle recognizable as individual hyphae); pseud., pseudoparenchymatous (hyphae of mantle simulating true parenchyma)
[b] Hartig net: Paraep., paraepidermal (penetrating only to the depth of the transverse walls of the epidermal cells); Periep., periepidermal (hyphae entirely encircle the epidermal cells) (follows Godbout and Fortin 1983)

Table 3. BLAST results of internal transcribed spacer (ITS) sequences obtained from two of the seven *Salix humboldtiana* ECM morphotypes described

GenBank accession no. and ECM voucher	Number of base pairs	Most similar sequence	Percent sequence similarity/bp overlap	*E* value
AY945291[a] AB 14-CORD	190	*Inocybe* sp. AJ889953.1	91% / 102	3.0 *E*–26
AY945290[b] AB 15-CORD	677	1: *Tomentella* sp. DQ068971	96% / 615	0.0
		2: Thelephoraceae, uncultured ECM, AY748885	95% / 633	0.0

[a] Partially amplified sequence; 10 best matches in the Cortinariaceae
[b] Ten best matches in the Thelephoraceae

Table 4. Differences in the percentage of each type of fungal colonization of *Salix humboldtiana* between sites (Hayke and La Calera) and sampling dates

	Sites	Sampling dates		
		April	May	June
% ECM	Hayke	44.12 ± 8.92 a	79.47 ± 18.69 bc	89.79 ± 9.96 bc
	La Calera	66.49 ± 2.54 ab	96.24 ± 2.54 c	96.19 ± 4.83 c
% AM	Hayke	8.35 ± 5.09 b	3.12 ± 2.51 ab	0.57 ± 0.45 a
	La Calera	3.37 ± 1.17 ab	6.56 ± 5.12 b	2.51 ± 3.93 a
% DSE	Hayke	8.25 ± 6.81 abc	14.87 ± 1.99 c	1.43 ± 0.33 ab
	La Calera	2.39 ± 1.04 a	12.04 ± 2.06 c	9.38 ± 5.06 bc

% ECM, percent ectomycorrhizae; % AM, percent arbuscular mycorrhizae; % DSE, percent dark-septate endophytes
Values within files of each variable followed by different letter differ at $P < 0.05$ according to Tukey test
Values are mean of five trees ± standard error

Table 5. Differences in the percentage of each ectomycorrhizal morphotype in both sites during autumn

Morphotype	Hayke			La Calera		
	April	May	June	April	May	June
Morphotype I: *Inocybe* sp.	19.18 ± 7.13	0.51 ± 1.14	0.44 ± 0.76*	36.05 ± 17.34	38.65 ± 30.96	52.89 ± 37.34
Morphotype II: *Tomentella* sp.	25.49 ± 16.92	67.15 ± 28.71	31.15 ± 6.19	19.90 ± 10.88	49.98 ± 29.57	20.20 ± 11.04*
Morphotype III	2.66 ± 2.68	17.96 ± 40.16	0.00 ± 0.00	7.05 ± 9.27	0.00 ± 0.00	0.00 ± 0.00*
Morphotype IV	5.95 ± 7.91	0.00 ± 0.00	57.50 ± 4.36**	4.05 ± 4.81	0.00 ± 0.00	15.54 ± 19.17
Morphotype V	0.00 ± 0.00	3.09 ± 4.39	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Morphotype VI	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.00 ± 17.89	0.00 ± 0.00*
Morphotype VII	0.00 ± 0.00	0.00 ± 0.00	2.10 ± 3.63	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values are means of 5 trees ± standard error (SE) for each season
* $P < 0.05$; ** $P < 0.0001$

The very few studies that have focused on the belowground ectomycorrhizal community of *Salix* reported low diversity of ectomycorrhizal types. Graf and Brunner (1996) defined 5 ECM types on *S. herbacea* L.; van der Heijden and Vosatka (1999) distinguished 15 ECM types on *S. repens* L.; Hashimoto and Higuchi (2003) distinguished 4 ECM types on *S. sachalinensis*; and Püttsepp et al. (2004) distinguished 9 ECM types on *S. dasyclados* and *S. viminalis*. Up to now, we have found 7 morphotypes associated with *S. humboldtiana*. These numbers are generally lower than those reported for coniferous trees such as *Pinus* sp. and *Picea* sp., which are associated with a higher number of ECM symbionts (Dahlberg et al. 1997; Jonsson et al. 1999; Taylor and Bruns 1999). The low richness registered could be related to a limited availability of ECM symbionts in the area, because in central Argentina *S. humboldtiana* populations are isolated and distant from other known native ectomycorrhizal host plants. Another possibility might be related to the low number of populations sampled, so further studies including an extended sampling of *S. humboldtiana* habitats are desirable to address the species richness question. On the other hand, an artifact of morphological similarity among the morphotypes produced in *Salix* sp. (Püttsepp et al. 2004), or multiple ECM morphotype colonization in single root tips of *S. repens* (van der Heijden and Vosatka 1999), could explain the low diversity of ECM observed in *S. humboldtiana*.

Two possible ECM symbionts associated with *S. humboldtiana*, *Tomentella* sp. in the Thelephoraceae and *Inocybe* sp. within Cortinariaceae, were identified from the seven ECM morphotypes. DNA amplification from ECM roots presented difficulties. Amplification failures of the remaining morphotypes were probably caused by old and inactive mycorrhizae, or mycorrhizae morphotypes with

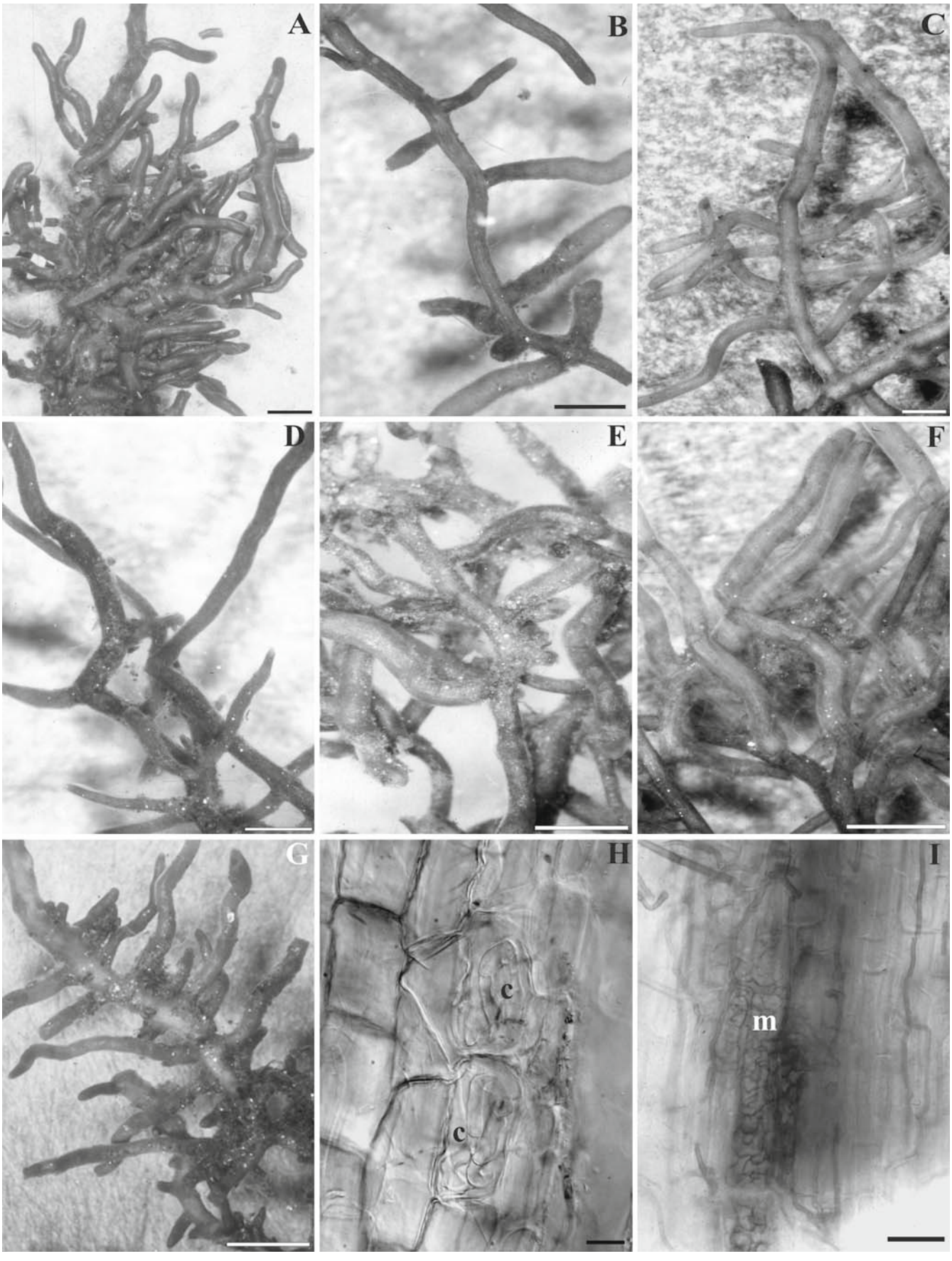

Fig. 1. Patterns of ectomycorrhizae (ECM), arbuscular mycorrhizae (AM), and dark-septate endophytic (DSE) root colonization of *Salix humboldtiana* from central Argentina. Simple (unramified) ectomycorrhizal root tip of *Inocybe* sp. (**A**), *Tomentella* sp. (**B**), morphotype III (**C**), morphotype V (**E**), and morphotype VI (**F**). Simple (unramified) ectomycorrhizal to monopodial pinnate of morphotype IV (**D**) and morphotype VII (**G**). Coils (*c*) of arbuscular mycorrhizal colonization (**H**). Dark-septate endophyte forming a microsclerotia (*m*)-like structure (**I**). *Bars* **A–G** 0.5 mm; **H** 10 µm; **I** 15.6 µm

poorly or discontinuously developed mantle layers, features that seem common in some species of *Salix* (Jones et al. 1990; Graf and Brunner 1996; Püttsepp et al. 2004; Trowbridge and Jumpponen 2004). Also, DNA amplification from *Salix* can be difficult, attributable to the coextracting inhibitors (possibly salicylic acid) (Herrera Medina et al. 2003). Lack of ECM fungal sporocarps in the field limited further mycorrhizal fungus identification using polymerase chain reaction-fragment length polymorphism (PCR-RFLP) techniques. Considering that gene databases are noticeably deficient in locally collected fungi for comparison, these preliminary data are a first contribution to increase our knowledge of the mycorrhizal fungal flora of the *S. humboldtiana* in the Argentinian Neotropics.

The percentage of ECM colonization of *S. humboldtiana* recorded in this study concurs with levels of colonization obtained for other *Salix* and *Populus* spp. (Visser et al. 1998; van der Heijden and Vosatka 1999; van der Heijden et al. 1999; Baum and Makeschin 2000; Khasa et al. 2002; Püttsepp et al. 2004; Trowbridge and Jumpponen 2004). However, Baum et al. (2002) and Hashimoto and Higuchi (2003) observed remarkably lower ECM colonization, less than 50%, in *S. viminalis* and *S. sachalinensis*, respectively. Total ECM colonization in *S. humboldtiana* differed between sites and sampling dates. This variation could be mediated by differences in temperature, soil moisture, and soil nutrient status, and physiological and phenological changes in the host plant, ultimately affecting the development of symbiosis (Harvey et al. 1978; Jones et al. 1990; Swaty et al. 1998; van der Heijden et al. 1999; Baum and Makeschin 2000; Baum et al. 2002; Püttsepp et al. 2004). Indeed, important changes in humidity and temperature usually occurred during the fall months (April to June), as was previously reported by Luti et al. (1979).

In relationship to the ECM morphotypes, morphotype I–*Inocybe* sp., morphotype II–*Tomentella* sp., and morphotype IV were found to be most abundant. Coincidentally, sporocarps and ECM morphotypes belonging to the Cortinariaceae often occurred in *Salix* populations (Graf 1994; Gardes and Dahlberg 1996; van der Heijden and Vosatka 1999; Baum et al. 2002; Nara et al. 2003). As Jumpponen and Trappe (1998) and Nara et al. (2003) suggested, species of *Inocybe* and Thelephoraceae are well-known colonizers of ECM plants in disturbed or primary habitats. The presence of these taxa would suggest that the ECM community is at an early successional stage, which could normally be characterized by a relatively small number of fungal taxa. In contrast to our study, Püttsepp et al. (2004) found *Tomentella* cfr. *lilacinogrisea* at low frequencies on the root tips of both *S. viminalis* and *S. dasyclados*. As these authors affirm, tomentelloid fungi (family Thelephoraceae) are widespread in ECM communities but seem to occur at low frequencies. In our study, *Tomentella* had one of the most abundant morphotypes, so further studies are needed to understand the behavior of *Tomentella* in *Salix* ECM communities. Another indigenous tree forming abundant ECM with *Tomentella* spp. is *Alnus acuminata* Kunth (Betulaceae) (Becerra et al. 2005b; Becerra et al., unpublished data).

The occurrence of morphotypes I–*Inocybe* sp., II–*Tomentella* sp., III, IV, and VI varied between sampling dates (see Table 5), suggesting seasonal changes in their relative abundance. The higher percentage of morphotype I–*Inocybe* sp. in the La Calera site than the Hayke site (Table 5) is probably the result of the soil conditions that benefit its dominance. Soil conditions directly affect mycorrhizal formation (Baar 1995). In our study, soil characteristics such as texture, organic matter, total N, available P, and some cation levels showed important differences between the sites (see Table 1) and possibly influenced the dominance of one particular symbiont. Diversity (Simpson's diversity index) of ECM morphotypes in the sampling dates and two sites did not differ significantly.

The low sporocarp numbers recorded, with no ECM species collected during the sampling season, limit conclusions on the ectomycorrhizal fungal community associated with *S. humboldtiana*. Baum et al. (2002) observed only one ectomycorrhizal fungus (*Inocybe glabripes*) under *S. viminalis*. In contrast, high ECM diversity (78 species) was recorded as fruit bodies in natural dune stands of *S. repens* (van der Heijden et al. 1999). In recent studies, Nara et al. (2003) reported 23 ECM fungi producing fruit bodies in association with *S. renii*. For instance, long-term monitoring would also be desirable to assess fungal species (Vogt et al. 1992), to minimize the chances that environmental conditions at a given time are not suitable to initiate fruiting (Jumpponen et al. 2002).

The occurrence of ECM and AM colonization in *S. humboldtiana* concurred with the observations of Lodge (1989), Khan (1993), Hashimoto and Higuchi (2003), and van der Heijden and Kuyper (2003). *Salix humboldtiana* showed low AM colonization that differed between sampling dates (Table 4). Our colonization rates resemble those observed by Trowbridge and Jumpponen (2004) on *S. commutata* and *S. planifolia* and van der Heijden and Vosatka (1999) on *S. repens*. Although we observed low levels of infection, AM fungi might still provide benefits to *S. humboldtiana*, as van der Heijden and Vosatka (1999) suggested.

This is the first report of DSE occurrence in *S. humboldtiana*, although these root symbionts have been previously identified in other species of *Salix* (Dhillion 1994; Fernando and Currah 1996; Trowbridge and Jumpponen 2004; Cázares et al. 2005). DSE colonization on *S. humboldtiana* presented similar features to previously described *Phialocephala fortinii* (Yu et al. 2001). Similar to the findings of O'Dell et al. (1993), DSE fungi failed to form a complete mantle or Hartig net when colonizing *S. humboldtiana* roots. In this work, DSE colonization showed differences in relation to sampling dates (see Table 4). As Jumpponen and Trappe (1998) and Fuchs and Haselwandter (2004) suggested, it might be possible that plant fitness benefits from colonization of DSE in a similar way as is known for AM. However, further information on the functional relationship of *S. humboldtiana* and DSE is needed to explain their conspicuous colonization.

Based on our results, the *S. humboldtiana* root system is likely to provide adequate noncolonized root space to allow simultaneous colonization by various types of fungi. Further

studies with extended time samplings and sites are necessary to elucidate other aspects of the mycorrhizal fungi associated with *S. humboldtiana* and their ecological role in the central Argentina ecosystems.

Acknowledgments This work was partially supported by Agencia Córdoba Ciencia (N° 1416/97; 1655/99). Special thanks to Drs. Joyce Eberhart, Efren Cázares, Hughes Massicotte and Ari Jumpponen for critical reading of the manuscript. We thank to Dr. Laura Domínguez and Bióls. Graciela Daniele for their help during the sampling. M.S. is grateful to CONICET for the fellowships provided. A.B. and E.N. are Researchers of CONICET.

References

Agerer R (1991) Characterization of ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Techniques for the study of mycorrhizae. Methods in microbiology, vol 23. Academic Press, London, pp 25–73

Agerer R (1999) Anatomical characteristics of identified ectomycorrhizas: an attempt towards a natural classification. In: Varma AK, Hock B (eds) Mycorrhiza. Structure, function, molecular biology and biotechnology, 2nd edn. Springer, Berlin, pp 633–682

Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W, Webb D (1997) Gapped BLAST and PSI BLAST: a new generation in protein database search programs. Nucleic Acids Res 25: 3389–3402

Antibus RK, Croxdale JG, Miller OK, Linkins AE (1980) Ectomycorrhizal fungi of *Salix rotundifolia* III. Resynthesis mycorrhizal complexes and their surface phosphatase activities. Can J Bot 59: 2458–2465

Baar J (1995) Ectomycorrhizal fungi of Scots pine as affected by litter and humus. PhD thesis, Wageningen University, Wageningen, the Netherlands

Baum C, Makeschin F (2000) Effects of nitrogen and phosphorus fertilization on mycorrhizal formation of two poplar clones (*Populus trichocapa* and *P. tremula* x *tremuloides*). J Plant Nutr Soil Sci 163:491–497

Baum C, Weih M, Verwijst T, Makeschin F (2002) The effects of nitrogen fertilization on mycorrhizal formation and soil properties on mycorrhizal formation of *Salix viminalis*. For Ecol Manag 160:35–43

Becerra A, Zak MR, Horton T, Micolini J (2005a) Ectomycorrhizal and arbuscular mycorrhizal colonization of *Alnus acuminata* from Calilegua National Park (Argentina). Mycorrhiza 15:525–531

Becerra A, Pritsch K, Arrigo N, Palma M, Bartoloni N (2005b) Ectomycorrhizal colonization of *Alnus acuminata* Kunth in northwestern Argentina in relation to season and soil parameters. Ann For Sci 62:325–332

Cázares E (1992) Mycorrhizal fungi and their relationship to plant succession in subalpine habitats. PhD thesis, Oregon State University, Corvallis

Cázares E, Trappe JM, Jumpponen A (2005) Mycorrhiza–plant colonization patterns on a subalpine glacier forefront: a model system of primary succession. Mycorrhiza 15:405–416

Dahlberg A, Jonsson L, Nylund JE (1997) Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in south Sweden. Can J Bot 75:1323–1335

Dhillion SS (1994) Ectomycorrhizae, arbuscular mycorrhizae, and *Rhizoctonia* sp. of alpine and boreal *Salix* spp. in Norway. Arct Alp Res 26:304–307

Di Rienzo JC, Robledo W, Casanoves F, Balzarini MG, González L, Guzmán A, Tablada E (2002) Infostat, versión beta. Estadística y Biometría, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina

Dimitri MJ, Leorardis RFJ, Biloni JS (1998) El nuevo libro del Árbol. Tomo II. Especies forestales de la Argentina oriental, 3rd edn. El Ateneo, Buenos Aires

Fernando AA, Currah RS (1996) A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. Can J Bot 74:1071–1078

Fuchs B, Haselwandter K (2004) Red list plants: colonization by arbuscular mycorrhizal fungi and dark septate endophytes. Mycorrhiza 14:277–281

Gardes M, Bruns T (1993) ITS primer with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gardes M, Bruns T (1996) ITS-RFLP matching for identification of fungi. Methods Mol Biol 50:177–186

Gardes M, Dahlberg A (1996) Mycorrhizal diversity in artic and alpine tundra: an open question. New Phytol 133:147–157

Gehring CA, Whitham TG (1994) Comparisons of ectomycorrhizae on pinyon pines (*Pinus edulis*; Pinaceae) across extremes of soil type and herbivory. Am J Bot 81:1509–1516

Godbout C, Fortin JA (1983) Morphological features of synthesized ectomycorrhizae of *Alnus crispa* and *Alnus rugosa*. New Phytol 94: 249–262

Grace C, Stribley DP (1991) A safer procedure for routine staining of vesicular arbuscular mycorrhizal fungi. Mycol Res 95:1160–1162

Graf F (1994) Ecology and sociology of macromycetes in snow-beds with *Salix herbaceae* L. in the alpine Valley of Radönt (Grisons, Switzerland). Diss Bot 235:1–242

Graf F, Brunner I (1996) Natural and synthesized ectomycorrhizas of the alpine dwarf willow *Salix herbacea*. Mycorrhiza 6:227–235

Harley JL, Harley EL (1987) A checklist of mycorrhiza in the British flora. New Phytol 105(suppl):1–102

Harvey AE, Jurgensen MF, Larsen MJ (1978) Seasonal distribution of ectomycorrhizae in a mature Douglas-fir/larch forest soil in western Montana. For Sci 24:203–208

Hashimoto Y, Higuchi R (2003) Ectomycorrhizal and arbuscular mycorrhizal colonization of two species of floodplain willows. Mycoscience 44:339–343

Helm DJ, Allen EB, Trappe JM (1996) Mycorrhizal chronosequence near Exit Glacier, Alaska. Can J Bot 74:1496–1506

Helm DJ, Allen EB, Trappe JM (1999) Plant growth and ectomycorrhiza formation by transplants on deglaciated land near Exit Glacier, Alaska. Mycorrhiza 8:297–304

Herrera Medina MJ, Gagnon H, Piché Y, Ocampo JA, García Garrido JM, Vierheilig H (2003) Root colonization by arbuscular mycorrhizal fungi is affected by the salicylic acid content of the plant. Plant Sci 164:993–998

Holmgren PK, Holmgren NH, Barnet LC (1990) Index herbariorum. Part I. The herbaria of the world, 8th edn. Regnum Veg 120:1–693

Jones MD, Durall DM, Tinker PB (1990) Phosphorus relationships and productions of balsam poplars, aspen and willows on former arable land in the Federal Republic of Germany. III. Soil ecological effects. For Ecol Manag 121:85–99

Jones M, Durall DM, Tinker PB (1998) A comparison of arbuscular and ectomycorrhizal *Eucalyptus coccifera*: growth response, phosphorus uptake efficiency and external hyphal production. New Phytol 140:125–134

Jonsson L, Dahlberg A, Nilsson MC, Zackrisson O, Karén O (1999) Ectomycorrhizal fungal communities in late-succession at Swedish boreal forest, and their composition following wildfire. Mol Ecol 8:205–215

Jumpponen A (2001) Dark septate endophytes–are they mycorrhizal. Mycorrhiza 11:207–211

Jumpponen A, Trappe JM (1998) Dark-septate root endophytes: a review with special reference to facultative biotrophic symbiosis. New Phytol 140:295–310

Jumpponen A, Trappe JM, Cázares E (1999) Ectomycorrhizal fungi in Lyman Lake Basin: a comparison between primary and secondary successional sites. Mycologia 91:575–582

Jumpponen A, Trappe JM, Cázares E (2002) Occurrence of ectomycorrhizal fungi on the forefront of retreating Lyman Glacier (Washington, USA) in relation to time since deglaciation. Mycorrhiza 12:43–49

Kahn A (1993) Occurrence and importance of mycorrhizae in aquatic trees of New South Wales, Australia. Mycorrhiza 3:31–38

Khasa PD, Chakravarty P, Robertson A, Thomas BR, Dancik BP (2002) The mycorrhizal status of selected poplar clones introduced in Alberta. Biomed Bioeng 22:99–104

Lapeyrie FF, Chilvers GA (1985) An endomycorrhiza-ectomycorrhiza succession associated with enhanced growth of *Eucalyptus domosa* seedlings planted in a calcareous soil. New Phytol 100:93–104

Largent DL (1986) How to identify mushrooms to genus. I: Macroscopic features. Mad River Press, Eureka, CA

Largent DL, Johnson D, Watling R (1986) How to identify mushrooms to genus. III: Microscopic features. Mad River Press, Eureka, CA

Lodge DJ (1989) The influence of soil moisture and flooding on formation of VA-endo- and ectomycorrhizae in *Populus* and *Salix*. Plant Soil 117:243–253

Luti R, Bertrán de Solís M, Galera F, Ferreira N, Nores M, Herrera M, Barrera JC (1979) Vegetación. In: Vázquez J, Miatello R, Roqué M (ed) Geografía Física de la Provincia de Córdoba. Bold, Buenos Aires, pp 1–464

Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. Stud Mycol 53:173–189

Marshall PE, Pattullo N (1981) Mycorrhizal occurrence in willows in a northern freshwater wetland. Plant Soil 59:525–532

McGonigle TP, Miller MH, Evans DG, Fairchild DL, Swan GA (1990) A new method which gives and objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

Molina R, Myrold D, Li CY (1994) Root symbiosis of red alder: technological opportunities for enhanced regeneration and soil improvement. In: Hibbs DE, DeBell DS, Tarrant RF (eds) The biology and management of red alder. Oregon State University Press, Corvallis, pp 23–46

Moreno G, García Manjón JL, Zugaza A (1986) La guía INCAFO de los hongos de la Península Ibérica, 2 vols. INCAFO, Madrid

Nara K, Nakaya H, Hogetsu T (2003) Ectomycorrhizal sporocarp succession and production during early primary succession on Mount Fuji. New Phytol 158:193–206

Nelson DW, Sommers LE (1982) Total carbon, organic carbon, and organic matter. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, part 2. American Society of Agronomy, Madison, WI, pp 639–577

O'Dell T, Massicotte HB, Trappe JM (1993) Root colonization of *Lupinus latifolius* Agradh. and *Pinus contorta* Dougl. by *Phialocephala fortinii* Wang & Wilcox. New Phytol 124:93–100

Püttsepp U, Rosling A, Taylor AFS (2004) Ectomycorrhizal fungal communities associated with *Salix viminalis* L. and *S. dasyclados* Wimm. clones in a short-rotation forestry plantation. For Ecol Manag 196:413–424

Ragonese A (1987) Familia Salicaceae. Flora Ilustrada de Entre Ríos III. Colección Científica del INTA. Tomo VI, Buenos Aires, pp 6–14

Read DJ (1989) Mycorrhizas and nutrient cycling in sand dune ecosystems. Proc R Soc Edinb Sect B (Biol Sci) 96:89–110

Read DJ, Haselwandter K (1981) Observations on the mycorrhizal status of some alpine plant communities. New Phytol 88:341–352

Roberston A (1984) Willow plantations in agroforestry. Span 27: 32–34

Sasaki A, Fujiyoshi M, Shidara S, Nakatsubo T (2001) Effects of nutrients and arbuscular mycorrhizal colonization on the growth of *Salix gracilistyla* seedlings in a nutrient-poor fluvial bar. Ecol Res 16: 165–172

Simpson EH (1949) Measurement of diversity. Nature (Lond) 163: 688

Singer R (1986) The Agaricales in modern taxonomy. Koeltz, Koenigstein

Swaty RL, Gehring CA, Van Ert M, Theimer TC, Keim P, Whitman TG (1998) Temporal variation in temperature and rainfall differentially affects ectomycorrhizal colonization at two contrasting sites. New Phytol 139:733–739

Taylor DL, Bruns TD (1999) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol Ecol 8:1837–1850

Trowbridge J, Jumpponen A (2004) Fungal colonization of shrub willow roots at the forefront of a receding glacier. Mycorrhiza 14:283–293

van der Heijden EW (2001) Differential benefits of arbuscular mycorrhizal and ectomycorrhizal infection of *Salix repens*. Mycorrhiza 10:185–193

van der Heijden EW, Kuyper TW (2003) Ecological strategies of ectomycorrhizal fungi of *Salix repens*: root manipulation versus root replacement. Oikos 103:668–680

van der Heijden EW, Vosatka M (1999) Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. II. Mycorrhizal dynamics and interactions of ectomycorrhizal and arbuscular mycorrhizal fungi. Can J Bot 77:1833–1841

van der Heijden EW, Vries FW, Kuyper TW (1999) Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems I. Above-ground and below-ground views of ectomycorrhizal fungi in relation to soil chemistry. Can J Bot 77:1821–1832

Visser S, Maynard D, Danielson RM (1998) Response of ecto- and arbuscular mycorrhizal fungi to clear-cutting and the application of chipped aspen wood in a mixed wood site in Alberta, Canada. App Soil Ecol 7:257–269

Vogt KA, Bloomfield J, Ammirati JF, Ammirati SR (1992) Sporocarp production by basidiomycetes, with emphasis on forest ecosystems. In: Carroll GC, Wicklow DT (eds) The fungal community: its organization and role in the ecosystem. Dekker, New York, pp 563–581

Walker C, McNabb HS (1984) Mycorrhizal symbionts associated with hybrid poplars from Iowa, USA. Eur J Pathol 14:282–296

White T, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D, Sninsky J, White T (eds) PCR protocols: guide to methods and applications. Academic Press, New York, pp 315–322

Wright JE, Albertó E (2002) Guía de los Hongos de la Región Pampeana. I. Hongos con laminillas. Edición L.O.L.A., Buenos Aires

Wright JE, Deschamps JR, Rovetta GS (1973) Basidiomicetos xilófilos de la región mesopotámica. I. Políporos trametoides. Revista de Investigaciones Agropecuarias, INTA Serie 5 10:117–179

Yu T, Nassuth A, Peterson RL (2001) Characterization of the interaction between the dark septate fungus *Phialocephala fortinii* and *Asparagus officinalis* roots. Can J Microbiol 47:741–753

Zar JH (1999) Biostatistical analysis, 4th edn. Prentice Hall, Upper Saddle River, NJ