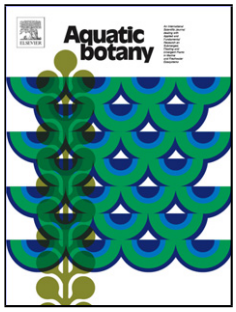

Title: Fungal root colonization of *Puccinellia frigida* (Phil.) Johnston, a dominant grass species inhabiting the margins of high-altitude hypersaline Andean wetlands

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Highligths

1-Diverse root fungal endophytes colonize the grass *Puccinellia frigida* in high Andean wetlands

2-Septate fungi and chytrids were observed in all root samples under extreme soil conditions

3-No variation in AM colonization was recorded between sampling seasons in two sites

4-In highly stressful sites AM fungi colonization was not was detected

5-Soil salinity and organic matter affect septate fungi and chytrids root colonization

Fungal root colonization of *Puccinellia frigida* (Phil.) Johnston, a dominant grass species inhabiting the margins of high-altitude hypersaline Andean wetlands

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Abstract

High-altitude hypersaline Andean wetlands are considered stressful environments by the prevalence of extreme abiotic conditions affecting both plant host and fungal root endophytes. *Puccinellia frigida* (Phil.) Johnston, a dominant plant species inhabiting the margins of these wetlands over 4000 m a.s.l. in Northwest Argentina, is frequently colonized by fungal root endophytes. Here we examined the nature and dynamics of fungal root colonization on this plant species in three different wetlands (six sampling sites) and two growing seasons. Morphologically diverse septate fungi and chytrids were observed in root samples of *P. frigida* in all sampling sites, whereas arbuscular mycorrhizal fungi were found only in two sites. The level of colonization of chytrids and septate fungi differed significantly both between sites and seasons. Soil organic matter and electrical conductivity were the only habitat parameters significantly related to the observed differences. The widespread occurrence of non-destructive fungal associations suggest an important role for plant survival in extreme environments.

Key words: mycorrhiza, stressful environment, salinity, Andean wetlands, *Puccinellia frigida*

1. Introduction

In the high Andean mountains of west and northwest Argentina, several hypersaline wetlands exist over 4000 m.a.s.l.; an elevation near the limit for plant life in the cold and arid central Andes (Arroyo et al., 2004; Squeo et al., 2006). These environments exhibit multiple extreme variables that limit the establishment and development of plant species (Zack and Wildman, 2004). These factors include high water and soil salinity, high UV irradiance, soil oligotrophy, high heavy metal content, hypoxia and low temperatures (Fernández Zenoff et al., 2006; Dorador et al., 2008; Dib et al., 2009).

Plants in extreme environments are specifically challenged to live on limited amounts of nutrients and water and to counteract soil toxicity problems. High salinity and low temperatures have a strong inhibitory effect on inorganic nitrogen and phosphorus uptake and availability (Clarkson et al., 1992; Navarro et al., 2001; Henry and Jefferies, 2003). Excessive Na^+ and Cl^- ions have toxic effects, including structural disruption of enzymes and other macromolecules, damage to cell organelles and plasma membrane, and disruption of photosynthesis, respiration and protein synthesis (Juniper and Abbott, 1993). Also, high heavy metal content in soil induces oxidative stress and can severely damage plant cell membranes (Barceló and Poschenrieder, 1990; Polle and Rennenberg, 1993), whereas low soil water activity is a key limiting factor for normal plant growth in hypersaline and cold environments (Morgan, 1984). Therefore, plant species that inhabit these environments must have developed efficient mechanisms and strategies in order to grow and survive under such conditions.

Plant symbiotic fungi are widespread and occur naturally in diverse ecosystems, from deserts to rainforests,as well as in boreal and austral regions just in the limit for plant growth (Brundrett, 1991; Fracchia et al., 2009). It is well known that symbiotic fungi

are important to the structure, function and health of plant communities, and may be responsible for the acclimation of plants to environmental stresses (Rodríguez and Redman 1997; Brundrett 2006; Porras-Alfaro et al., 2008). Specifically, it has been demonstrated that root-fungal associations can be determinant to alleviate diverse biotic and abiotic stresses for plant growth (Rodríguez et al., 2004). Understanding the nature and dynamics of these associations could be a first approach for the management and conservation of native plants living in these extreme habitats as well as to understand their ecological interactions (Barrow et al., 1997).

In preliminary studies, we found patches of the grass *Puccinellia frigida* growing as a dominant species in the margins of several high-altitude hypersaline Andean wetlands of Northwest Argentina. We observed this species in a wide range of soil conditions, being the only plant species able to grow between salt crusts but also growing close to freshwater springs in association with other macrophytes (Nicora, 1999, Fracchia, pers. obs)

The objective of this work is to describe the nature and extent of the fungal root colonization on *P. frigida* in three high-altitude Andean wetlands of Northwest Argentina, exploring differences in the level of colonization between six sampling sites and two growing seasons. Additionally, we analysed the main soil chemical characteristics of the six sites and set up the hypothesis that the colonization level of each fungal type is influenced by the soil chemical features at each site.

2. Material and Methods

2.1. Site description

The three wetlands studied (Laguna Brava, Laguna Mulas Muertas and Laguna Veladero) belong to the Central Andes mountains of La Rioja Province, Northwest Argentina, and are located within the protected area of the Reserva Provincial Laguna Brava (Fig. 1). The region comprises a network of hypersaline shallow lagoons associated with small communities of *bofedales* (wet-marshy meadows; Carrizo et al., 1997). These lands correspond to the Altoandina phytogeographic region, which is characterized by altitudes over 4000 m.a.s.l. (Cabrera, 1971). The wetlands are included in the List of Wetlands of International Importance (RAMSAR). Climatic conditions are similar for the three water systems (Table 1): precipitation is scarce (annual average 200 mm), falling mostly as snow during the winter (Squeo et al, 2006), and air temperatures can reach -25°C in winter and 15°C in summer (annual average 0.8°C). Strong western winds with snow storms occur along the year, but mostly during fall and winter. Maximal UV-B irradiance for a nearby Andean wetland (Laguna Azul, Catamarca Province, 4400 m.a.s.l.) has been found to reach 10.8 Wm^{-2} for the 300 to 325 nm range, measured at noon in the austral summer (Dib et al., 2009). Soils are sandy and rocky, with low organic matter content (Table 2), and are classified as entisols (Soil Survey Staff. 2010. Keys to Soil Taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington, DC).

Recent analyzes have detected high heavy metal concentrations (As:3.5, Cd:6, Cu:20, Pb:9, Cr:35, Ni:25 µg/g) in the margins soils from Laguna Brava and Mulas Muertas wetlands (Fracchia, pers. com.).

2.2. Field sampling and plant identification

Six different sampling sites were selected on the margins of the three wetlands (Fig. 1, Table 1). Sites A, B and C belong to Laguna Brava, site A has salt crusts on the surface

and *P. frigida* grow there in the form of compact patches (Fig. 2A); site B has *P. frigida* as the only growing plant species and site C is a small *bofedal* with several macrophytes. Site D is on the margin of the small shallow hypersaline lagoon Veladero and has only small patches of *P. frigida. S*ites E and F belong to Mulas Muertas, site E is close to a freshwater spring and *P. frigida* is found there associated with a few macrophytes whereas site F has salt crusts on the surface and very few and inconspicuous individuals of *P. frigida*. At each sampling site, six to ten *P. frigida* individuals of similar size (8-10 cm tall; 2 cm wide at the base) were randomly collected at the beginning (November 2009) and at the end (March 2010) of the growing season. Around each plant, a hole of 20 cm diameter and 15 cm deep was dug and the entire plant and soil surrounding it was collected, in order to obtain their root system as complete as possible (Fig. 2B). Individual plants were sealed in polyethylene bags and kept in a cool chamber until their analysis in the laboratory.

The taxonomical identification of the plant species was based on published regional Floras (Kiesling, 2009, Zuloaga et al., 1994; Zuloaga and Morrone, 1996). Voucher specimens were deposited in the Darwinion Botanical Institute, Buenos Aires.

2.3. Root preparation and staining

Root samples of each *P. frigida* individual were carefully washed in running tap water to remove soil debris, and observed with a stereo binocular microscope (Leica MZ12) to detect external hyphal cover and extraradical mycelium. About 20 to 30 healthy root segments 1.5 cm long of each plant sample were processed according to the method of Barrow (2003) for dual staining (sudan IV and trypan blue). With this method, chitin stained dark blue with trypan blue and fungal and plant lipids stained bright red with sudan IV.

2.4. Root colonization

About 20 to 30 stained root segments per plant individual were screened for the presence/absence of the different endophytic fungal types with a binocular microscope (Leica DMLB) at a x1000 magnification. Arbuscular micorrhiza (AM) fungi, septate fungi and Chytridiomycetes were analyzed in each root fragment. Colonization level of target fungi was obtained with the colonization frequency formula (Ai-Rong and Kai-Yun, 2007). For AM fungi, the colonization type (Paris, Arum or intermediate) was determined as defined by Dickson (2004). Root samples were placed in lactic acid and deposited at the CRILAR (Centro Regional de Investigaciones La Rioja) in Anillaco, La Rioja, Argentina.

2.5. Soil chemical analysis

For soil chemical analysis, five subsamples were taken from the top 20 cm at each sampling site and bulked into a composite pooled sample of about 1 kg. The soil was analyzed as follows: % humidity was determined for 10 g soil samples by subtracting the weight of the soil dried overnight at 105°C from its fresh weight; electric conductivity (EC) and pH were determined in 1:2.5 suspension of soil in water; organic matter (OM) content followed the Walkley and Black method (Walkley and Black, 1934); available P followed the Kurtz and Bray method (Kurtz and Bray, 1945) and soluble Na^+ content was determined by the direct flame photometer method (Halstead and Chairen, 1950). All measurements were carried out in triplicate.

2.6. Statistical analysis

A two-way Analysis of Covariance (ANCOVA) was carried out separately for each fungal type to test for differences in the percent of fungal colonization (dependent variable) between sites and seasons (fixed factors), and the influence of environmental soil parameters (treated as covariates) on the dependent variable. When differences were significant, means were compared with a *post hoc* Tukey's test. Simple linear regression analyses were used to determine correlations between those significant covariates and the fungal colonization percentage. Percentage values were arcsine transformed prior the analyses to normalize variability. The p-value for hypothesis tests was set at 0.05. All the analyses were carried out with the STATISTICA program.

3. Results

Root colonization morphology

All *P. frigida* individuals sampled in the three wetlands were highly colonized by diverse root fungal endophytes. Plant defense-related symptoms (i.e. cell wall thickening and gall formation, cell death) were observed associated with fungal structures only in a few root samples. Before root staining, solitary external hyphae were scarcely observed on the root surface of individuals from sites A, B, D and F, whereas an inconspicuous mycelial network attached to sand particles and soil aggregates was observed in the roots of individuals from sites C and E.

The co-occurrence of septate fungi and chytrids was observed in all root samples from the six sampling sites (Table 3),), whereas AM fungal structures were only present in the roots of *P. frigida* from site C (Laguna Brava) and site E (Mulas Muertas); in both cases with low colonization levels (Table 3). In site C, two AM fungal colonization types were distinguished: 1) typical arum colonization (Fig. 3 A-B) with arbuscules, vesicles and intra- and intercellular hyphae in the cortical tissue, and 2) a fine endophyte

type (Fig. 3C), with a few small and round vesicles, very thin hyphae and arbuscule-like structures inside the cortical cells.

All *P. frigida* individuals from the six sites and both sampling seasons were colonized by septate fungi of variable morphology. Septate fungi were characterized by microsclerotia of variable appearance and structure inside the root tissue (Fig. 3 D-F) and diverse hyaline and melanized hyphae (Fig. 3 G-L). Some septate hyaline hyphae colonized the cortex, intra- and intercellularly, and in some root sections, hyaline fungal structures inside the vascular tissue were observed. In this case, septate fungi were characterized by the presence of profuse lipid stained bodies and a very thin wall that was not stained with trypan blue (Fig. 3G). Also, intracellular hyaline hyphae with negative reaction to the dual staining method used were scarcely visible (Fig. 3H), but observed in the root system from almost all sampled specimens from all sampling sites. A continuum between melanized and hyaline septate hyphae, as well as between globose and thin hyaline hyphae, was commonly observed (Fig. 3I). The colonization of some septate endophyte fungi seemed to spread from one cortical cell to another by narrow penetrating hyphae (Fig. 3J). Globose and septate hyaline mycelia that stained positively with sudan IV but not with trypan blue (Fig. 3K) were also visible in sampling roots from sites A, B and C. Finally, a septate mycelium that stained partially pale blue with trypan blue and colonized the cortical cells was also observed in *P. frigida* individuals from all sampling sites of Brava and Mulas Muertas wetlands (Fig. 3L).

Chytridiomycetes were observed in all sampling sites and were characterized by resting sporangia of variable size and structure inside root cortical cells of *P. frigida* (Fig. 3 M-O). No evidence of tissue disruption or necrosis was observed in the roots colonized by this fungal type.

Root colonization dynamics

Root colonization levels by chytrids and septate fungi differed significantly between sites and seasons (Table 4). Individuals from sites C and E were more heavily colonized by both fungal types compared to the other sites (post hoc Tukey's test $p < 0.0001$) with no significant differences between these both sites. During March the colonization level of both fungal types was higher compared to September. The statistical analysis showed a significant interaction term between sites and season: during March colonization was higher at sites D and E for chytrids and at sites E and F for septate fungi (Table 3).

Also for chytrids and septate fungi, soil organic matter and electrical conductivity were the only significant soil parameters that were correlated to the observed differences in their level of colonization (Table 4). Simple linear regressions showed that colonization was positively correlated with the organic matter (chytridio: $F = 108.38$, $p < 0.0001$, $r = 0.87$, septate fungi: $F = 65.28$, $p < 0.0001$, $r = 0.81$) and negatively correlated with the electrical conductivity (chytrids:$F = 85.87$, $p < 0.0001$, $r = -0.84$, septate fungi: $F = 41.66$, $p < 0.0001$, $r = -0.74$). For Glomeromycetes, differences between sites were significant, but not between seasons or their interaction term. None of the tested habitat parameters had a significant influence on the differences in colonization percentage for this fungal type.

4. Discussion

The results of our study show that *P. frigida*, a plant species that grows in the margins of high-altitude hypersaline Andean wetlands, presents widespread fungal-root colonization by septate and chytridiomycetes endophytes. In contrast, we observed low values of AM colonization: AM fungi were recorded only in two sampling sites near

freshwater springs, where the plants were associated with other macrophytes. In sites where *P. frigida* grew as the only plant species and under very stressful conditions (i.e. high EC and Na^+ content), AM fungi were not observed. It is commonly accepted that AM fungi are prevalent in mesic conditions but nearly absent in stressful environments (Barrow and Aaltonen, 2001). It is interesting to note that we observed active AM colonization with arbuscules, vesicles and hyphal coils in *P. frigida* roots, even at EC values over 50 (dS/m) (site E). AM colonization was recorded at this salinity content only in a few other surveys (Juniper and Abbott, 1993; Evelin et al., 2009). Given that is well known that the establishment of specific mycorrhizal symbiosis allows plant species to protect or alleviate soil stress conditions (Schulz, 2006), the isolation, identification and characterization of these AM fungal strains could be useful to evaluate their protective role under salinity stress.

No fluctuations in AM colonization were recorded between sampling seasons in both sites where AM fungi were observed. Seasonal fluctuation in AM colonization has been reported to be correlated mainly with the soil moisture/dry conditions, nutrient availability and seasonal climatic differences (Schulz et al., 1999; Barrow et al., 2001; Ruotsalainen et al., 2002); all variables that did not show significant seasonal changes in the high-altitude Andean wetlands of this study.

Diverse septate hyphae morphotypes were observed in the roots of all sampling sites. Septate fungal endophytes could have a wider adaptability to the soil conditions than AM fungi. However, colonization fluctuations were observed between sites; particularly soil salinity and organic matter seem to be determinant soil variables that condition the root colonization by these fungi. It has been suggested that, in the absence of arbuscular mycorrhizas, septate fungi might act as surrogate root mutualists in cold-stressed soils

(Bledsoe et al., 1990; Urcelay et al., 2011), as well as in soils with high EC or Na^+ content (Mandyam and Jumpponen, 2005).

Although some of the fungal morphologies were recognizable and clearly different from each other, we could not discriminate in separate categories, mainly due to the difficult to discern the nature of some structures belonging to a particular fungal morphotype. The observed continuum of melanized and hyaline hyphae, as well as between hyaline globose and thin hyphae, and the overlap of different fungal hyphae in the same root section and even inside the same cell, were the main causes that did not allow us to discriminate between different morphotypes. It is clear that a consortium of septate fungal taxonomic identities colonizes the plant species *P. frigida,* and these different strains might be less or better adapted to specific soil conditions. Using molecular tools, Porras-Alfaro et al. (2008) identified several orders of fungal taxa colonizing the roots of the grass species *Bouteloua gracilis* in semiarid grasslands. The orders included Hypocreales, Xylariales, Sordariales, Agaricales, Pleosporales and Pezizales, all of which are taxa with septate hyphae species.

The extraradical mycelium of root endophytic fungi regularly explores the soil surrounding the root system (Brundrett, 2006). In our study, although we observed high root fungal colonization levels, we did not detect a well-developed extraradical mycelium network. The reduction of this mycelial network was notorious at sites where the most stressful soil conditions prevail (sites A, B, D, and F). These observations suggest that soil stressful conditions could be an obstacle for the development of the extraradical phase of endophytic fungi. However, obligate fungal species are known to inhabit the roots and not to extend outside the root tissue in their whole life cycle (Lucero et al., 2006). Thus, we can not exclude that some septate fungi with these characteristics are also present in the roots of *P. frigida.*

Chytrid sporangia were observed in all root samples of *P. frigida* and in sites with very high soil salinity levels. In all cases there was no evidence of harm to the host plant, even in roots that were highly colonized. True symbiotic interactions between plants and chytrids are not known, and species of some chytrid genera such as *Synchytrium* and *Physoderma* have been described as plant pathogens (Laidlaw, 1985). Nonetheless, it has been suggested that these fungi could regulate fungal colonization and nutrient uptake by the host (Barrow et al, 1997; Gleason et al, 2010). The regular, non-symptomatic colonization of *P. frigida* roots by chytrids suggest that they could play a significant but unknown function in the life cycle of *P. frigida* at least in wetland ecosystems.

Future research should be directed towards the isolation and identification of fungal strains associated with the roots of *P. frigida* of natural ecosystems. Some of these isolates could be of crucial importance for the establishment and survival of the plant species growing in these extreme environments. Furthermore, considering that extreme environments are a good source of microorganisms with exceptional phenotypic and genotypic characteristics (Dib et al., 2009), the detection of beneficial fungi for plant stress alleviation could be very interesting not only for basic research purposes, but also as valuable tools for biotechnological applications.

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Figure legends

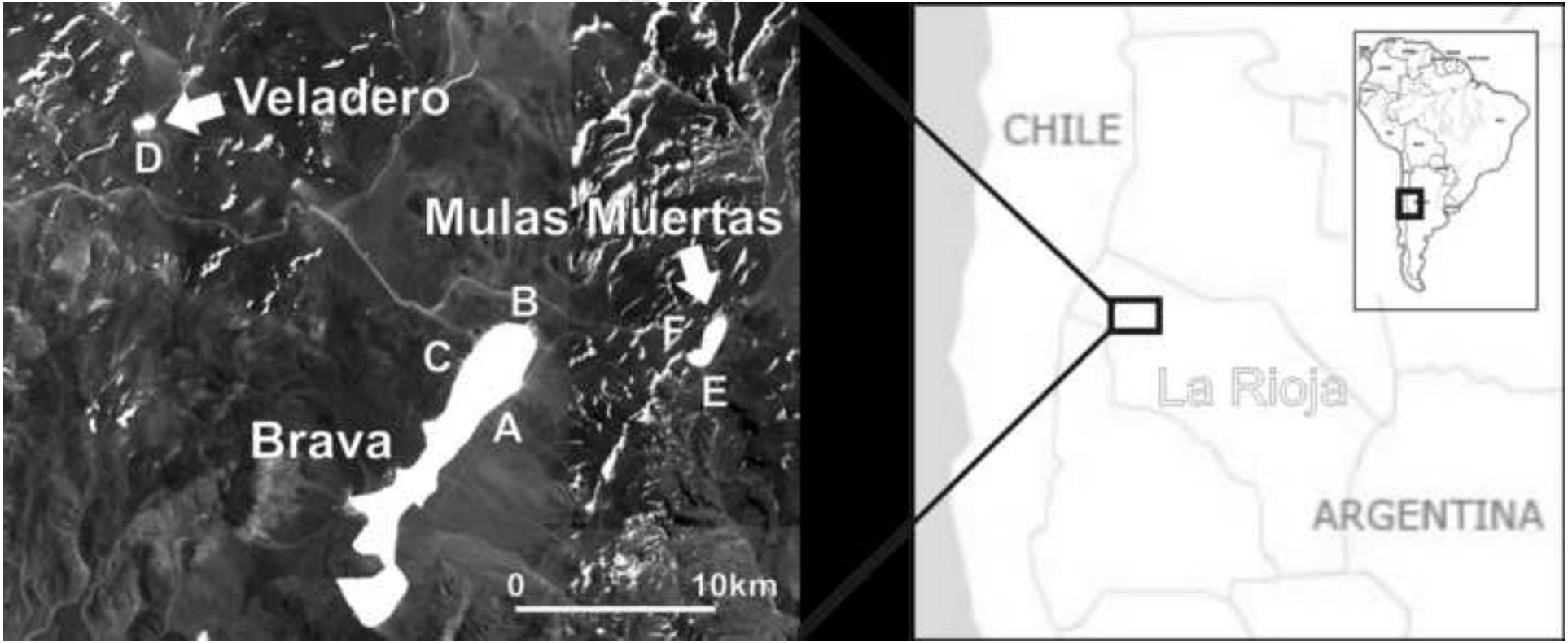
Fig. 1. Locations of the three high-altitude Andean wetlands and the six sampling sites (A-F)

Fig. 2 A, view of Laguna Brava at sampling site A with patches of *P. frigida* (arrow); B, whole sample of *P. frigida* from site C

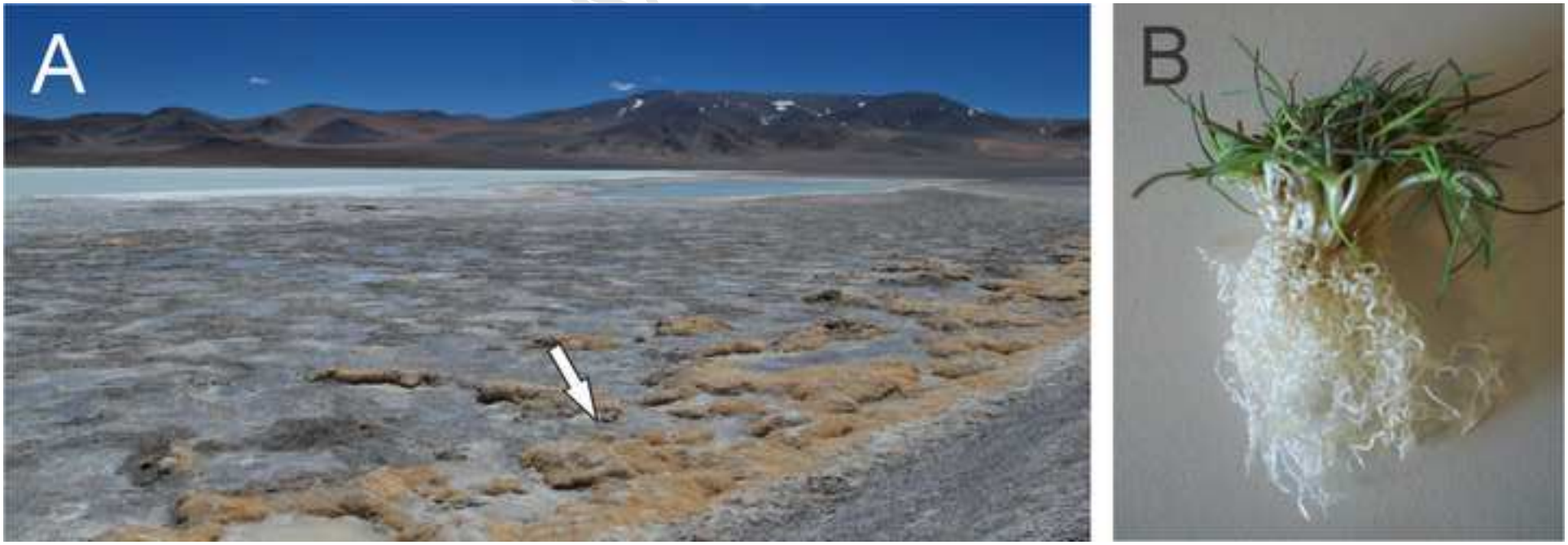
Fig 3. *P. frigida* root fungal structures observed under binocular microscope after dual staining with tripan blue and sudan IV. A-C Arbuscular mycorrhizal structures in root samples from site C (A: arum type colonization with arbuscules and hyphae, B: arbuscule, C: hyphae of a fine endophyte); D-F Microsclerotia of different size and morphology (D: laxus and melanized microsclerotium structure, E: compact and melanized microsclerotium, F: non melanized microsclerotium with lipid content); G-L Septate fungi hyphae (G: hyaline hyphae inside the vascular tissue with positive stained

lipids, H: hyaline hyphae inside cortical cells with no reaction to the dual staining method, I: a continuum between melanized and hyaline septate hyphae, J: cell to cell colonization by a septate melanized hyphae, K: globose and septate hyphae with stained lipids in cortical tissue, L: Intracellular hyphae partially stained with tripan blue); M-O Chytridiomycetes sporangia of variable size and morphology in root cortical cells. Bar: 25µm.

Figure


Veladero
D
Mulas Muertas
B
C
A
F
E
Brava
0
10km
CHILE
La Rioja
ARGENTINA

Figure



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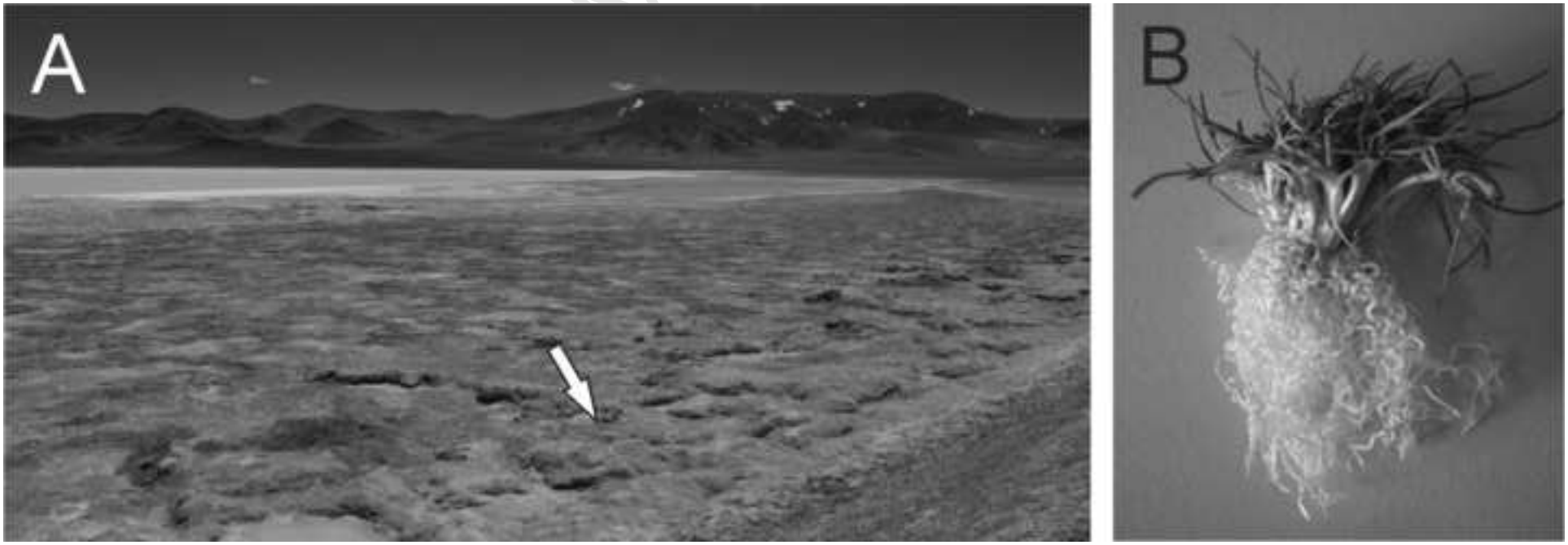

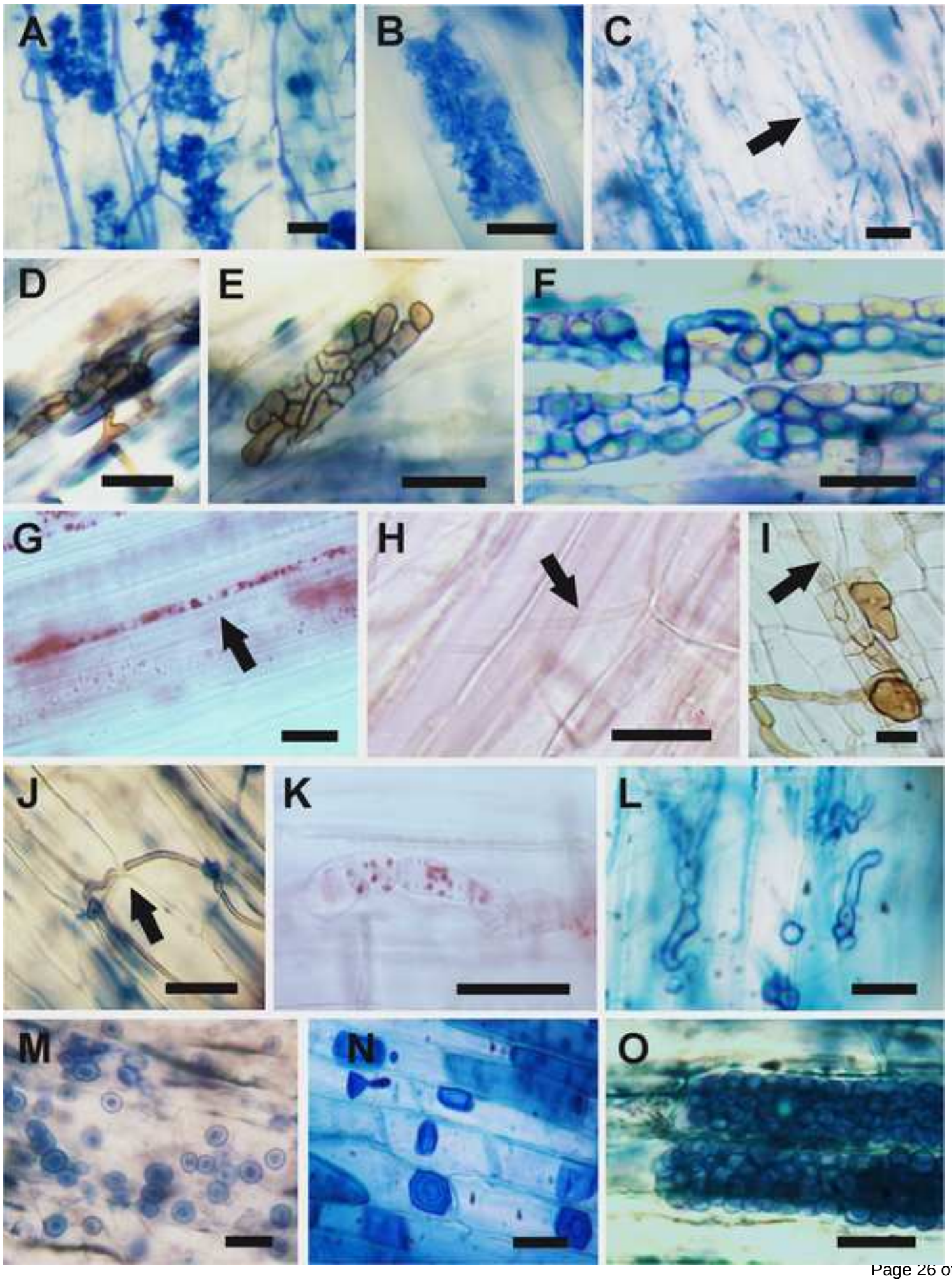

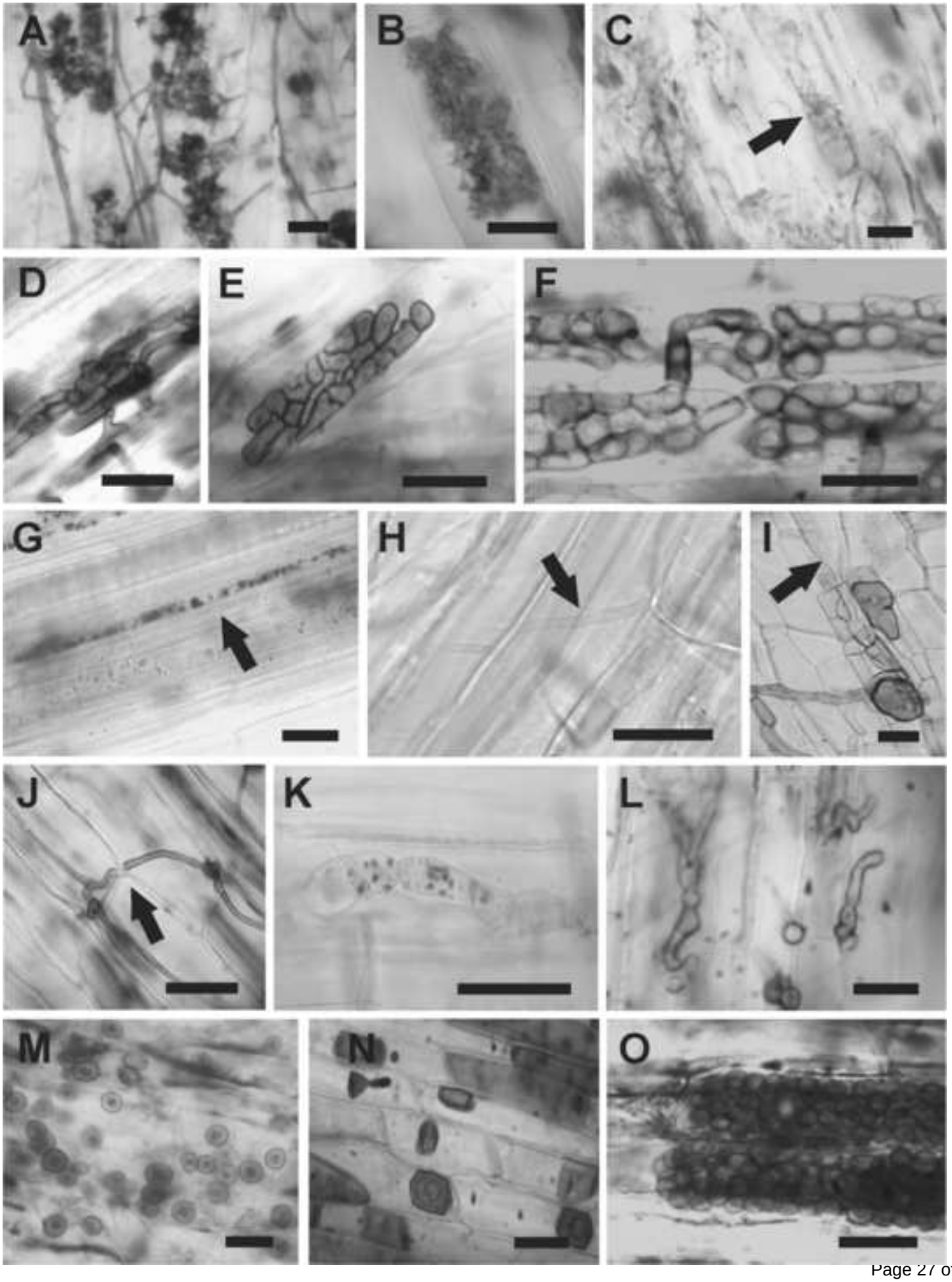

Table 1. Sampling sites for *P. frigida*, associated plant species, water salinity (means ± SD) and geographical position in three high-altitude Andean wetlands of Northwest Argentina.

Wetland*	Sampling site	Associate plant species	Total dissolved salts (g L-1)	Altitude (masl)	Global position
Brava (5100)	A	--	188 (±55)	4260	28° 18' 29" S 68° 49' 14" W
	B	--			28° 16' 27" S 68° 49' 15" W
	C	*Deyeuxia curvula* *Zameioscirpus atacamensis* *Ruppia sp.* *Patosia clandestina* *Nastanthus caespitosus* *Ranunculus exilis* *Calandrinia compacta*			28° 16' 49" S 68° 50' 23" W
Veladero (12)	D	--	191 (±13)	4327	28° 11' 19" S 69° 00' 25" W
Mulas Muertas (210)	E	*Zameioscirpus atacamensis* *Deyeuxia curvula* *Ranunculus exilis*	160 (±39)	4123	28° 17' 24" S 68° 44' 03" W
	F	--			28° 16' 56" S 68° 43' 51" W

*wetland surface in ha.

Table 2: Main soil chemical characteristics at six sampling sites in three high-altitude Andean wetlands of Northwest Argentina

Wetland	Brava			Veladero	Mulas Muertas	
Sampling site	A	B	C	D	E	F
Soil Parameter						
Moisture (%)**	26.1	25.3	35.7	33.1	29.9	25.5
pH**	9.1	9.5	9.0	9.9	8.7	8.0
EC (dS/m)**	141	96	37	112	56	117
OM (%)*	0.43	0.55	2.45	0.32	1,01	0.34
Available P (ppm)*	13.8	15.0	29.5	10.9	19.3	20.8
Na^+ (meq/l) *	459	400	347	433	398	400

*determined in march

**mean of both sampling seasons

Table 3: Mean frequency colonization (F%) of glomeromycetes, septate fungi and chytridiomycetes in *Puccinellia frigida* roots at six ampling sites in three high-altitude Andean wetlands of Northwest Argentina

Fungal type	Glomeromycetes		Septate fungi		Chytridiomycetes	
Sampling site	November	March	November	March	November	March
Brava						
A	0	0	48.6±9.0	46.0±11.3	35.0±9.1	35.6±11.0
B	0	0	40.6±21.3	45.0±7.5	32.0±3.6	31.0±20.8
C	3.0±2.6	4.3±2.0	86.3±14.2	100±0.0	89.6±3.5	97.3±4.6
Veladero						
D	0	0	40.0±14.4	44.6±8.0	19.3±4.7	33.3±8.3
Mulas Muertas						
E	5.3±4.0	2.0±1.7	75.3±7.6	98.3±2.8	68.6±7.0	90.3±3.5
F	0	0	25.3±4.5	50.0±14.9	18.3±4.0	25.3±5.0
Mean[1]	1.3±2.7	1.0±1.9	52.7±24.4	64.0±26.7	44.0±27.4	52.1±31.8
Fungal mean	1.2±2.3		58.3±25.9		48.0±29.5	

Values are means ± SD (n = 6-10). [1] Mean for each fungal type at each sampling season

Table 4: Two way ANCOVA for each fungal type with Season (March and September) and Sites (A-F) as categorical factors, frequency of colonization as dependent factor, and habitat variables as covariates. P-values are considered significant at the 0.05 level.

	df	Chytrids		Septate fungi		Glomeromycetes	
		F	p	F	p	F	p
Site	5	5.76	**0.0023**	18.56	**<0.0001**	12.24	**0.0001**
Season	1	28.86	**<0.0001**	11.39	**0.0033**	0.91	0.35
Site x season	5	14.12	**<0.0001**	5.25	**0.0181**	1.83	0.15
OM	1	4.88	**0.0403**	4.95	**0.0345**	0.83	0.37
EC	1	4.61	**0.0456**	4.78	**0.0417**	0.27	0.61
Moisture	1	2.86	0.11	0.004	0.94	0.3	0.59
pH	1	0.01	0.91	0.01	0.89	1.08	0.31
Available P	1	0.86	0.36	0.78	0.38	0.95	0.34
Na	1	0.04	0.83	0.005	0.94	0.28	0.59
Error	18						

(When the Interaction is significant, means that the season has a different influence at each site on the percent of colonization by each fungal type.)