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Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations

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Abstract The oil sand industry in northeastern Alberta produces vast areas of severely disturbed land. The sodicity of these anthropic soils is one of the principal constraints that impede their revegetation. Previous in vitro studies have shown that the ectomycorrhizal fungi *Laccaria bicolor* (Maire) Orton UAMH 8232 and *Hebeloma crustuliniforme* (Bull) Quel. UAMH 5247 have certain salt-resistant traits and thus are candidate species for the inoculation of tree seedlings to be outplanted on salt-affected soil. In this study, the in vitro development of these fungi was compared to that of three mycorrhizal fungi [*Suillus tomentosus* (Kauff.) Sing., Snell and Dick; *Hymenoscyphus* sp. and *Phialocephala* sp.] isolated from a sodic site created by Syncrude Canada Ltd. Their growth, osmotica and Na/Cl contents were assessed over a range (0, 50, 100, 200 mM) of NaCl concentrations. After 21 days, the two ascomycetes (*Hymenoscyphus* sp. and *Phialocephala* sp.) were shown to be more resistant to the NaCl treatments than the three basidiomycete species. Of the basidiomycetes, *L. bicolor* was the most sensitive to NaCl stress, while *H. crustuliniforme* showed greater water stress resistance, and the *S. tomentosus* isolate exhibited greater Na and Cl filtering capacities and had a better biomass yield over the NaCl gradient tested. Both ascomycetes used mechanisms other than carbohydrate accumulation to palliate NaCl stress. While the *Hymenoscyphus* isolate accumulated proline in response to NaCl treatments, the darker *Phialocephala* isolate may have used compounds such as melanin. The basidiomycete species accumulated mainly mannitol and/or proline in response to increasing concentrations of NaCl.

Keywords NaCl stress · Ectomycorrhizal fungi · Growth · Compatible solutes

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Introduction

Naturally or anthropically created soil sodicity is a cosmopolitan edaphic stress that reduces soil fertility and that drastically affects growth and survival of glycophytes (Brady and Weil 2002; Essington 2004). Living cells are affected by salt stress through disturbance of their osmotic homeostasis. Hyperosmotic stress in plants and fungi is associated with inhibition of cell wall extension and cellular expansion, leading to a reduction in growth (Yeo 1983; Luard 1985; Coleman et al. 1989; Clipson and Jennings 1992; Niu et al. 1995; Hasegawa et al. 2000). Cells under salt stress initially accumulate salts as free osmotica; however, a toxic specific ion effect appears once a certain threshold level of Na and/or Cl accumulation has been reached. An excess of these ions may alter membrane integrity, enzymatic activity, and protein and nucleic metabolism (Yeo 1983; Gadd 1993; Niu et al. 1995; Niu et al. 1997; Hasegawa et al. 2000; Posas et al. 2000; Zhu 2001, 2002; Mansour and Salama 2004). A living organism is considered resistant by avoidance to a given physicochemical stress if it is able to exclude, either partially or completely, the intracellular penetration of the toxicant. By contrast, providing that the organism grows and survives, it is resistant by tolerance if it allows penetration of the toxicant and uses this process to reduce the external stress effects on its metabolism (Levitt 1980; Segner and

Braunbeck 1998). Strategies conveying salt tolerance are thought to involve protection of cell metabolism functions by compartmentalization (e.g. vacuolization) of excess ions while using the available salts (in a controlled amount) and small organic solutes for osmotic adjustment (Clipson and Jennings 1992; Niu et al. 1995; Niu et al. 1997; Yeo 1998; Posas et al. 2000). Organic compounds used as osmotica are often referred to as compatible solutes because they can accumulate to high concentrations, but do not interfere with cell metabolism (Brown and Simpson 1972). Polyols, such as mannitol, arabitol and glycerol, and non-reducing saccharides, such as trehalose, are the main soluble carbohydrates found in basidiomycetes and ascomycetes (Lewis and Smith 1967) including those species forming ectomycorrhiza (Lewis and Harley 1965; Söderström et al. 1988). Levels of mannitol, glycerol and trehalose and some other metabolites, such as the proline amino acid, have been observed to increase in fungi under osmotic stress (Lewis and Smith 1967; Jennings and Burke 1990; Shen et al. 1999) and freezing (Takagi et al. 1997; Tibbett et al. 2002). In fungi, trehalose is used as a reserve carbohydrate. However, it also serves as a protectant against cell damage induced by nutrient limitation, heat shock, oxidative stress and reduced osmotic potential (Arguelles 2000), as it favors stabilization of membranes and proteins (Crowe et al. 1984; Wiemken 1990). Mannitol and glycerol are also potential osmoprotectors (Shen et al. 1999; Tibbett et al. 2002). The latter is known to accumulate in the yeast *Saccharomyces roxii* in response to decreased external osmotic potential (Brown and Simpson 1972) and in response to salinity in a number of other fungi (Jennings 1983, 1984). The water-like hydroxyl group of polyols allows them to form an artificial sphere of hydratation around macromolecules, thus preventing metabolic inactivation under conditions of low osmotic potential (Galinsky and Truper 1994).

The majority of higher plants forms mutualistic symbiosis with mycorrhizal fungi. In return for a carbohydrate supply from the host, the root-inhabiting fungi enhance plant mineral nutrition (Read 1991) and facilitate plant establishment in sites subjected to various edaphic stressors. Osmotic stress (e.g. drought) or toxicity by certain elements (e.g. heavy metals) in the soil can be alleviated by associations with ectomycorrhizal (ECM) fungi (Kropp and Langlois 1990; Kottke 1992; Malajczuk et al. 1994; Marschner and Dell 1994; Pfleger et al. 1994; Smith and Read 1997; Jentschke and Goldbold 2000). In northeastern Alberta (Canada), the mining activities of the oil sand industry create vast amounts of sodic sand tailings. The latter are reclaimed by overlaying with local topsoil material and are then revegetated by planting nursery-grown trees. However, due to manipulations and stockpiling, the organic material used to amend the sites has a low ECM inoculum potential (Bois et al. 2005). Therefore, the inoculation of coniferous trees with salt-resistant ECM fungi prior to outplanting on salt-affected sites could favor host growth and survival. Dixon et al. (1993) showed that other ECM basidiomycetes such as *Laccaria laccata* (Scop.: Fr.) Cooke and *Pisolithus tinctorius* (Mich.: Pers.) Coker & Couch could help improve host tolerance to saline conditions. In addition, Chen et al. (2001) showed, in vitro, that most of 18 isolates of Australian *Pisolithus* spp. tested were tolerant of concentrations of NaCl and Na_2SO_4 exceeding 200 mM.

Several studies investigating metal tolerance of mycorrhizal fungi have shown inter- and intra-specific growth differences between ECM fungal isolates from unpolluted and polluted sites (Colpaert et al. 2000; Sharples et al. 2000; Formina et al. 2005). By contrast, little work has been done on ECM fungal ecotypes from salt-affected sites. An in vitro study by Kernaghan et al. (2002) suggested that *Laccaria bicolor* (Maire) Orton UAMH 8232 and *Hebeloma crustuliniforme* (Bull) Quel. UAMH 5247 were potential candidate species for use under conditions of salt stress. Nonetheless, these species were selected from isolates originating from non-saline sites. In a preliminary study, we isolated three ECM/ectendomycorrhizal fungal species from a sodic site at Syncrude Canada Ltd.: *Suillus tomentosus* (Kauff.) Sing., Snell and Dick; *Hymenoscyphus* sp. and *Phialocephala* sp. As these latter three fungal isolates have potentially undergone selection for adaptation to excess salt and other stresses found on sodic sites, it was hypothesized that these isolates are similarly or more resistant to NaCl than the two previous species selected from an existing in vitro collection (Kernaghan et al. 2002). To test this hypothesis, growth of all fungi was assessed over a range of NaCl concentrations. The species were compared using in vitro radial growth and biomass yields. Furthermore, their individual salt-resistance strategies were characterized by evaluating the water, Na, Cl, trehalose, mannitol, glycerol and proline content of the mycelia at the different NaCl concentrations used.

Material and methods

Origin and growth conditions of the fungi

L. bicolor UAMH 8232 and *H. crustuliniforme* UAMH 5247 were obtained from the University of Alberta Microfungus Collection and Herbarium (Sigler and Flis 1998). The *Suillus*, *Hymenoscyphus* and *Phialocephala* isolates were obtained from mycorrhizal root tips of jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) bait plants grown under greenhouse conditions in soil collected from a sodic site at Syncrude Canada Ltd. (AB, Canada). Control plants that were grown in sterile soil with the same conditions were non-mycorrhizal. The genera were identified by sequencing purified amplicons using DNA extracts from pure mycelium amplified with the primers ITS1 and ITS4 (Operon-Qiagen, Alameda, CA, USA). Sequencing was done using the BigDye Terminator Sequencing Kit (Applied Biosystems, CA, USA) and an ABI genotype 3100 automated sequencer (Applied Biosystems). The sequences obtained were subjected to the Genbank (http://www.ncbi.nlm.nih.gov) sequence homologies search engine. The *Suillus* isolate showed 99% homology with *S. tomentosus* (accession number U74614) and 98% homol-

ogy with *Suillus varietagus* (Schwein.) Kuntze (accession number AJ272418). Phylogenetic analyses confirmed this species to be *S. tomentosus*, a species morphologically and genetically (ITS) very similar to the European taxon *S. varietagus* (den Bakker, personal communication). The *Hymenoscyphus* isolate showed 93% homology with an unidentified *Hymenoscyphus* sp. (accession number AF08 1435). The *Phialocephala* isolate showed 99% homology with *Phialocephala fortinii* Wang & Wilcox (accession number AY078134).

The fungi were grown on modified Melin–Nokrans medium (MNM) (Marx 1969), which contains in 1 l of distilled water: 0.05 g $CaCl_2$, 0.025 g NaCl, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.03 g $FeCl_3$, 100 μg thiamine-HCl, 3 g malt extract, 10 g glucose and 10 g agar. To allow sampling of the mycelia without contamination by sugars or NaCl contained within the medium, a disc of sterilized cellophane was placed on the solidified medium prior to inoculation with a plug of mycelium. The sodic stress was applied by amending MNM to either 0, 50, 100, 200 or 300 mM NaCl. These concentrations are in addition to the 0.025 g of NaCl (or 0.5 mM) found in the MNM medium. Fungal cultures were incubated for 21 days in the dark at 23°C.

Radial growth and biomass yield

The radial growth of each species was monitored on four replicates every 3 days for 21 days. For each colony, radii were measured on two perpendicular axes bisecting the center of the colony and the mean calculated. At the end of the experimental period, all mycelia were detached from the cellophane and the fresh biomass measured. Each sample was oven-dried at 65°C for 3 days and the dry biomass recorded. Colony density was calculated as a function of the dry biomass and of the average surface area of the colony for each NaCl treatment. The surface area was calculated using the mean radius of the colony. Growth of the latter was assumed to be circular. The weight of the inoculum plug was included in dry biomass measures but was considered a negligible bias for between-treatments comparisons.

Tissue analyses

Trehalose, mannitol, glycerol and proline contents were evaluated from 0.3 g of fresh mycelium. The mycelium from three colonies was pooled to give a single replicate, and three replicates per fungus were analyzed for each NaCl treatment. The mycelium was ground in liquid N prior to extraction in 6 ml of a methanol:chloroform:water (12:5:3, v/v/v) solution. The samples were incubated (30 min) at 65°C to stop enzymatic activity. Tubes were centrifuged (10 min at 14,000×*g*), and 1 ml of the supernatant was collected. To induce phase separation, a 0.250-ml aliquot of water was added to the extract. After shaking, the tubes were centrifuged (10 min at 13,000×*g*) and the aqueous phase collected. A 1-ml subsample was collected for proline determination, and a second was evaporated to dryness on a rotary evaporator, solubilized in 1 ml of water and stored at −80°C prior to analysis by high-pressure liquid chromatography (HPLC). Samples were centrifuged (3 min at 13,000×*g*) prior to HPLC analysis. The HPLC analytic system was controlled by WATERS Millennium^{32} software (WATERS, Milford, MA, USA) and comprised a Model 515 pump and a Model 717^{plus} autosampler. Sugars were separated on a WATERS Sugar-Pak column (6.5×300 mm) eluted isocratically at 85°C at a flow rate of 0.5 ml min^{-1} with EDTA (Na^+, Ca^{+2}, 50 mg l^{-1}) and detected on a refractive index detector (Waters, Model 2410). The WATERS Sugar-Pak column could not easily discriminate between sucrose and trehalose; thus, all samples were subsequently separated on a Bio-Rad Aminex HPX-87P column (7.8×300 mm) eluted isocratically with water at 85°C and detected on a refractive index detector (Waters, Model 2410). Peak identity and sugar quantity were determined by comparison to standards.

For proline determination, a 500-μl aliquot of the 1-ml subsample was mixed with 300 μl of a ninhydrine solution [0.125 g of ninhydrine dissolved in 5 ml of H_3PO_4 (6M): glacial CH_3COOH, 2:3, v/v) and 200 μl glacial CH_3-COOH] (Paquin and Lechasseur 1979). Each sample was thoroughly mixed and incubated at 100°C for 45 min. Samples were cooled prior to the addition of 800 μl of toluene. After 45 min, the optical density (OD) of the upper phase (toluene) was assessed by spectrophotometry at 515 nm. The proline content was calculated from the regression curve of OD obtained from standard solutions of pure proline, ranging from 0 to 10 μg.

The mycelium of two dried colonies were pooled for each of three replicates, and their Na and Cl contents were analyzed. Each replicate was crushed in 25 ml of distilled water and incubated for 1 h at room temperature. The Na and Cl content of the water was measured using an Optima 4300DV ICP (Perkin-Elmer).

Statistical analyses

The five fungal species and the five NaCl treatments were distributed according to a completely randomised factorial design. Data from response variables were analyzed as a two-way ANOVA using PROC GLM (SAS system, The SAS Institute, Cary, NC, USA). Assumptions of the ANOVA were checked prior to all analyses. Analysis of radial measurements included time repeat measurements. Contrast analyses were used to compare the different species responses along the sodicity gradient used. The NaCl effect was analyzed using polynomial contrasts. The fungal isolates were analyzed using the following contrasts: (a) *L. bicolor*, *H. crustuliniforme* (from an existing in vitro collection) vs *S. tomentosus*, *Hymenoscyphus* sp., *Phialocephala* sp. (isolated from a sodic site), (b) *L. bicolor* vs *H. crustuliniforme*, (c) *S. tomentosus* (basidiomycete) vs *Phia-*

loxcephala sp. and *Hymenoscyphus* sp. (ascomycetes) and (d) *Phialocephala* sp. vs *Hymenoscyphus* sp.

Results

Growth and water content

All five species exhibited growth over all NaCl concentrations tested; however, there was a significant difference in colony size ($P<0.001$), biomass yield ($P<0.01$), mycelium density ($P<0.001$) and water content ($P<0.001$) (Fig. 1, Table 1). The order in radial size (Fig. 1a) for the different species did not change over time and was identical to that observed at the time of harvest. Time had a significant ($P<0.001$) linear interaction with the fungal isolate and NaCl concentration. Growth parameters measurements indicated that *L. bicolor* was the most sensitive fungus, with a strong decrease in radial growth and biomass yield in response to increasing NaCl concentrations. By contrast, the two ascomycete isolates showed a high resistance over all NaCl treatments. *H. crustuliniforme* and the *S. tomentosus* isolate exhibited similar growth response patterns to the NaCl gradient and were less sensitive to NaCl than *L. bicolor*.

H. crustuliniforme, which, like the *S. tomentosus* isolate, showed planar as well as aerial hyphal development, exhibited the lowest radial growth. By contrast, the remaining three species only exhibited planar growth. In treatments with less than 200 mM NaCl, *L. bicolor* exhibited a higher or similar radial growth to that of the *Hymenoscyphus* isolate and a much lower growth than that of the *Phialocephala* isolate. In the 200- and 300-mM NaCl treatments, radial growth of *L. bicolor* declined and became similar to, or lower than, that of the other basidiomycetes. Both of the ascomycete isolates maintained a high radial growth over all treatments compared to the basidiomycetes. The *Phialocephala* isolate tested exhibited the highest radial growth in all treatments. With the exception of *H. crustuliniforme*, the radial growth of the

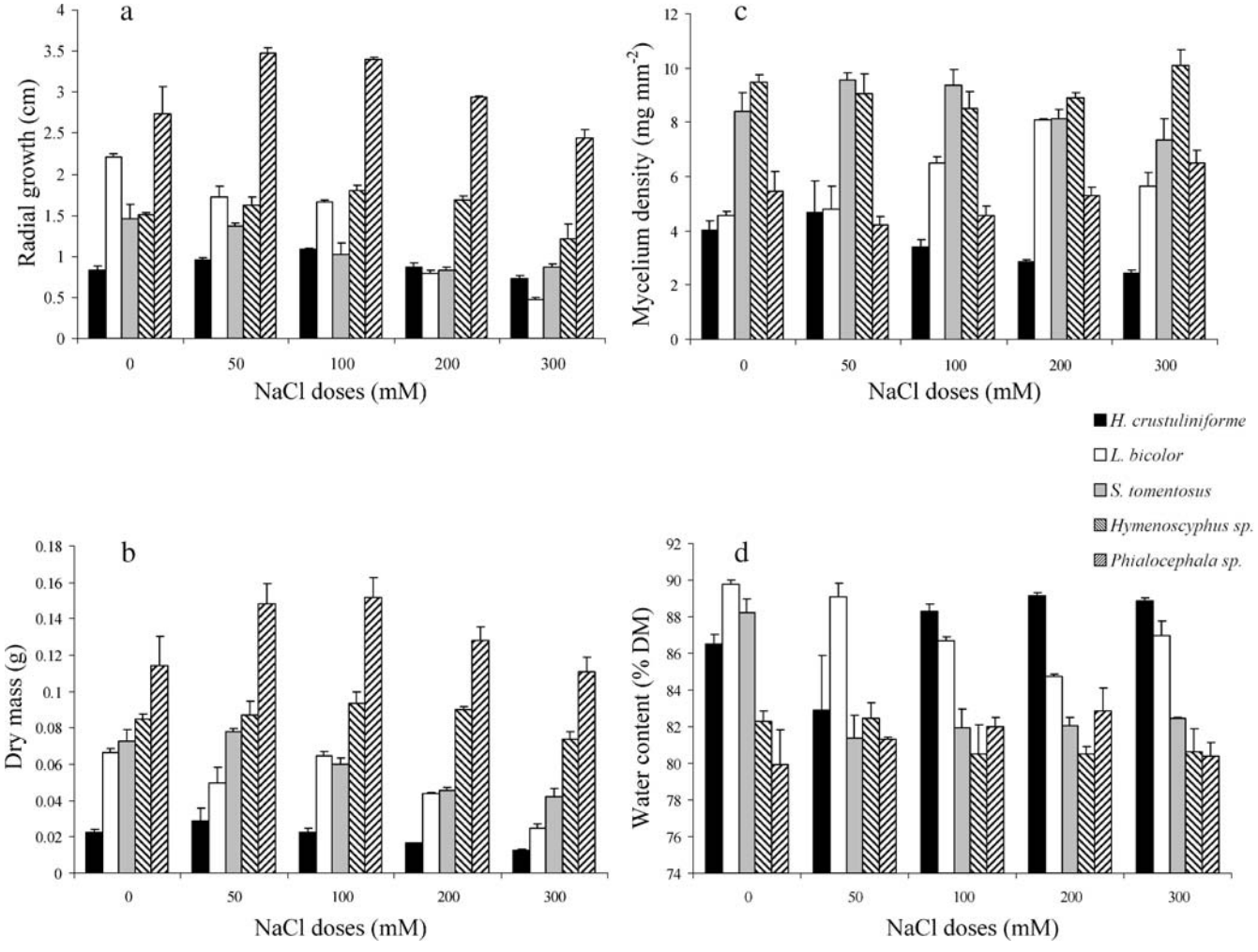

Fig. 1 Growth and water content response of five mycorrhizal species (*H. crustuliniforme*, *L. bicolor*, *S.tomentosus*, *Hymenoscyphus* sp., *Phialocephala* sp.) growing at five NaCl concentrations during 21 days. **a** Maximum radial growth (means±standard errors, $n=4$). **b** Dry mass (DM) (means±standard errors, $n=3$). **c** Density (means± standard errors, $n=3$). **d** Water content (means±standard errors, $n=3$)

Table 1 Two-way analysis of variance of radial growth (n=4), dry mass (DM), water content and mycelium density (n=3) of five mycorrhizal species (*H. crustuliniforme*, *L. bicolor*, *S. tomentosus*, *Hymenoscyphus* sp., *Phialocephala* sp.) at five NaCl concentrations

Sources of variation	*df*	Radial growth	DM	Water content	Density
Between-subject variables					
Species (Sp)	4	372.64***	223.28***	48.01***	105.59***
C1	1	693.07***	502.35***	179.07***	195.64***
C2	1	22.26***	55.59***	NS	55.28***
C3	1	220.83***	204.87***	12.82***	22.74***
C4	1	581.62***	130.31***	NS	148.69***
Salt (Sa)	4	75.74***	15.17***	NS	NS
S1	1	287.77***	45.08***	NS	NS
S2	1	9**	11.66**	NS	NS
S3	1	6.12*	3.91*	NS	NS
S4	1	NS	NS	NS	NS
Sp × Sa	16	15.25***	2.67**	4.62***	4.48***
C1*S1	1	38.16***	NS	NS	NS
C1*S2	1	14.6***	NS	NS	9.67**
C1*S3	1	NS	NS	NS	NS
C1*S4	1	NS	NS	NS	NS
C2*S1	1	15.04***	4.81*	18.14***	17.82***
C2*S2	1	8.7**	NS	5.02*	8.48**
C2*S3	1	NS	NS	5.06*	5.13*
C2*S4	1	6.17*	NS	6.43*	NS
C3*S1	1	108.48***	5.62*	NS	11.98***
C3*S2	1	25.62***	10.35**	13.45***	10.22**
C3*S3	1	NS	NS	5.15*	NS
C3*S4	1	NS	NS	4.36*	NS
C4*S1	1	13.68***	NS	NS	NS
C4*S2	1	NS	NS	5.77*	NS
C4*S3	1	NS	6.13*	NS	NS
C4*S4	1	NS	NS	NS	NS
Residual	72 (50)				
Within-subject variables					
Time	6	2548.27***			
Time*Sp	24	150.64***			
Time*Sa	24	11.59***			
Time*Sp*Sa	96	5.68***			
Residual (time)	432				

df residual: 50 for DM, water content, mycelium density. Contrast on species effect: C1: selected species vs isolated species; C2: *L. bicolor* vs *H. crustuliniforme*; C3: *S. tomentosus* (basidiomycete) vs *Phialocephala* sp. and *Hymenoscyphus* sp. (ascomycetes); C4: *Phialocephala* sp. vs *Hymenoscyphus* sp. Contrast on salt effect: S1: linear; S2: quadratic; S3: cubic; S4: quartic
NS Not significant
*P<0.05; **P<0.01; ***P<0.001

basidiomycete species was reduced with increasing concentration of NaCl. The radial growth of the *Hymenoscyphus* isolate tested was increased with increasing NaCl concentration, and it exhibited the highest growth in the 100-mM NaCl treatment. The *Phialocephala* isolate showed the highest radial growth in the 50-mM NaCl treatment. All fungi exhibited reduced radial growth rates in the 300-mM NaCl treatment compared to that in the control treatment.

H. crustuliniforme had the lowest dry biomass (Fig. 1b) production in all NaCl treatments. The *S. tomentosus* isolate and *L. bicolor* had similar and intermediate dry biomass values, and the two ascomycetes had the highest biomass yield. Dry biomass production of *H. crustuliniforme* and that of the *S. tomentosus* isolate were stimulated in the 50-mM NaCl treatment but were inhibited in the 100-, 200- and 300-mM NaCl treatments. The dry biomass yield of *L. bicolor* declined with increasing NaCl concentrations. By contrast, the two ascomycetes were only inhibited in the 300-mM treatment. In the context of this study, growth stimulation occurred when the amount of dry biomass produced was superior to that of the control; inhibition indicated a reduction in the dry biomass produced compared to the control.

H. crustuliniforme had the lowest density (Fig. 1c) of all fungi, while the *S. tomentosus* and *Hymenoscyphus* isolates had the highest. Mycelium densities of *H. crustuliniforme* and of the *S. tomentosus* isolate were highest in the 50-mM treatment, while that of *L. bicolor* was highest in the 200-mM treatment. The highest density values for ascomycete isolates were obtained in the 300-mM treatment.

On average, the three basidiomycetes tested had a higher water content (Fig. 1d) than the ascomycete isolates tested, over all NaCl concentrations. In the 50-mM treatment, *H. crustuliniforme* had a lower water content than *L. bicolor*; however, in the other treatments, the reverse was observed. The water content of *L. bicolor* generally decreased with

increasing NaCl. In the control treatment, the water content of the *S. tomentosus* isolate was similar to that of *L. bicolor* and *H. crustuliniforme*. However, for the other treatments, its water content decreased and stabilized at 81% and was similar to the values obtained for the ascomycete isolates. The water content of the *Hymenoscyphus* and *Phialocephala* isolates were significantly different ($P<0.05$). For the *Hymenoscyphus* isolate, the water content tended to decrease with increasing salinity, while the water content of the species of *Phialocephala* rose slightly with increasing NaCl concentrations up to 200 mM (significant interaction C4 × S2, $P<0.05$, Table 1).

Stress indicators

Trehalose and mannitol were the two main carbohydrates detected in the tissue samples. In addition, significant amounts of glucose were detected in all five fungi, and therefore, glucose concentrations were included in the analyses. Small amounts of glycerol and fructose were recorded in most of the species, but concentrations were too low for accurate analysis. Compared to the three isolates from the sodic site, both *L. bicolor* and *H. crustuliniforme* had a significantly ($P<0.001$) higher level of trehalose (Fig. 2a, Table 2). *L. bicolor* had a significantly ($P<0.0001$) higher trehalose content than *H. crustuliniforme*, although this pattern was reversed in the 300-mM NaCl treatment. Trehalose content of species isolated from the field was not affected by changes in NaCl concentration and were similar for the three fungi. *H. crustuliniforme* had the highest mannitol (Fig. 2b, Table 2) content, and the amount present increased sharply with increasing NaCl concentrations. By contrast, no mannitol was detected in *L. bicolor* tissues. The *S. tomentosus* isolate had the second highest mannitol content and also exhibited increased content with increasing NaCl concentrations. The *Hymenoscyphus* and *Phialocephala* isolates showed similar and constant mannitol levels over the NaCl gradient tested. All three isolates from the sodic site had a basal level of mannitol at the 0 mM treatment. By contrast, at this level, no mannitol was detected in either *L. bicolor* or *H. crustuliniforme*. Although glucose

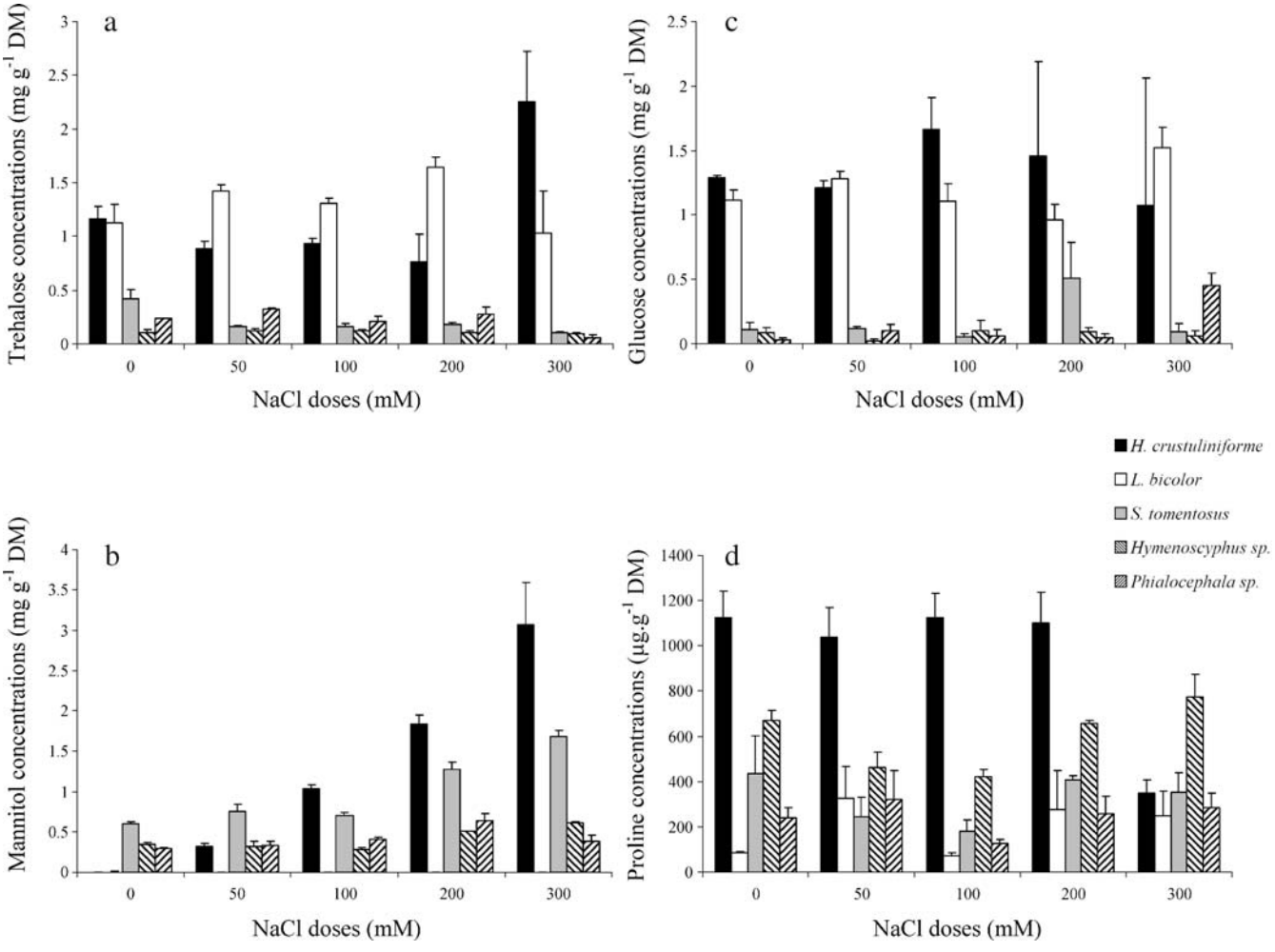

Fig. 2 Carbohydrate and proline content of mycorrhizal ECM species (*H. crustuliniforme*, *L. bicolor*, *S.tomentosus*, *Hymenoscyphus* sp., *Phialocephala* sp.) growing at five NaCl concentrations during 21 days. **a** Trehalose content (means±standard errors, $n=3$). **b** Mannitol content (means±standard errors, $n=3$). **c** Glucose content (means±standard error, $n=3$). **d** Proline content (means±standard errors, $n=3$)

was present in tissues of all species and at all NaCl concentrations, the levels were not affected by the different treatment (Fig. 2c, Table 2). *L. bicolor* and *H. crustuliniforme* had a significantly higher glucose content than the fungi isolated from the sodic site.

The proline content of tissues differed significantly ($P<0.001$) between *L. bicolor* and *H. crustuliniforme*, and the three fungi isolated from the sodic site (Fig. 2d, Table 2). This difference was explained mainly by the proline content of *H. crustuliniforme*, which ranked the highest at all NaCl treatments except 300 mM. The lowest proline content occurred in the *L. bicolor* and *Phialocephala* isolates, and both had in average the lowest proline contents. Although the proline content of the *S. tomentosus* isolate did not differ from that of the ascomycete isolates, it was closer to the values in the *Phialocephala* isolate than to those in the *Hymenoscyphus* isolate. The latter had a significantly higher proline content ($P<0.001$) compared to that of the *Phialocephala* isolate. The three isolates from the sodic site tended to exhibit reduced levels of proline between the control and the 100-mM NaCl treatments. However, levels increased at higher NaCl concentrations. The highest proline content occurred in the *Hymenoscyphus* isolate, in the 300 mM NaCl treatment.

Na and Cl content

All species accumulated increasing amounts of Na and Cl with increasing concentration of NaCl in the growth media (Fig. 3a and b respectively, Table 2). *L. bicolor* and *H. crustuliniforme* accumulated significantly more Na ($P<0.0001$) and Cl ($P<0.001$) than the isolates from the sodic site. *H. crustuliniforme* accumulated more Na and Cl than *L. bicolor*. In all treatments, *H. crustuliniforme* had between two and five times as much Cl as any of the other fungi. All three isolates from the sodic site showed similar Na and Cl accumulation levels.

Table 2 Two-way analysis of variance of trehalose, mannitol, glucose, proline, Na and Cl content ($n=3$) of five mycorrhizal species (*H. crustuliniforme*, *L. bicolor*, *S.tomentosus*, *Hymenoscyphus* sp., *Phialocephala* sp.) at five NaCl concentrations

Sources of variations	*df*	Trehalose	Mannitol	Glucose	Proline	Na	Cl
Species (Sp)	4	83.4***	98.19***	27.89***	55.94***	76.78***	13.98***
C1	1	330.61***	NS	110.47***	23.77***	230.3***	51.43***
C2	1	NS	303.55***	NS	160.71***	63.34***	4.43*
C3	1	NS	89.04***	NS	NS	3.81*	NS
C4	1	NS	NS	NS	35.66***	NS	NS
Salt (Sa)	4	NS	54.53***	NS	2.59*	96.59***	771.78***
S1	1	NS	216.72***	NS	NS	385.96***	2531.45***
S2	1	NS	NS	NS	NS	NS	373.86***
S3	1	NS	NS	NS	6.54*	NS	115.22***
S4	1	NS	NS	NS	NS	NS	9.51**
Sp × Sa	16	5.17***	20.95***	NS	4.14***	2.37*	3.04**
C1*S1	1	12.99***	60.91***	NS	11.65**	6.47*	NS
C1*S2	1	NS	NS	NS	13.74***	NS	7.63**
C1*S3	1	NS	NS	NS	NS	6.72*	NS
C1*S4	1	NS	NS	NS	NS	NS	NS
C2*S1	1	15.47***	237.11***	NS	21.97***	NS	15.47***
C2*S2	1	38.74***	NS	NS	6.61*	NS	NS
C2*S3	1	5.4*	NS	NS	NS	NS	NS
C2*S4	1	NS	NS	NS	NS	NS	NS
C3*S1	1	NS	25.23***	NS	NS	NS	NS
C3*S2	1	NS	NS	NS	NS	NS	NS
C3*S3	1	NS	NS	NS	NS	6.43*	NS
C3*S4	1	NS	NS	NS	NS	NS	NS
C4*S1	1	NS	NS	NS	NS	NS	8.31**
C4*S2	1	NS	NS	NS	NS	NS	9.77**
C4*S3	1	NS	NS	NS	NS	11.13**	NS
C4*S4	1	NS	NS	NS	NS	NS	NS
Residual	50						

Na and Cl values were log-transformed to fit with the assumptions of the ANOVA. Contrast on species effect: C1: selected species vs isolated species; C2: *L. bicolor* vs *H. crustuliniforme*; C3: *S. tomentosus* (basidiomycete) vs *Phialocephala* sp. and *Hymenoscyphus* sp. (ascomycetes); C4: *Phialocephala* sp. vs *Hymenoscyphus* sp. Contrast on salt effect: S1: linear; S2: quadratic; S3: cubic; S4: quartic
NS not significant
*$P<0.05$; **$P<0.01$; ***$P<0.001$

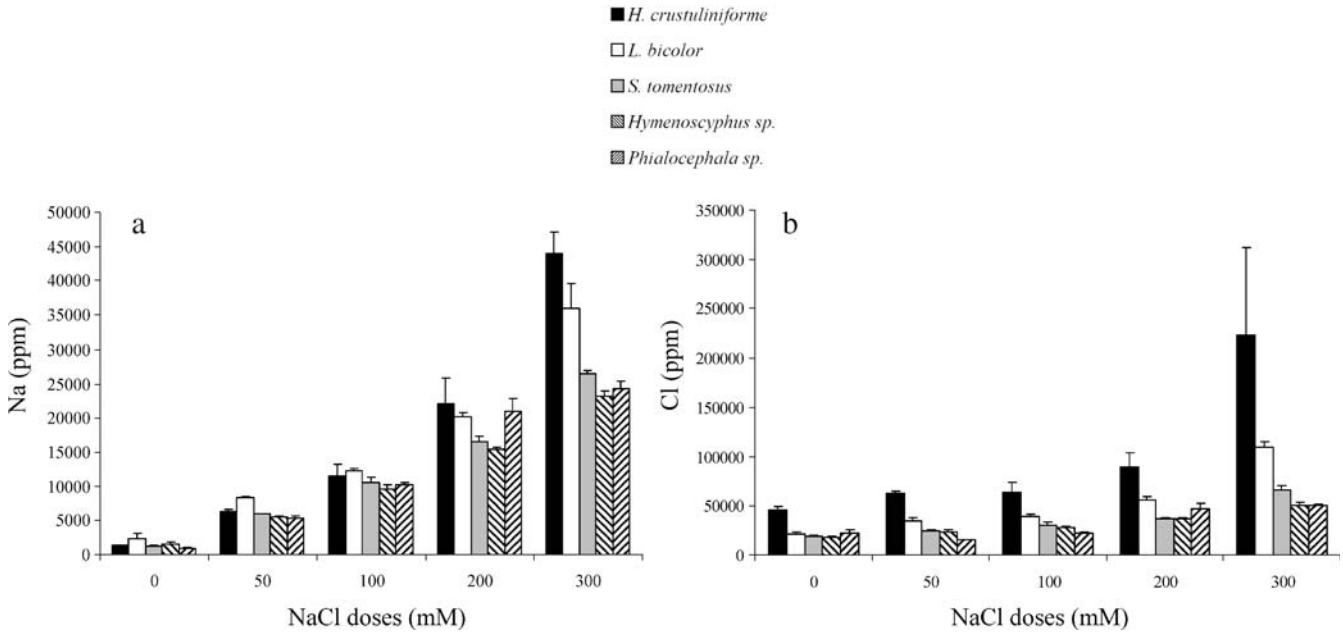

Fig. 3 **a** Na accumulation (means±standard errors, *n*=3) and **b** Cl accumulation (means±standard errors, *n*=3) of five mycorrhizal species (*H. crustuliniforme*, *L. bicolor*, *S.tomentosus*, *Hymenoscyphus* sp., *Phialocephala* sp.) growing at five NaCl concentrations during 21 days

Discussion

Our results clearly show that the three isolates from the sodic site were more resistant to NaCl than *L. bicolor* UAMH 8232 and *H. crustuliniforme* UAMH 5247. The former three species isolates exhibited a significantly higher dry biomass yield and accumulated less Na and Cl than those of the two fungal isolates selected from an existing in vitro collection. The isolates from the sodic site showed a strategy of toxic ion avoidance when subjected to osmotic stress. By contrast, *L. bicolor* and *H. crustuliniforme* exhibited a preferential ion accumulation tolerance mechanism to osmotic stress. The Na and/or Cl toxicity threshold level for the fungi tested could not be determined as all species grew at the highest NaCl concentration tested. Nevertheless, the NaCl threshold concentration between stimulation and inhibition of growth could be approximated. Considering dry biomass yield results, the inhibitory NaCl level of each species should indicate the outset of ionic toxicity. At the inhibiting NaCl levels, water stress cannot be compensated for with available inorganic ions from the external environment, and cells start using organic compounds as osmotica at the expense of growth (Clipson and Jennings 1992; Niu et al. 1997).

Our results showed that the two ascomycete isolates did not counteract the osmotic effect of NaCl by increasing the concentration of sugars. The *Hymenoscyphus* isolate used proline as an osmotica to prevent NaCl stress at 200 and 300 mM. At these concentrations, growth declined, and Na and Cl content of the tissues reached their highest levels. In treatments below 200 mM NaCl, the proline content declined gradually from the control level to 100 mM NaCl. If inorganic osmotica such as Na or K are not present in the growth medium in amounts sufficient to achieve osmotic homeostasis, the fungus might compensate by accumulating proline but at the expense of growth. Therefore, under the conditions tested, 100 mM NaCl appeared to offer the optimum concentration of inorganic osmotica for growth of the *Hymenoscyphus* isolate: proline content was the lowest at this NaCl concentration and biomass yield was maximum. The *Phialocephala* isolate exhibited melanized cell walls. Therefore, this species possibly used other stress metabolites such as melanin as observed by Gadd (1993) on fungal species in response to toxic metals. Fungal melanins in cell walls are known to act as a protective interface between fungal metabolism and biotic and abiotic environmental stressors (Bell and Wheeler 1986; Butler and Day 1998). The *Hymenoscyphus* isolate was a white morphotype but may have also produced lipidic stress metabolites related to melanin as other members of this genus produces such compounds (Vrålstad et al. 2002). Other mechanisms including ion transport and compartmentalization, and oxidative protection (Yeo 1998) may also explain the resistance showed by these isolates. The most abundant carbohydrate in both ascomycete isolates was mannitol. However, the carbohydrate reserves remained low compared to the three basidiomycetes, even without addition of NaCl in the growing media. As fast-growing species, both fungi may have rapidly converted carbohydrates into new organic matter. Sugar from the growing medium was either rapidly used for growth or stored by complexation/conjugation with other cell compounds (e.g. melanin or phenolic stress metabolites). By contrast, the *S. tomentosus* isolate, the only basidiomycete isolated from the sodic site, relied mainly on mannitol and trehalose as reserve carbohydrates. When subjected to

increased NaCl levels, the trehalose content of the *S. tomentosus* isolate decreased, whereas the mannitol content increased. The latter was possibly used as a compatible solute to counteract osmotic stress. Sun et al. (1999) described mannitol as an osmotically potent polyol that accumulates in active hyphal tips of *Suillus bovines* (L.: Fr.) Kuntze. The active mannitol loading and the consequent build up of osmotic pressure drive translocation of water to hyphal tips and therefore promote growth. The surplus water and its solutes would be discriminately exuded from the tips and released into the medium (Unestam and Sun 1995; Sun et al. 1999). The *S. tomentosus* isolate also accumulated proline in conjunction with mannitol at NaCl treatments of 200 and 300 mM NaCl. As discussed above on the *Hymenoscyphus* isolate, the stimulatory effect of NaCl on the growth of the *S. tomentosus* isolate could be related to the reduced production of proline, or other unmeasured organic osmotica, with accumulation of inorganic osmotica. In the present study, the *S. tomentosus* isolate released yellowish phenolic-like compounds (e.g. extracellular melanin (Gadd 1993; Bell and Wheeler 1986)) into the medium around the mycelium. Visual estimation suggested that the concentration of this substance increased with increased NaCl concentrations. Droplets of the same color appeared on the surface of the mycelium. The production and exudation of metabolites could be used for external osmotic adjustment to avoid the need of internal adjustment by the accumulation of Na and/or Cl. Sun et al. (1999) showed evidence that *S. bovinus* exudes mannitol and other osmotica, thereby conditioning the hyphal environment. This exudation–absorption process could possibly be common to a number of *Suillus* species.

Trehalose was abundant in *L. bicolor* and *H. crustuliniforme*. Its concentration was at least three times higher in both species than in the three isolates from the sodic site. This sugar is most likely a reserve carbohydrate as its content varied little in response to NaCl increase for *L. bicolor* and *H. crustuliniforme* in all treatments except the 300-mM treatment. In *L. bicolor*, the trehalose content increased slightly with increasing NaCl concentration, which could enhance cell protection in this species. In the 200- and 300-mM NaCl treatments, the proline content of *L. bicolor* increased and may be used as a complementary osmoticum to counteract NaCl stress. As for the other four fungal isolates, *L. bicolor* accumulated Na and/or Cl as free osmotica but without stimulatory effects. This suggests that, in this fungus, Na and/or Cl was toxic even at the 50-mM treatment and/or that other unmeasured stress metabolites were produced that resulted in reduced growth potential. As water stress is the primary stress prior to the onset of any ion specific effect, most of the response of *L. bicolor* may have been due to its sensitivity to water stress as shown by Coleman et al. (1989). Therefore, the cytoplasmic protection potential of *L. bicolor* against osmotic stress (e.g., organic osmotica) was constrained by species-specific limitations; however, its metabolism was highly sensitive to Na and/or Cl specific ion effect. Under in vitro conditions, *L. bicolor* may have relatively few means of overcoming water stress, but its response may be different when in symbiosis (e.g. different carbohydrates sources). By contrast, *H. crustuliniforme* used mannitol as an osmoprotectant to combat NaCl stress. This is supported by the fact that the mycelium of this species readily accumulated mannitol in response to increasing NaCl levels. Furthermore, in *H. crustuliniforme*, trehalose reserves were reduced in response to increasing NaCl levels, and these only increased in the 300-mM NaCl treatment. In the latter treatment, the proline content of *H. crustuliniforme* fell drastically, indicating a probable switch of resource allocation to sugars that may be better adapted as organic osmotica. Protein synthesis involves more energy than production of carbohydrates (Niu et al. 1997), and the latter may represent better cost/benefits for the fungal growth at high NaCl concentrations. This result is in accordance with Tibbett et al. (2002), who observed increasing level of mannitol and trehalose in several isolates of *Hebeloma* under increasing cold stress.

The composition of the growth medium had an important influence on the response of the mycelia (Lewis and Smith 1967; Pfyffer and Rast 1988) to NaCl stress, and although these fungi may react differently when in symbiosis, we can still make inferences about their potential use for inoculation of seedlings produced for the revegetation of sodic sites. Among the three fungi isolated from the sodic site, the *Hymenoscyphus* and the *Phialocephala* isolates exhibited the best NaCl resistance potential. This confirms the salt stress tolerance abilities of pioneer symbiotic species and suggests that certain *Hymenoscyphus* species may have a number of physiological and biochemical attributes allowing them to withstand a wide range of environmental stresses (Hashem 1995; Read and Kerley 1995; Read 1996; Souto et al. 2000; Vrålstad et al. 2000). However, the *Hymenoscyphus* and *Phialocephala* isolates obtained in the present study are probably not of primary interest for revegetation purposes. Firstly, their mutualistic association with coniferous trees is not clear (Jumpponen and Trappe 1998; Jumpponen 1999; Vrålstad et al. 2000; Vrålstad et al. 2002): species of *Hymenoscyphus* are known to associate with pioneer plant species (e.g. species of the Ericacae), and species of *Phialocephala* show little host specificity (Jumpponen and Trappe 1998; Jumpponen et al. 1998; Jumpponen 1999, Vrålstad et al. 2000). Nevertheless, Jumpponen et al. (1998) showed that *Pinus contorta* colonized by *Phialocephala fortinii* gave a typical mycorrhizal growth response on an environmentally stressed (low N and organic matter) glacier forefront. Secondly, these two pioneer species might not persist on coniferous roots in the long term. By contrast, the *S. tomentosus* isolate is of greater interest. *Suillus* species are known to be an ECM associate of pine and spruce species during early and middle successional stages and thus would likely persist on transplanted seedling roots. In addition, its biomass production under NaCl stress was similar to or better than that of *L. bicolor* and *H. crustuliniforme*. Moreover, like the other fungi isolated from the sodic site, it might also have certain abilities to tolerate other stresses found on the study site. It is likely that strains of ECM fungi surviving in oil sand tailings have undergone severe selection pressure and

eventually eco-adaptation (e.g. epigenetic processes and genome rearrangement; Niu et al. 1997; Lerner 1999; Arnholdt-Schmitt 2004) to many of the physico-chemical stresses characterizing such environments (e.g. excess salt, water stress, temperature extremes and toxic oil residues). Although, the *S. tomentosus* isolate would probably have better Na and Cl filtering capacities when in symbiosis compared to *L. bicolor* and *H. crustuliniforme*, the latter showed a high osmotic adjustment potential that allowed it to maintain its water content. This capacity came possibly from the apparent diversity of organic and inorganic osmotica accumulated by this species. Indeed, different osmotica work in coordination avoiding crystallization caused by excessively high concentrations of a single osmoticum in case of severe stress (Niu et al. 1997). This observation tends to confirm the conclusion of Mushin and Zwiazek (2002) that *H. crustuliniforme* improves the water flux of its host. Such a characteristic makes this fungus another good candidate for inoculation of nursery seedlings. Nevertheless, it readily accumulated Na and Cl, which might be deleterious for its host in the long term. From the result obtained, *L. bicolor* UAMH 8232 may not have the qualities necessary for revegetation of sodic sites: it accumulated more Na and Cl than the *S. tomentosus* isolate and was the most sensitive fungi to increasing NaCl concentrations. To confirm these results, the NaCl resistance of the three basidiomycetes must be tested in symbiosis.

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