Mycorrhiza (2011) 21:117–129
DOI 10.1007/s00572-010-0316-4

ORIGINAL PAPER

Elemental composition of arbuscular mycorrhizal fungi at high salinity

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Received: 14 January 2010 / Accepted: 26 April 2010 / Published online: 25 May 2010

Abstract We investigated the elemental composition of spores and hyphae of arbuscular mycorrhizal fungi (AMF) collected from two saline sites at the desert border in Tunisia, and of *Glomus intraradices* grown in vitro with or without addition of NaCl to the medium, by proton-induced X-ray emission. We compared the elemental composition of the field AMF to those of the soil and the associated plants. The spores and hyphae from the saline soils showed strongly elevated levels of Ca, Cl, Mg, Fe, Si, and K compared to their growth environment. In contrast, the spores of both the field-derived AMF and the in vitro grown *G. intraradices* contained lower or not elevated Na levels compared to their growth environment. This resulted in higher K:Na and Ca:Na ratios in spores than in soil, but lower than in the associated plants for the field AMF. The K:Na and Ca:Na ratios of *G. intraradices* grown in monoxenic cultures were also in the same range as those of the field AMF and did not change even when those ratios in the growth medium were lowered several orders of magnitude by adding NaCl. These results indicate that AMF can selectively take up elements such as K and Ca, which act as osmotic equivalents while they avoid uptake of toxic Na. This could make them important in the alleviation of salinity stress in their plant hosts.

Keywords PIXE · Saline soils · Arid · Sodium · Potassium · Chloride

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Introduction

The salinity of soils is a considerable problem in many parts of the world. This is particularly the case in regions with high rates of evaporation, where salts are easily accumulated in the topsoil. Increased irrigation is needed to combat the spread of deserts and to meet the greater demand for food of a growing world population, but at the same time, inappropriate irrigation management leads to the accumulation of salt in poorly drained soils. According to the UN, more than 20% of the world's irrigated land is contaminated by salt (FAO 2005). Most crops grow poorly under saline conditions, making the pursuit of agriculture difficult.

Salinity affects organisms primarily in three ways: (1) through water shortage due to the low water potential of the saline soil solution, (2) by ion imbalance due to a relative shortage of essential nutrients compared to salt ions, and (3) by cell toxicity caused by the accumulation of salt ions (Evelin et al. 2009). To cope with the water shortage resulting from salinity, plants can either accumulate low molecular weight organic molecules (Kogej et al. 2007), or non-toxic ions (Läuchli and Lüttge 2002). Salt-tolerant

plants can be categorized into excluders or includers according to the strategy employed: excluders try to prevent excessive uptake of salt ions, while includers take up salt but store it outside the cytosol, for example, in vacuoles, or secrete it through special salt glands (Munns 2002). Salt tolerance levels have been studied in halophytic and glycophytic fungi (Tresner and Hayes 1971), but, to the best of our knowledge, no studies have been carried out to elucidate the strategies employed by arbuscular mycorrhizal fungi to cope with excessive levels of salt.

Arbuscular mycorrhizal fungi (AMF) can be found under severe saline conditions in nature, both in saline inlands (Aliasgharzadeh et al. 2001; Nasr 2003) and in salt marshes (Wolfe et al. 2007; Wilde et al. 2009). Many greenhouse experiments have shown that they can promote plant growth under salinity stress (e.g., Zuccarini and Okurowska 2008; Wu et al. 2010) by providing nutrients (Ruiz-Lozano and Azcón 2000) and alleviating water stress (Augé 2001; Sheng et al. 2008). Improved plant growth under conditions of excess salinity is often explained by better P nutrition leading to higher plant growth, and the fact that a larger plant biomass results in dilution of the toxic ions (Hirrel and Gerdemann 1980; Al-Karaki and Hammad 2001). Mycorrhizal plants grown under saline conditions often have a higher K:Na ratio (Giri et al. 2007; Sannazzaro et al. 2006; Rabie and Almadini 2005), and a lower shoot Na concentration (Al-Karaki and Hammad 2001) than non-mycorrhizal plants. Plant cultivars with higher salt tolerance usually have a greater ability to maintain low Na levels in the cytoplasm (Hajibagheri et al. 1989). Na can easily enter plant cells via non-selective K^+ channels (Demidchik et al. 2002), but generally, K is the preferred ion in the cytoplasm, providing a reactive surrounding for enzymes, being an osmotic equivalent under conditions of water stress and high salinity. The K:Na ratio reflects the level of salt stress of an organism, and its ability to discriminate against the toxic Na ions in favor of the essential and chemically similar nutrient K. Also the Ca:Na ratio is an important measure of salinity stress since Na replaces Ca in the plasma membrane and cell wall when Na levels are high, reducing cell turgor and hydraulic conductivity, and disturbing Ca signaling (Läuchli and Lüttge 2002). Therefore, additional Ca can alleviate salinity-induced growth reduction (Lazof and Bernstein 1999). Hence, sodic soils are defined based on the ratio of Na to Ca and Mg, since the electric conductivity (EC) alone does not provide sufficient information about the potential salinity stress in a soil (Brady and Weil 2008).

Important morphological barriers for ion selection in plants are the root hair membrane, the Casparian strip and, before transfer to the shoot, the xylem membrane (Kramer 1983; Tester and Davenport 2003). Mycorrhizal fungi may act as a very first barrier for ion selection, but this has not been studied previously. Ion discrimination could take place during the fungal uptake of nutrients from the soil or during transfer to the plant host. Similar discrimination of ions has been reported for heavy metals, whose concentration in plants can be reduced by colonization by AMF. The main aim of this study was to investigate whether AMF can prevent uptake of toxic Na in saline areas. In addition, we wanted to examine whether AMF accumulated elements that could act as osmotic equivalents in response to salinity, such as Ca and K. We quantified the elemental composition of AMF spores and hyphae extracted from saline field soils (Study 1), and those of *Glomus intraradices* grown in vitro with no or high additional NaCl (Study 2) using proton-induced X-ray emission (PIXE), combined with scanning transmission ion microscopy (STIM). We treated AMF as a functional group, examining a native species mixture, potentially colonizing the plants in the studied habitats. In order to relate the elemental content of the AMF sampled in the field to its environment, we also analyzed the elemental composition of the irrigation water, the surrounding soil, and the associated plants. We expected the elemental composition of the fungi to reveal information about their salinity defense mechanisms, and to shed light on the question of whether AMF can alleviate salt stress in plants by pre-selecting nutrients to prevent toxic salt ions from entering the plant. We particularly hypothesized that AMF may discriminate against Na, and in contrast, disproportionally accumulate mineral ions capable of osmoregulation, such as K and Ca, in their tissues. PIXE analysis has been used successfully for the investigation of plant (Scheloske et al. 2004; Orlic et al. 2002) and fungal material (Wallander and Pallon 2005; Pallon et al. 2007; Král et al. 2005; Olsson et al. 2008), especially for the investigation of heavy metal accumulation (Scheloske et al. 2001; Turnau et al. 2001; and reviewed in Turnau and Kottke 2005). This is the first time that the elemental composition of AMF sampled in the field has been quantified, and the first time Na and Mg are included in the PIXE analysis of AMF tissue.

Materials and methods

Field study: experimental sites

The main experiment was performed at the Experimental Farm of Biosaline Agriculture in El Hicha, Gabes, southern Tunisia (34°42′N; 7°58′E) (Site 1). The area is arid with 164 mm yearly precipitation and a mean annual temperature of 20.2°C. The soil is alkaline (pH 8.6, Table 1), saline, and contains gypsum; the structure is dominated by silt, and it has a low organic matter content (1.3% dry weight loss on ignition at 600°C). The site is irrigated with saline water

Table 1 Soil characteristics and amounts of the AMF signature fatty acid 16:1ω5 at the two investigated saline sites in El Hicha (Site 1) and in Mahdia (Site 2) in Tunisia

	Site 1	Site 2
Soil structure	Silty loam	Sandy loam
pH	8.6	8.3
EC 1:5 [dS/m]	2–3	5–13
Loss on ignition	1.3%	4%
PLFA 16:1ω5 [μg g^{-1} soil]±SE	6.0±0.3	0.8±0.09
NLFA 16:1ω5 [μg g^{-1} soil]± SE	55±10	1.9±0.3

from wells in shallow aquifers. The EC of the irrigation water from the different wells varied from 5.6 to 11.5 dS/m; and the soil salinity at the time of sampling varied from 2 to 3 dS/m in 1:5 soil:water slurry ($EC_{1/5}$). The natural vegetation is dominated by shrub halophytes and xerophytes. Soil samples were taken from an *Acacia cyanophylla* plantation in eight spatially distant plots. Samples from the eight plots were used for fatty acid analysis, four of them for elemental analysis of the soil and plants, while all eight samples were pooled into one composite sample for spore analysis. *A. cyanophylla* is a tree of the family Fabaceae that can form mutualistic association with the nitrogen fixating bacteria rhizobia (Nasr et al. 1999). A tripartite symbiosis, where a plant is associated with both AMF and rhizobia, can synergistically enhance plant performance (Chalk et al. 2006). AMF and rhizobia colonize the plant simultaneously and generally do not compete for infection sites (Barea et al. 2005). *A. cyanophylla* trees of this study were nodulated, but no further quantification of N fixation was performed in this experiment.

Samples were also collected from a second site near Mahdia (Site 2) in a salinity gradient facing Sebkhat Sidi El Hani (35°29′N 10°28′E), a seasonal salt lake. The area is used for livestock grazing. Soil samples were taken from a section of the gradient where the salinity varies from 5 and 13 dS/m ($EC_{1/5}$), n=8. Those eight samples were used for fatty acid analysis, four of those for soil elemental composition, while spores were extracted from a pooled composite sample. The soil structure is a sandy loam with a pH of 8.3 (Table 1). The vegetation was dominated by halophytes such as *Atriplex halimus, Atriplex glauca, Arthrocnemum perenne*, and *Suaeda aegyptica*. No data for plant elemental composition is available for Site 2.

Extractable ions in the soil samples were analyzed after 0.2 M $BaCl_2$ extraction of Al, Ca, Fe, K, Mg, Na, P, S, and Si with inductively coupled plasma atomic emission spectroscopy (ICP-AES) and with ion chromatography for Cl after water extraction. At Site 1, we also analyzed the dissolved ions of the irrigation water, and the roots and leaves of four randomly selected *Acacia* plants growing in the four subplots. The total amounts of the same elements in roots and leaves were determined using ICP-AES after high pressure microwave digestion in hot HNO_3. The P-fixation capacity of the pooled soil samples from Site 1 was determined separately by adding 20 mL g^{-1} soil of a 5.0 mM PO_4 solution, followed by extraction using a solution of 0.05 M HCl and 0.025 M H_2SO_4 (Mehlich method, Black et al. 1965).

Fatty acid analysis Samples of 2 g freeze-dried soil were analyzed for their fatty acid content. Lipid extraction was performed according to Frostegård et al. (1991). The extracted lipids were fractionated into neutral lipids, glycolipids, and polar lipids on a silicic acid column (Bond Elut, Varian Inc., Palo Alto, CA, USA) by successively eluting with chloroform, acetone, and methanol. The chloroform fraction (containing the neutral lipids) and the methanol fraction (containing the phospholipids) were subjected to mild alkaline methanolysis to transform the PLFAs and the NLFAs into free fatty acid methyl esters. These were analyzed on a gas chromatograph with a flame ionization detector and a 50-m HP5 capillary column according to Frostegård et al. (1993). The amounts of the PLFA 16:1ω5 and the NLFA 16:1ω5 in the different experimental plots were determined and used as indicators of the AM fungal biomass. The PLFA 16:1ω5 is not entirely specific to AM fungi since it can occur in some bacterial groups, which though rarely produce NLFA 16:1ω5. Thus, the majority of this PLFA is considered to originate from AM fungi when the ratio of NLFA and PLFA 16:1ω5 is above 1 (Olsson 1999).

AMF species At Site 1, mycorrhization of the roots of *A. cyanophylla* was confirmed microscopically after clearing in 80°C KOH and staining with Trypan Blue (Phillips and Hayman 1970). Spores were extracted from the soil by wet sieving and glucose gradient centrifugation according to Brundrett et al. (1996). Spores were analyzed morphologically according to Schenk and Perez (1990) and the INVAM database (www.invam.caf.wvu.edu). The spore biovolume was calculated according to the equation $V = 3/4\pi r^3$, using mean spore radii in five categories (12.5–100 μm).

Laboratory study: in vitro cultures

For examination of the elemental composition of a non-salt stressed AMF, and of changes especially in Na, K, and Ca caused by high NaCl addition to its growth medium, we

grew *G. intraradices* Schenck and Smith (DAOM 197198; Biosystematics Research Center, Ottawa, Canada) in root organ cultures of *Daucus carota* L. (Bécard and Fortin 1988) under sterile conditions in two-compartment Petri dishes. The transformed *D. carota* roots are a clone of the DC1 line, transformed with T-DNA from the Ri plasmid of *Agrobacterium rhizogenes* (Bécard and Fortin 1988), originally established by St. Arnaud et al. (1996). Plates were inoculated by plug transfer from monoxenic mother cultures, and cultures were maintained on a minimal nutrient medium (Bécard and Fortin 1988) containing 10 g/L sucrose, a low phosphorus concentration of 35 µM (4.8 mg/L KH_2PO_4) and 0.3% Phytagel™ for stabilization (Sigma Chemical Co., St. Louis, MO, USA). For PIXE analysis, AMF hyphae and spores were harvested after 300 days of growth. To test for the salinity tolerance of *G. intraradices*, we used split plates with a fungal compartment containing liquid medium without sucrose and phytagel. Control plates received plain M-medium into the fungal compartment, and treatment plates received additional NaCl at a concentration of 250 mM.

PIXE analysis With PIXE, in combination with STIM, it is possible to identify, quantify, and locate the elements in a sample. PIXE is a multi-elemental analytical technique based on the detection of characteristic X-rays produced by bombardment with MeV ions (protons or heavier ions). It is analogous to electron-induced energy-dispersive X-ray analysis (EDX), but its sensitivity is much higher allowing elements to be detected at the ppm level. The analyses were performed at the Lund Nuclear Microprobe Laboratory, where a focused, micrometer-sized beam was scanned across the sample. Data were acquired for elemental analysis at several points on the sample. STIM measurements (Overley et al. 1988; Lefevre et al. 1987) were performed in on-axis/off-axis geometry simultaneously with the PIXE measurements (Pallon et al. 2003; Pallon 1987), which has the advantage that it permits simultaneous PIXE and STIM measurements. The STIM data were used to create images of the sample and to determine the mass per unit area of the sample. STIM is based on the detection of energy loss when protons pass through the sample.

AMF spores and their attached hyphae were extracted from the soil by wet sieving and sucrose centrifugation, as described by Brundrett et al. (1996). Extracted spores were mounted between two layers of KimFoil© brazed to a specimen ring. Mycelium from monoxenic cultures were treated as described by Olsson et al. (2008). Twelve spores from Site 1, three spores from Site 2, and each four monoxenically grown spores were analyzed. Proton beams of 1.8 and 2.5 MeV were used for the analysis of the main experiment, with a beam current of 300 pA focused to a spot of about 5 µm. Each sample was typically scanned accumulatively over an area of 256×256 µm for 25–35 min, or until the data collected gave sufficiently detailed elemental maps of at least P, S, K, Ca, and Fe. In some cases, several spores on each mounted sample were analyzed.

After analysis, quantitative images were created using the CSIRO Dynamic Analysis method (http://www.nmp.csiro.au/dynamic.html), which enables true elemental images to be resolved from the complex PIXE energy spectrum (Ryan and Jamieson 1993). The program is based on the software package IDL 6.2 (Research Systems, 2002: www.ResearchSystems.com). The elemental images were inspected to identify regions for precise quantitative evaluation. Detailed PIXE spectra were constructed by re-sorting the data, and the spectra were fitted to yield quantitative information. The local mass per unit area of the sample was determined from the corresponding STIM spectra, and the elemental content per unit dry weight (freeze-dried) was calculated.

Spore # 4 (Fig. 1a) from Site 1 was found to be empty after PIXE analysis. It showed an exceptionally high P content per unit biomass, but mass and most other elements were present at very low amounts. Data from this spore were therefore excluded from the analysis. Spore # 3 (Fig. 1b) from Site 2 was contaminated by mineral material, easily identifiable by the high amount of Si. Data from the contaminated areas were excluded from elemental quantification.

Experimental comparison of PIXE and ICP-AES We compared the results obtained with PIXE and ICP-AES using elemental composition data of in vitro cultures of *G. intraradices* Schenck and Smith. We harvested 2.4± 0.1 mg mycelium per plate (mean±SE, n=4) of 60-day-old mycelium from the hyphal compartment, which is sufficient for elemental analysis in ICP-AES. Each four spores and attached hyphae from two plates were prepared according to Olsson et al. (2008) and analyzed with PIXE using a 2.0 MeV proton beam. PIXE can distinguish between the two different types of tissue, i.e. hyphae and spores, which may differ in their elemental composition, while ICP-AES cannot. Therefore, we calculated a proxy assuming that the spores to hyphae weight ratio was 4:1.

Statistics ANOVA, correlations, and multivariate principal component analysis were performed employing JMP 7 (SAS Institute Inc.). Due to inhomogeneity in variation, tests were performed on log-transformed data. To test for differences between more than two means, Tukey's all pairs Honestly Significant Difference test was performed. Error bars show the standard error (SE).

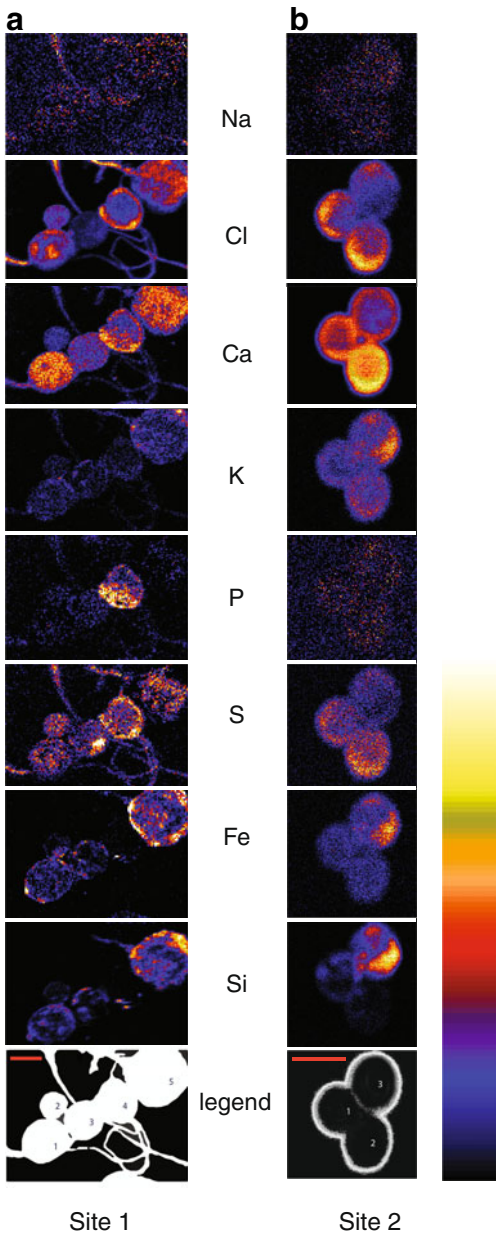

Fig. 1 PIXE generated elemental maps: scanned areas of spores and hyphae from the saline fields of Site 1 (**a**) and spores from Site 2 (**b**), selected specifically to illustrate the distribution of Na, Cl, Ca, K, P, S, Fe, and Si within the tissue. The bar to the right shows the gradient from low to high elemental concentration. The gradient scale indicates the variation in elemental concentration within a specific graph and should not be used to compare between different graphs. Size scale bars=100 μm

Results

Field study

PIXE analysis revealed high levels of several elements in the AMF tissue from the saline sites, especially Ca and Cl (Fig. 1; Table 2). Na concentrations, in contrast, were much lower than its counterion Cl. No accumulation of Na was found in AMF spores from either of the saline sites compared to the irrigation water or soil (p=0.13 for spores vs. irrigation water, with a tendency to be lower; p=0.14 and 0.96 for spores vs. soil at Site 1 and Site 2, respectively; Tables 2 and 3).

The K:Na ratio in the fungal spores was about 20 times higher than that in the irrigation water and 2.2 times higher than the soil at Site 1 (Fig. 2a). There was another fourfold increase between AMF and associated plant roots. The Ca:Na ratio was increased 36 times in spores compared to the irrigation water, or 3.8 times compared to the soil in Site 1 (Fig. 2d). Hyphae and roots had the same Ca:Na ratio (12 times higher than in the irrigation water), while plant leaves showed another fivefold increase in the Ca:Na ratio. At Site 2, we found a 60-fold increase in the K:Na ratio and an 11-fold increase in the Ca:Na ratio in spores compared to soil (Fig. 2b and e). The differences in the K:Na and Ca:Na ratios between plants and AMF were mainly due to the increase in the K and Ca concentrations in plants, while the Na concentration remained in the range of 1,000–2,000 ppm in both AMF and plants.

The contents of Cl, K, and Ca (possible osmotic equivalents) were strongly enhanced in AMF tissue compared to the soil at Site 1 (Fig. 3). AMF spores at Site 2 also accumulated significant amounts of Cl (threefold), but even more so for K (60-fold), and high levels of Ca and Mg were also found (both tenfold). While Na and Cl concentrations in irrigation water and soil were rather balanced, 13 and 4 times more Cl was accumulated in AMF, compared to the levels of Na in spores, at Sites 1 and 2, respectively. AMF at both sites and plant roots at Site 1 were rich in Si (Table 2, Table 3). The plants accumulated mainly K, Cl, and Ca in their roots and leaves. No accumulation of Mg was found in AMF tissue at Site 1, while it was significantly enhanced in the plant roots. The soil from Site 2 had a higher electrolytic conductivity, and in general a higher content of all measured ions (Table 3). Spores from Site 2 contained higher amounts of all elements than of those from Site 1.

The detectable elements with the lowest concentration in AMF were Mn and P. The soils from both sites were extremely low in P; the amounts being close to or below the detection limit with ICP-AES after 0.2 M $BaCl_2$ extraction, and we found a P-fixing capacity of above 99% absorption. The level of P in the hyphae from Site 1 was in the same range as that in the associated plant roots, while P content of the spores was lower at both sites (Fig. 4a, b).

The highest variability within the spores from Site 1 was found for Fe (coefficient of variation (CV 48%), while the lowest variability between the different AMF spores was found for the Ca contents (CV 14%). Images of the spatial distribution of the different elements revealed even distri-

Table 2 Elemental composition of spores and hyphae from different AMF at Site 1 and Site 2, as revealed by PIXE [μg g^{-1}]. b.d. = below detection limit

		Na	Mg	Al	Si	P	S	Cl	K	Ca	Ti	Fe	Mn	Cu	Zn
Site 1															
Spores	(n=12)	780	500	900	3,200	52	390	10,200	640	9,600	100	800	35	60	160
SE		64	48	100	400	5	18	1,100	71	380	12	110	3	5	11
Hyphae	(n=4)	2,100	92	460	1,600	310	1,600	50,000	1,500	8,700	25	220	b.d.	b.d.	b.d.
SE		260	46	230	760	54	152	5,800	180	1,000	13	67			
Site 2															
Spores	(n=3)	4,900	9,600	7,900	10,000	190	7,200	19,000	8,900	120,000	320	1,900	260	14,000	610
SE		360	1,200	5,300	9,800	36	730	2,100	950	11,000	321	4,000	16	150	37

butions for many elements, such as Na, and K at both sites, and Ca at Site 1. In contrast, Cl was accumulated in certain areas within the AM fungal spores, and especially in the hyphae (Fig. 1a). Ca occurred in the structure of the spore wall, and there was a co-location of Cl and S in the spores from Site 2 (Fig. 1b).

Laboratory study: in vitro cultures of *G. intraradices*

Na levels in the non-salt-stressed monoxenically grown *G. intraradices* were around 350 μg/g dry weight, i.e., lower than the AMF isolated from the saline fields (Table 4). Na concentration was higher than Mg or Cl concentrations, but lower than K or Ca concentrations. Under conditions without NaCl addition, the K:Na and Ca:Na ratios were in the same range as in AMF isolated from saline soils (Fig. 2c and f). Even though those ratios within the growth environment were lowered several orders of magnitude by the addition of 250 mM NaCl to the medium, the internal K:Na and Ca:Na ratios in spores of *G. intraradices* remained rather constant (Fig. 2c and f).

AMF abundance and diversity The amounts of the AMF signature fatty acids PLFA 16:1ω5 and NLFA 16:1ω5 in the soil were 6 and 55 nmol g^{-1} soil, respectively, at Site 1 and 0.8 and 1.9 nmol g^{-1} soil, respectively, at Site 2 (Table 1). At Site 1, the spore density was 110 spores g^{-1} soil. Spores were separated into 17 spore morphotypes, 10 of which were determined to species level (Table 5). The most frequent among these morphotypes were the small spores of *G. cerebriformae*, with a relative abundance of 34.5% based on spore number. However, based on spore volume, *G. aggregatum* and *G. mosseae* and an unidentified morphotypes were most abundant, each of them contributing to between 10 and 20% of the total spore biovolume. *G. cerebriformae* on the other hand, contributed only 1.1% to the spore biovolume.

Comparison between PIXE and ICP-AES The elemental data obtained by two methods PIXE and ICP-AES was strongly correlated (R^2=0.77, p=0.004, Fig. 5). A few elements (S, Ca, and P) seemed to be overestimated by PIXE or underestimated by ICP-AES. There was in general greater variation in the PIXE measurements. This may be explained by the fact that we used several magnitude smaller samples for PIXE than for ICP-AES, due to the different sensitivities of the methods.

The significant correlation between the methods allowed the comparison between the elemental compositions of the AMF and their growth environment, i.e. the elemental composition of the irrigation water, the soil, and the associated plants.

Table 3 Elemental composition [μg g^{-1}] of the soil, plant roots and leaves at Site 1, and the soil at Site 2, according to ICP-AES analysis (n=4). $BaCl_2$ extraction was applied to the soil and hot HNO_3 microwave digestion was applied to plant and root material before analysis. n.d. = not determined, b.d. = below detection limit

	Na	Mg	Al	Si	P	S	Cl	K	Ca	Fe
Site 1										
Soil	340	190	0.05	20	0.2	1,900	340	130	1,100	0.003
Roots	2,100	2,220	b.d.	3,010	330	2,340	6,210	7,110	10,500	970
Leaves	1,700	5,180	b.d.	680	480	12,000	5,480	9,080	39,700	210
Site 2										
Soil	4,950	900	8.7	n.d.	b.d	9,410	6,870	150	11,200	4

Table 4 Elemental composition of spores and hyphae of *G. intraradices* grown in root organ culture, as revealed by PIXE [μg g^{-1}]. b.d.= below detection limit

		Na	Mg	Al	Si	P	S	Cl	K	Ca	Ti	Fe	Mn	Cu	Zn
Spores	(*n*=8)	320	160	210	140	810	140	23	1,090	1,200	15	48	9	15	65
SE		12	6	27	18	69	8	2	81	78	4	3	2	3	9
Hyphae	(*n*=5)	360	140	b.d.	21	120	50	12	530	2,500	b.d.	b.d.	b.d.	b.d.	b.d.
SE		170	28	0	4	23	10	2	106	510	0	0	0	0	0

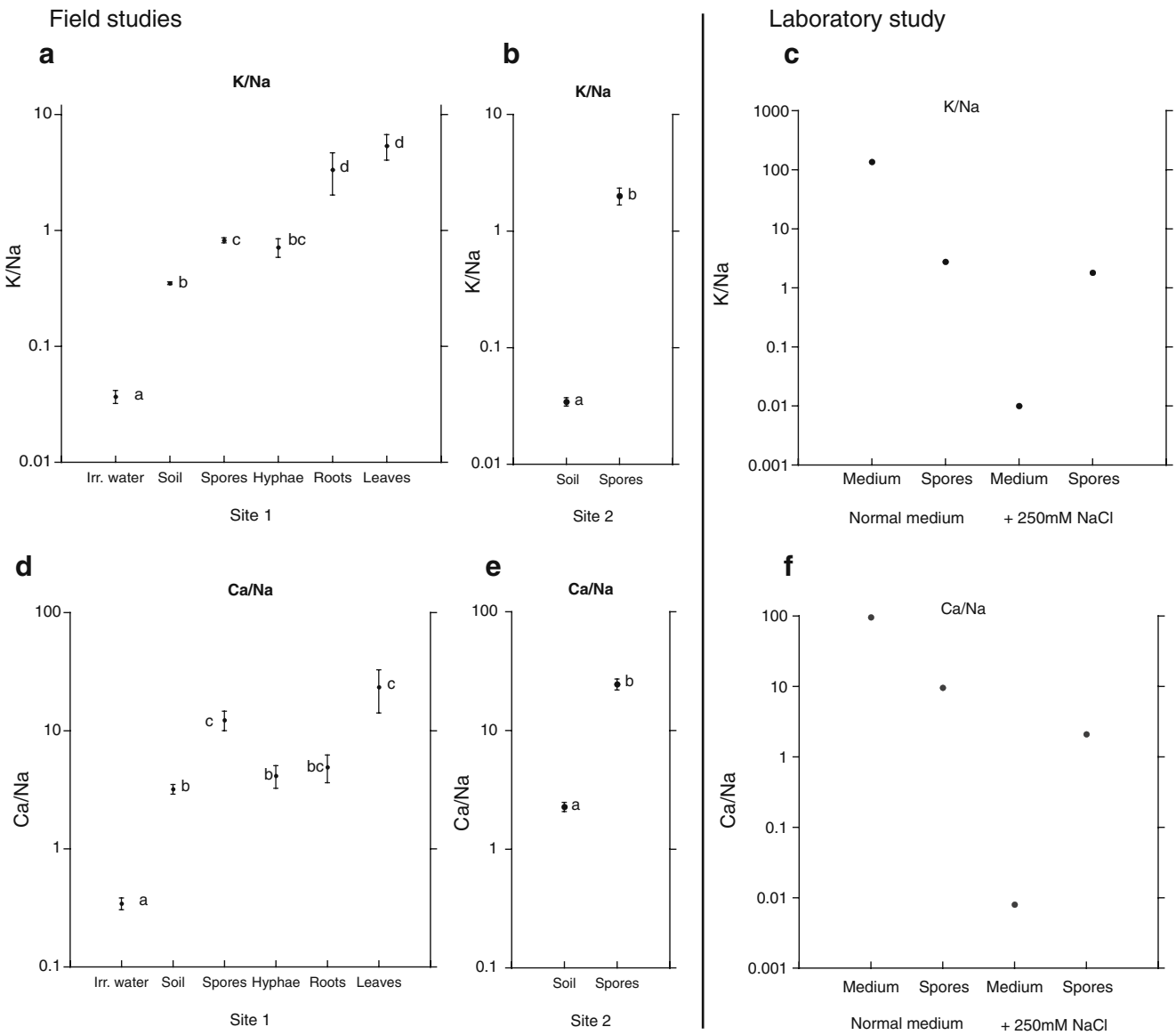

Fig. 2 K:Na ratios (**a**, **b**, **c**) and Ca:Na ratios (**d**, **e**, **f**) of the irrigation water, soil, AMF spores and hyphae and associated plant roots and leaves for Site 1 (**a**, **d**); of the soil and AMF spores for Site 2 (**b**, **e**); and for the medium and for spores of *G. intraradices* grown under monoxenic conditions, with or without addition of 250 mM NaCl to the growth medium (**c**, **f**). Different letters indicate significantly different values at $p \leq 0.05$

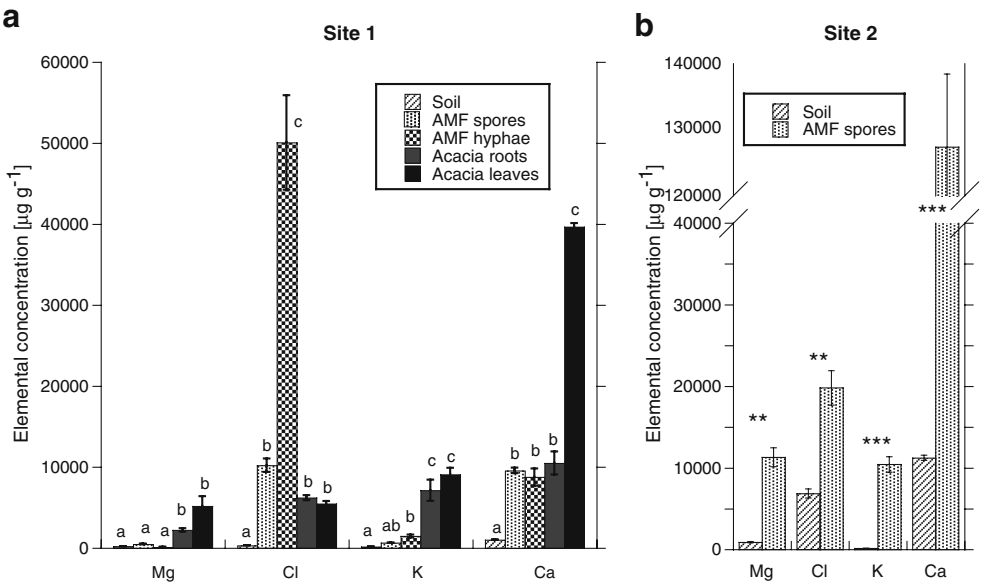

Fig. 3 The content of the possible osmotic equivalent ions Mg, Cl, K, and Ca of the soil, of the AMF spores and hyphae and associated plant roots and leaves for Site 1 (**a**), and of the soil and AMF spores for Site 2 (**b**). The elements in the soil were measured after a $BaCl_2$ extraction for Mg, K, and Ca and after a water extraction for Cl. For AMF and plants, total concentrations are shown. Different letters indicate significantly different values at $p \leq 0.05$

Discussion

We could confirm the hypothesis that AMF discriminate against Na, as Na was found at moderate levels in AMF. Having the Na concentrations of the monoxenically grown, non-salinity stressed fungi as a reference, it was surprising that even though the external Na concentrations of the field-isolated AMF was several orders of magnitude higher, their internal Na concentrations were not strongly elevated compared to the non-salinity stressed fungus. Na was homogeneously distributed within the spores and hyphae from both sites, with no indication of excretion into the vacuole-like structures, for example. Both the AM fungi from the saline soils and the salinity stressed *G. intraradices* had a high K:Na ratio, preferring the more favorable K within their tissue. Moreover, Ca was accumulated to a very high extent in spores and hyphae, which can alleviate salinity stress in plants (Cramer 2002). Thus, the second hypothesis of a

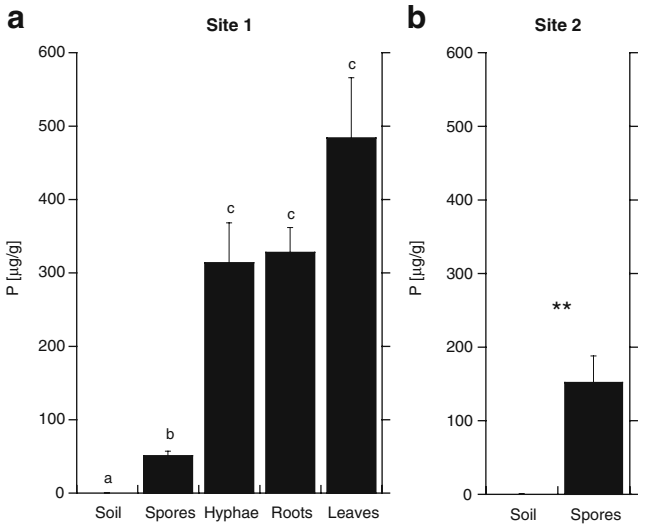

Fig. 4 Available P content of the soil ($BaCl_2$ extracted), total P content of the AMF spores, hyphae and associated plant roots and leaves for Site 1 (**a**); available P content of the soil ($BaCl_2$ extracted) and total P content of AMF spores for Site 2 (**b**). Different letters indicate significantly different values at $p \leq 0.05$

Table 5 Relative abundance and percentage of the total spore biovolume of the most common AM species/spore morphotype groups found in the samples

Species/morphotype group	Relative abundance [%]	% of total spore biovolume
A. trappei	6.3	4.2
G. aggregatum	25.2	19.6
G. ambisporum	4.8	2.8
G. cerebriformae	34.5	1.1
G. clarum	5.2	2.8
G. constrictum	0.7	2.8
G. deserticola	3.1	0.7
G. etunicatum	1.5	1.0
G. intraradices	0.6	0.4
G. mosseae	1.1	10.7
Brown ornamented	0.5	0.5
Clear	3.7	0.7
Orange thick-walled detritus	0.8	4.7
Red thick-walled	2.5	9.6
Yellow rough thin-walled	2.2	14.5
Yellow smooth, shiny thin-walled	3.0	20.5
Others	4.3	3.5

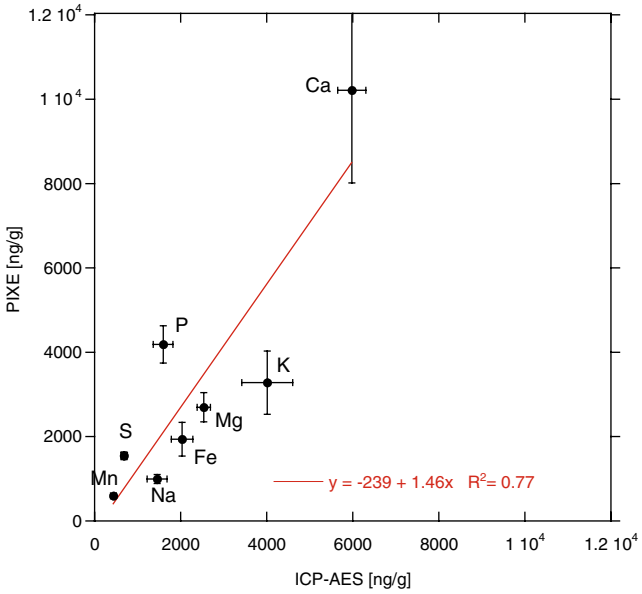

Fig. 5 Comparison of the results for elemental concentration of spores of *G. intraradices* measured with PIXE and ICP-AES

disproportional accumulation of mineral ions capable of osmoregulation in AMF was also confirmed.

The distribution of Cl was patchy at high levels. It is unlikely that the measured Cl level was an artifact since all Cl compounds are very soluble. The concentrations and distributions of Na and Cl within the fungal tissue suggest that the AMF examined in this study act as excluders regarding Na, but as includers for Cl. Little is however known about the physiological effects of Cl in plants. Some plant species are very sensitive to Cl, such as *Citrus sinensis* (Bañuls and Primomillo 1992) and *Vaccinium ashei* (Wright et al. 1993). On the other hand, in salt-tolerant plants such as barley or sugar beet, Cl can be found at concentrations of 20–30 mg g^{-1} dry weight, which are typically the levels of macronutrients, where it has been confirmed to play an important role in plant osmoregulation (Marschner 1995). The high levels of Cl found in the AMF suggest that Cl also might act as an osmotic equivalent. Although we could not measure the actual transfer of Cl to the host plant, it does not seem to be significantly transferred, as the Cl levels in both the roots and shoots were moderate.

Ion discrimination, as here seen for Na, could take place during the fungal uptake of nutrients from the soil or during transfer to the plant host. AMF have been shown to be important for revegetation of heavy metal-polluted soils, where they were found to reduce heavy metal uptake of the associated plant, either by avoiding heavy metal uptake (Weissenhorn and Leyval 1995; Orlowska et al. 2008), or discriminating heavy metals during nutrient transfer to the plant, leading to the accumulation of toxic ions in the fungus (Christie et al. 2004).

However, there is no great concordance in the literature since AMF have also been found to increase the uptake of heavy metals in plants (Ortiz-Cano et al. 2009; Arias et al. 2010), and also Na can be found in enhanced levels in mycorrhized plants (Scheloske et al. 2004). Not all studies could determine whether the ions remained in fungal structures within the roots, or if they were transferred to the plant. Strains might differ in their heavy metal tolerance and their ability to alleviate plant stress, and adapted strains may lose heavy metal adaptation when exposure is disrupted (Sudova et al. 2007).

It is interesting to note that Si was also accumulated in the AMF. It has been reported that Si improves plant tolerance to salt stress by limiting Na uptake in plants, improving photochemical efficiency, protecting cell membrane integrity, and increasing antioxidant enzyme activity (Liang 1999; Ahmad et al. 1992; Zhu et al. 2004). Si can induce precipitation of Na on cell walls (Saqib et al. 2008), and thereby reduce Na concentrations in plant shoots and roots growing in saline soils (Ashraf et al. 2010). Si polymerizes on cell cuticules, and can by this stabilize tissue structures, and was therefore suggested to decrease water loss (Romero-Aranda et al. 2006). Si can be mobilized by ectomycorrhizal fungi (van Hees et al. 2006). Si concentrations in plants were found to be enhanced by AMF in host plants growing in acidic soil (Clark and Zeto 2000), under heavy metal stress (Turnau et al. 2007) or elevated manganese levels (Nogueira et al. 2002). It would be of great interest to further investigate the role of Si in AMF and in mycorrhizal plants under salinity.

Olsson et al. (2008) suggested that spores were storage organs for mineral elements transferred to the plant by AMF, especially P. However, only low levels of P were found in spores in the present study. P levels in the soils were very low, possibly preventing the accumulation of high amounts of P in the fungus. On the other hand, considerable amounts of Fe, Cu, and Zn were found in the spores. Even though getting less attention in literature, the transfer of those ions has also been reported to be important in the mycorrhizal symbiosis (Smith and Read 2008). These elements, Fe in particular, can be very immobile at high pH as in the study sites. Other fungi can exude siderophors in order to enhance Fe uptake (Kornitzer 2009). The elemental maps obtained in the present study revealed that the measured high levels of Fe might be partly due to mineral precipitation, since small spots of high levels of Fe were found on the spore surface (e.g., Fig. 1a spore 1; 5). Thus, the levels of Fe measured in this study might be slightly overrated, and higher than those actually active in the cells. However, otherwise there is an even distribution with moderate concentration within the whole spores, not accumulating at the spore wall, suggesting that there is also a considerable amount located inside the spores (e.g.,

Fig. 1b, spore 1; 2). Despite the apparently high concentrations of ions measured in the spores, cytosolic concentrations of all salt ions are regulated within a narrow range in most organisms (Läuchli and Lüttge 2002). Active Ca ions, e.g., can interact with cytosolic P, and therefore cytosolic levels do not exceed 10^{-7}M. Also K inhibits enzymes at levels above 200 mM in the cytoplasm. The high levels of Ca and other elements found in the AMF in this study may therefore be located in the vacuolar system. To confirm this assumption, a higher resolution in the elemental analysis is required. It is not possible to determine the exact proportion of active vs. precipitated ions from the present measurements, but all K and Cl compounds are very soluble, and precipitation of ions like Ca, Fe, or Si on the outer surface of the spores would be visible in the elemental maps.

In this study of elemental composition, we did not separate the AMF into species. From the plant's point of view, AMF constitute a functional group for nutrient uptake, since the root system of each plant can potentially be colonized by the majority of the fungi present in its rhizosphere, and by several different species at the same time (Clapp et al. 1995; Abbott 1982). We analyzed a mixture of different spore morphotypes, and common traits in elemental composition should indicate important physiological adaptations, while differences in the composition of other elements might indicate different strategies for coping with salinity or nutrient delivery among different AMF species. The low variation in the Na content in all spores suggests that this physiological adjustment is crucial to AMF in saline soils, and that Na ions are generally prevented from entering the AMF cells. Also, Ca was found at similar levels in all spores, but at high concentrations, indicating its use as an osmotic equivalent is essential. In contrast, the high variability of Fe and P concentrations could indicate differences in the efficiency of acquisition of nutrients by different AMF species, but this might also be explained by differences in the age of the spores. The accumulation of Cl was also more variable, and some spores contained very low amounts of Cl. Other possible osmotic equivalents than mineral ions are low molecular weight carbon compounds such as glycerol, proline, and mannitol (Kogej et al. 2007), which are not detected by PIXE. They have also been shown to be produced in some ectomycorrhizal basidiomycetes and ascomycetes (Bois et al. 2006). But their production is energetically more costly than the use of mineral ions (Raven 1985), and they are carbon sinks. They are therefore no low-cost option for AMF, which are dependent on C supply from their host plant.

The amounts of PLFA 16:1ω5 and NLFA 16:1ω5 in the soil from Site 1 were in the same range or higher than in many temperate soils, despite the low amounts of organic matter (van Diepen et al. 2007; Hedlund 2002), whereas the amounts at the highly saline site (Site 2) were about an order of magnitude lower, indicating low amounts of AMF (Olsson 1999). The NLFA:PLFA ratio at Site 1 was 9.1, and at Site 2 it was 2.4, indicating either lower amounts of AMF or that the AMF produced less storage lipids (Olsson 1999).

We found a higher AMF species diversity than in saline soils in Europe (Landwehr et al. 2002; Carvalho et al. 2001; Grzybowska 2004) and Asia (Mohammad et al 2003), where a strong dominance of a single species, *G. geosporum*, was reported. Here, we found *G. mossae* and *G. cerebriformae* to be the most abundant species, but no single species dominated the AMF community. The AMF genus diversity was lower at the present study sites than in a saline dryland in central Tunisia, where four AMF genera were identified; *Glomus* spp. and *Gigaspora* spp. being the most salt-tolerant (Nasr 2003). However, the high abundance of a species in soil does not necessarily imply a high symbiotic potential, as the highly abundant *G. geosporum* failed to show a positive effect on plant performance in numerous experiments (Füzy et al. 2008). The symbiotic effectivity of the strains examined in this study would need to be tested in green house trials if one wishes to produce a salinity-tolerant AMF inoculum.

The accumulation of K, Ca, and Mg in plant tissues prevents Na toxicity and improves both cell membrane stability and nutrient uptake under salt stress (Yildirim et al. 2009). The considerable differences between the AMF tissue and its environment in Na concentration and of other ions possibly acting as osmotic equivalents (such as Mg, Cl, Ca, and K), demonstrate the ability of selective ion uptake by AMF. They kept their internal K:Na and Ca:Na ratios within narrow limits despite changes of several orders of magnitude in their growth environments. We found similar results at the field sites and in the monoxenically grown *G. intraradices*. If a significant proportion of the elemental uptake in plants occurs via the mycorrhizal fungi, this could explain the often higher K:Na ratios in mycorrhized plants (Giri et al. 2007; Sannazzaro et al. 2006) and indicate that the AM fungal mycelium might have the possibility to pre-select nutrients for the plants. These findings show that stimulating the growth of AMF, either by introducing selected inocula or by applying suitable agricultural practices to enhance native AMF growth, can be an important tool to sustainable and productive management of salt-affected soils.

Acknowledgments We gratefully thank Nancy Collins Johnson for the help with species identification of the spores. This study was made possible through financial support from the Swedish International Development Cooperation Agency (SIDA) and from The Swedish Research Council for the Environment, Agriculture Sciences and Spatial Planning (FORMAS). We acknowledge support from the National Research Institute for Rural Engineering, Water and Forest of Tunisia.

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