ARBUSCULAR MYCORRHIZA OF HERBS COLONIZING A SALT AFFECTED AREA NEAR KRAKÓW (POLAND)

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ABSTRACT

The arbuscular mycorrhizal (AM) status of plants colonizing an area affected by leakage of salty water (Barycz near Kraków, Poland) was studied in 2000 and 2001. The occurrence of plants typical for soils of increased salinity was observed. Among the 13 examined plant species 7 were mycorrhizal. The abundance of mycorrhizal plant populations was increased in the second year of study. Strains of 4 species of AMF, including *Glomus caledonium*, *G. claroideum*, *G. geosporum* and *G. intraradices* were isolated using trap cultures. On the basis of morphological characters the presence of *G. tenue* was detected in plant roots of several species from the study area. The efficiency of mycorrhizal colonization and arbuscule formation by two strains of *G. geosporum* isolated from a saline area and a strain of *G. intraradices* from unaffected sites was tested in an experiment carried out on *Plantago lanceolata* cultivated on substrata of different salinity levels. The increase in mycorrhizal parameters with growing salt content was observed in the case of strains originating from the salt-affected area. At the highest salt level these strains formed better developed mycorrhiza than the strain from the non-saline site, suggesting a better adaptation of the strains from the saline area. The data on vitality (alkaline phosphatase test) of intraradical AM fungi gave a clearer picture than those obtained by the conventional aniline blue staining.

KEY WORDS: arbuscular mycorrhiza, AMF, salinity, halophytes, glycophytes, alkaline phosphatase test.

INTRODUCTION

Salt-affected soils cover almost 10% of the global land surface (Ruiz-Lozano et al. 1996), including salt marshes of the temperate zones, mangrove swamps of the tropics and subtropics and saline soils of arid and semiarid zones where the amount of rainfall is insufficient for substantial leaching. The origin of soil salinity can be natural, due to climate conditions, or caused by human activity, such as mining or application of salt for deicing (Blassius and Merritt 2002). Salinity inhibits growth and development of most plants. It affects growth, morphology and physiology of roots; however, adaptation to this particular stress condition can be observed in plants occurring on soils of increased salinity (Kopcewicz and Lewak 1998; Volkmar et al. 1998). The development of a symbiosis with microorganisms such as arbuscular mycorrhizal fungi (AMF) may be one of the mechanisms that can attenuate the stress by compensation of the soil volume for nutrient acquisition, reduction of the negative osmotic pressure and imbalance of essential cations and anions, alteration of the pH and soil structure (Schwarz and Gale 1984; Hasegawa et al. 1986). Rhizospheric microorganisms have been found in soils containing up to 30% of NaCl (Zahran 1996). Arbuscular mycorrhizal fungi isolated from such soils have been found to improve growth and fitness of the plants (Hirrel and Gerdemann 1980; Ojala et al. 1983; Singh et al. 1997; Al-Karaki and Hammad 2001; Feng et al. 2002). Inoculum containing selected strains can be used e.g. for alleviation of stress to grasses colonizing road sides that have been treated with salt during winter. Production of such inoculum should include strains originating from the same geographic region and adapted to the particular climatic and soil conditions.

The present investigation was carried out in the area affected by leakage of salt from tubes transporting waste-water from the salt mine near Kraków and water pumped out from the salt mine during the failure that happened a few years ago. The salt effect was still visible, as characteristic halophytic vegetation was present at this site. The aims of this research were: 1) evaluation of the mycorrhizal status of herbs to check the ability of the fungal strains to form functional symbiosis under a range of soil salinity levels; 2) isolation and identification of the AMF strains; 3) evaluation of the ability of selected strains to form mycorrhiza under salt stress.

MATERIALS AND METHODS

Site description and sample collection

The salt-affected region is located on the northern slope of Pogórze Wielickie (southern Poland), between two villages Barycz and Soboniowice (in the vicinity of Kraków), near the communal rubbish dump. The area of approximately 1000 m^2, developed in the place where originally oak-hornbeam wood Tilio-Carpinetum was present on brown soil (Dubiel 1988). The salinity of soil at the sampling area was caused by brine leak from the pipeline delivering the saline water from the salt mine to the tank (reservoir) located at the site. The vegetation on the area affected by the saline water was dominated by halophytic plant species such as *Puccinellia distans* (Jacq.) Parl and *Spergularia salina* Presl. They were accompanied by glycophytes.

The study was carried out during the flowering period in June 2000 and 2001. The plant material was collected from 6 plots (about 16 m^2 each) chosen randomly at the site. The identification of plant species was carried out during the first vegetative season. The plant percentage cover was estimated according to the 6-degree Braun-Blanquet scale (Szafer and Zarzycki 1970). Roots of 5 specimens of each plant species were collected from the individual plots and were pooled into a single bulk sample. Plant roots with adhering soil were collected and used for the establishment of trap cultures. Samples for soil analysis were collected from three randomly chosen points of each plot to the depth of 15 cm, and mixed thoroughly. Table 1 summarizes some of the soil chemical and physical properties: electrical conductivity (EC) of the saturation paste extract (Richards 1954), pH with H_2O (1:2.5 w/v) (Kowalkowski et al. 1973), concentration of the Cl^-, Na^+, K^+, Ca^{+2} ions (Ostrowska et al. 1991), organic carbon and organic matter content (Lityński et al. 1968).

Estimation of mycorrhizal colonization

Roots were rinsed with water, cleared in 10% KOH for 24 hours, acidified in 5% lactic acid for 1 hour, stained in 0.01% aniline blue (in 5% lactic acid) for 24 hours and stored in pure lactic acid. The procedure was followed at room temperature. The roots were cut into 1 cm pieces (45 pieces per sample), mounted on slides in glycerol and analysed with the Nikon Eclipse 800C light microscope. The following mycorrhizal parameters were assessed: mycorrhizal frequency (F%), relative (M%) and absolute (m%) mycorrhizal root length, relative (A%) and absolute (a%) arbuscule richness (Trouvelot et al. 1986), http: //www.dijon.inra.fr.bbceipm/Mychintec/Mycocalc-prg/download.html.

Isolation of AMF strains

Soil samples (including plant roots) collected from the rhizosphere of plants growing in the investigated area were placed in pots containing sterile substratum (sand: expanded clay 3:1, v/v) containing 50 g of phosphate rock per 1l of the substratum and covered by 1cm layer of the substratum. The cultures were kept in Sigma sunbags in greenhouse conditions using *Plantago lanceolata* L. as host plant (10 seedlings per pot). Pots were watered to the point of saturation and the humidity was maintained according to Walker (personal communication). After three months spores were isolated using wet filtering (mesh size 50 μm).

Identification of the AM fungi was carried out on material obtained from single spore cultures. Isolated spores were germinated on cellulose filters on a layer of wet sterile sand in Petri dishes. A single germinating spore was attached to the root of a *Plantago lanceolata* seedling and they were put together in a small pot containing sterile substratum (sand and expanded clay 1:1, v/v), and watered. After 3 months the substratum with AMF mycelium from the small pots was moved to bigger pots with the same sterile substratum and more seedlings were planted to multiply the AMF inoculum for next three months. Spores isolated from monocultures were mounted in a drop of PVLG and Meltzer's reagent on a slide and crushed to examine the wall structure by applying gentle pressure to the coverslip (Omar et al. 1979). Slides with isolated spores were deposited in the slide collection of the Institute of Botany of the Jagiellonian University.

Estimation of mycorrhizal efficiency of AMF strains

Three different AMF strains: two strains of *Glomus geosporum* originating from the saline soil near Barycz and *Glomus intraradices* (BEG Rej. E-1 99/Lav.) from non-saline soil, were used to inoculate *Plantago lanceolata*. This species was selected as most commonly occurring at road sides affected by salt stress. Additionally, this plant forms mycorrhiza with a wide range of AMF strains/species and is easily grown under laboratory conditions. Four growth substrates differing in salt content (EC) were applied: saline soil collected at the site (24.70 mS/m), nonsaline soil (3.92 mS/m) and two other substrata consisting of saline soil diluted by the nonsaline soil in proportions 3:1 v/v (9.98 mS/m) and 1:1 v/v (11.82 mS/m). One liter of expanded clay with 200 g of phosphate rock were added per each three liters of the growth substratum. 10 seedlings were cultivated in each pot (volume 500 cm^3). Pots were kept in Sigma sunbags and the humidity maintained as described above. After 8 weeks the plants were removed from the pots. Roots of 3 randomly chosen plants growing

TABLE 1. Physical and chemical properties of soil in the salt-affected area near Barycz.

Plot number	pH[a]	EC[b] mS/m	Cl^-	Na^+	K^+	Ca^{2+}	Organic matter	Organic carbon
			mg/100 g of dry soil				%	
1	6.45	10.62	68	125	5	200	3.37	1.96
2	6.87	11.77	43	155	15	570	4.58	2.65
3	6.89	12.29	28	125	12	500	4.14	2.40
4	6,40	18.95	42	150	20	1000	3.51	2.07
5	6.75	26.30	42	185	15	310	2.88	1.67
6	6.89	31.20	76	210	20	870	5.21	3.02

[a] with H_2O

[b] electric conductivity

at each salinity level were stained for the activity of alkaline phosphatase. Remaining plant roots were stained in aniline blue as described above. The roots for the estimation of the activity of alkaline phosphatase were stained according to Van Aarle et al. (2001). The roots were washed in tap water, cut into 1 cm pieces (45 per sample), mounted on slides and stained for 15 min with the ELF 97 Endogenous Phosphatase Detection Kit (Molecular Probes). After staining the roots were washed in the wash buffer (30 mM Tris, 1.5 M NaCl and 0.05% Triton X-100, pH 8) for 15 min before the mounting medium from the kit was applied and the slides were closed with coverslips. The roots were analysed with a Nikon Eclipse 800C fluorescence microscope equipped with a mercury lamp and the mycorrhizal parameters were assessed as described above.

Statistics

Data for mycorrhizal parameters were analysed with the non-parametric U-Mann Whitney test ($p<0.05$). The analyses were performed using STATISTICA (version 5.0) software.

RESULTS

The vegetation of the investigated plots consisted of 13 species (Table 2). Comparing the species composition on the plots during the two seasons of investigation, 2000 and 2001, it was found that the population of some mycorrhizal species increased and appeared on a higher number of plots (e.g. *Matricaria chamomilla* L., *Plantago maior* L., *Trifolium repens* L. and *Tussilago farfara* L.). The new mycorrhizal species in 2001, not occurring in the previous season, was *Ranunculus repens* L. In contrast, *Lotus* spp. L. disappeared. Non-mycorrhizal species such as *Agropyron repens* (L.) P. B., *Atriplex hastatum* L. var. *salinum* and *Lepidium ruderale* L. were absent on the site in the second year of the study (Table 2).

On the selected plots, similarly to the whole area, the most abundant plants were *Puccinellia distans* and *Spergularia salina* (Table 3). In both cases no mycorrhizal structures were found. Nonmycorrhizal were also other species of Gramineae, Chenopodiaceae, Brassicaceae and Caryophyllaceae which were, however, less common. Mycorrhizal structures were observed in roots of Asteraceae, Fabaceae, Ranunculaceae and Plantaginaceae (Table 2). *Plantago maior* was one of the most abundant mycorrhizal plants, followed by *Trifolium repens* and *Tussilago farfara*. (Table 3). The degree of mycorrhizal frequency (F%) in roots of

TABLE 2. Species composition of plots and mycorrhizal status of plants at the salt-affected site near Barycz; np – not present in this year.

Plant species	Family	Plot number where occurred	
		in 2000	in 2001
non-mycorrhizal			
Agropyron repens	Graminae	1	np
Atriplex hastatum var. *salinum*	Chenopodiaceae	1	np
Lepidium ruderale	Brassicaceae	1	np
Puccinellia distans	Gramineae	1,2,3,4,5,6	1,2,3,4,5,6
Spergularia salina	Caryophyllaceae	2,3,5,6	2,3,5,6
Typha latifolia	Typhaceae	4	4
mycorrhizal			
Lotus spp.	Fabaceae	1	np
Ranunculus repens	Ranunculaceae	np	1,3
Matricaria chamomilla	Asteraceae	3	2,3,6
Melilotus officinalis.	Fabaceae	1	3
Plantago maior	Plantaginaceae	1,2,3,4,5	1,2,3,4,5,6
Trifolium repens	Fabaceae	4,6	2,4,5,6
Tussilago farfara	Asteraceae	3	1,3,4,5,6

TABLE 3. Species abundance, relative mycorrhizal root length (M%) and relative arbuscule richness (A%) of plants on different plots at the salt-affected site near Barycz (values listed are means ± standard deviation, number of samples from 4 to 6 depending on availability of material). Each species abundance in the plot was estimated according to the 6-degree Braun-Blanquet scale: ± the species covers up to 1% of the area; 1 – the species covers up to 5%; 2 – the species covers 5 to 25%; 3 – the species covers 25 to 50%; 4 – the species covers 50 to 75%; 5 – the species covers 75 to 100%.

Plant species	Species abundance	M %	A %
Plot 1			
Agropyron repens	+	0.0	0.0
Atriplex hastatum var. *salinum*	1	0.0	0.0
Lepidium ruderale	+	0.0	0.0
Puccinellia distans	3	0.0	0.0
Spergularia salina	3	0.0	0.0
Lotus spp.	+	71.6±19.5	32.4±14.5
Melilotus officinalis	+	60.1±11.4	34.0±6.5
Plantago maior	2	81.0±6.5	42.0±23.8
Ranunculus repens	+	80.4±3.4	18.2±9.1
Tussilago farfara	1	52.5±9.8	23.0±5.6
Plot 2			
Puccinellia distans	3	0.0	0.0
Spergularia salina	2	0.0	0.0
Matricaria chamomilla	2	67.6±17.6	32.2±17.5
Melilotus officinalis	+	56.3±17.5	18.1±6.1
Plantago maior	2	66.7±19.8	36.6±9.4
Trifolium repens	2	90.0±2.9	42.0±9.0
Plot 3			
Puccinellia distans	3	0.0	0.0
Spergularia salina	3	0.0	0.0
Plantago maior	2	74.2±15.8	96.6±5.9
Ranunculus repens	+	52.3±10.1	27.2±4.3
Tussilago farfara	2	52.6±12.0	19.2±8.0
Plot 4			
Puccinellia distans	2	0.0	0.0
Typha latifolia	1	0.0	0.0
Plantago maior	1	69.1±19.1	31.7±11.3
Trifolium repens	2	77.7±1.6	37.2±7.6
Tussilago farfara	1	67.7±7.3	23.6±6.3
Plot 5			
Puccinellia distans	3	0.0	0.0
Spergularia salina	2	0.0	0.0
Plantago maior	1	66.3±11.7	26.4±17.7
Trifolium repens	2	65.9±4.7	27.4±3.8
Tussilago farfara	2	43.2±31.1	16.8±12.6
Plot 6			
Puccinellia distans	3	0.0	0.0
Spergularia salina	3	0.0	0.0
Matricaria chamomilla	1	63.7±13.6	44.7±10.7
Plantago maior	2	61.3±13.6	49.0±35.9
Trifolium repens	2	86.8±6.4	50.7±19.9
Tussilago farfara	2	60.5±23.4	32.4±11.1

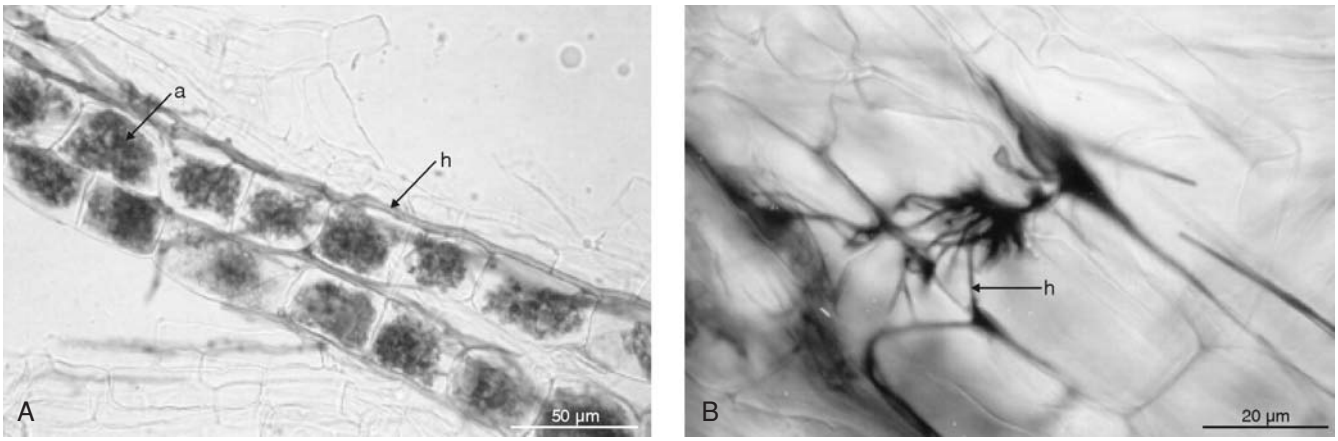

Fig. 1. Mycorrhizal colonization of *Plantago maior* collected from the salt affected area near Barycz: A – coarse endophyte with arbuscules (a) and intracellular hyphae (h); B – fine endophyte with typical finger-like branching of hyphae (h).

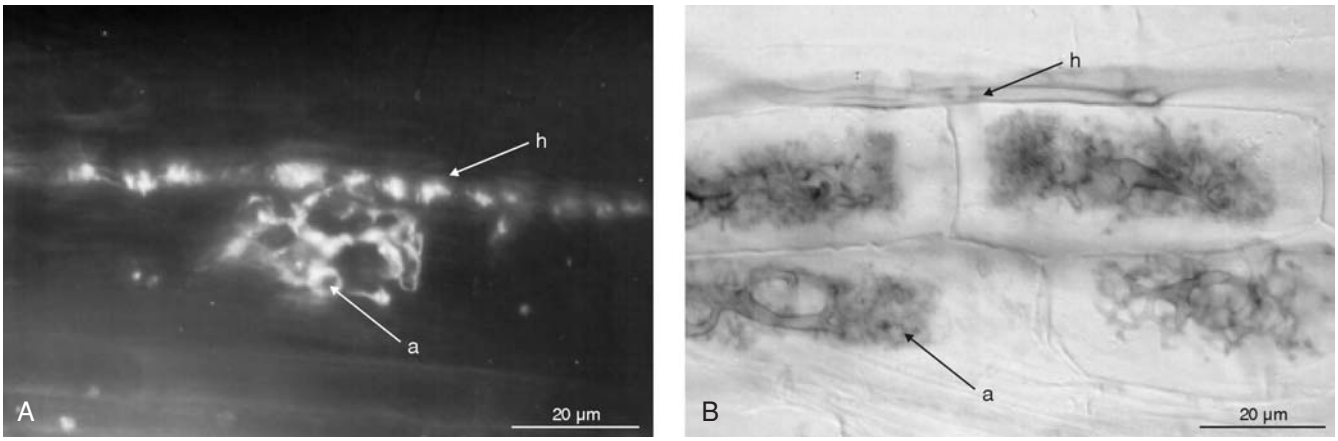

Fig. 2. Comparison between two staining methods used to detect mycorrhiza of *Plantago lanceolata* grown experimentally in salt affected soil: A – alkaline phosphatase test showing arbuscules (a) and hyphae (h) due to positive reaction of vacuoles; B – conventional aniline blue staining visualising mycelial walls.

all species varied between 86 and 100% in 2000 and 67 to 100% in 2001. Relative mycorrhizal root length (M%) and intensity of colonization in individual mycorrhizal roots (m%) were the highest in *Plantago maior* in 2000 and in *Trifolium repens* in 2001. Arbuscules were most abundant (a%) in *Melilotus officinalis* (L.) Lam in 2000 and in *Plantago maior* in 2001 (data not shown).

Within roots of plants collected from the investigated area both coarse (Fig. 1A) and fine mycorrhizal fungi (Fig. 1B) were observed. On the basis of mycelium characteristics (finger-like branching of mycelium of about 2 µm diameter with abundant, small vesicles) the fungus was identified as *Glomus tenue* (Greenhall) Hall. The species was noticed in *Lotus* spp. and *Trifolium repens* in the first year and additionally in *Matricaria chamomilla* and *Melilotus officinalis* in the second year. The fine endophyte was not obtained from the trap cultures. On the contrary, this method resulted in obtaining isolates of *G. caledonium* (Nicol. & Gerd.) Trappe & Gerd., *G. claroideum* Schenck & Smith; *G. geosporum* (Nicol. & Gerd.) Trappe & Gerd.; *G. intraradices* Schenck & Smith. The most frequently isolated were strains of *G. geosporum*.

The results of the experiment in which two strains of *G. geosporum* isolated from salt affected area near Barycz and a strain of *G. intraradices* originating from non-affected soils were used to inoculate *Plantago lanceolata* seedlings cultivated at different salinity levels are presented in Figures 3 and 4. Some differences among strains were shown in mycorrhizal parameters, although no visible differences in growth were found between plants inoculated with different strains and cultivated at various salt concentrations (data not shown). Almost no differences were found in mycorrhizal colonization levels in samples stained with aniline blue. Statistically higher mycorrhizal colonization was found in the case of strains originating from the salt affected site than from the nonsaline soil, especially at the highest salt concentration; the differences were more pronounced when the phosphatase activity method was used (Figs 2A and 4). The relative arbuscule richness estimated after aniline blue staining (Figs 2B and 3) has shown differences between fungal strains of different origin only at the highest salt content and was much lower in the case of the strain from non-affected place. Similar tendencies were visible in data for the alkaline phosphatase activity (Fig. 4). In the case of both staining methods differences were found also between the two strains from the salt affected site and in addition the tendency to increase mycorrhizal colonization and arbuscule richness by the strains from salt affected areas was observed (Figs 3 and 4).

DISCUSSION

Arbuscular mycorrhiza (AM) represents a common root-fungus symbiosis in 80% of the higher plants in a wide range of environments (Smith and Read 1997). The AM

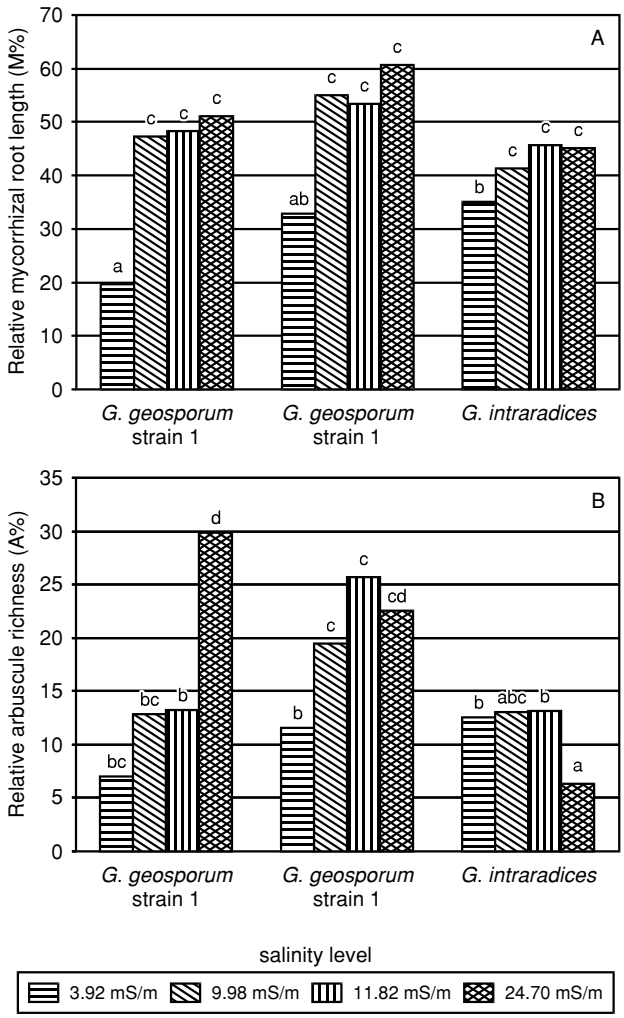

Fig. 3. Mycorrhizal parameters of *Plantago lanceolata* inoculated by *G. geosporum* strains originating from the salt affected soil and *G. intraradices* from non-affected soils, cultivated in substrata of increasing salt level – data estimated on the basis of aniline blue staining: A – relative mycorrhizal root length (M%) and B – relative arbuscule richness (A%). Different letters above bars indicate statistically important differences between all bars ($p < 0.05$).

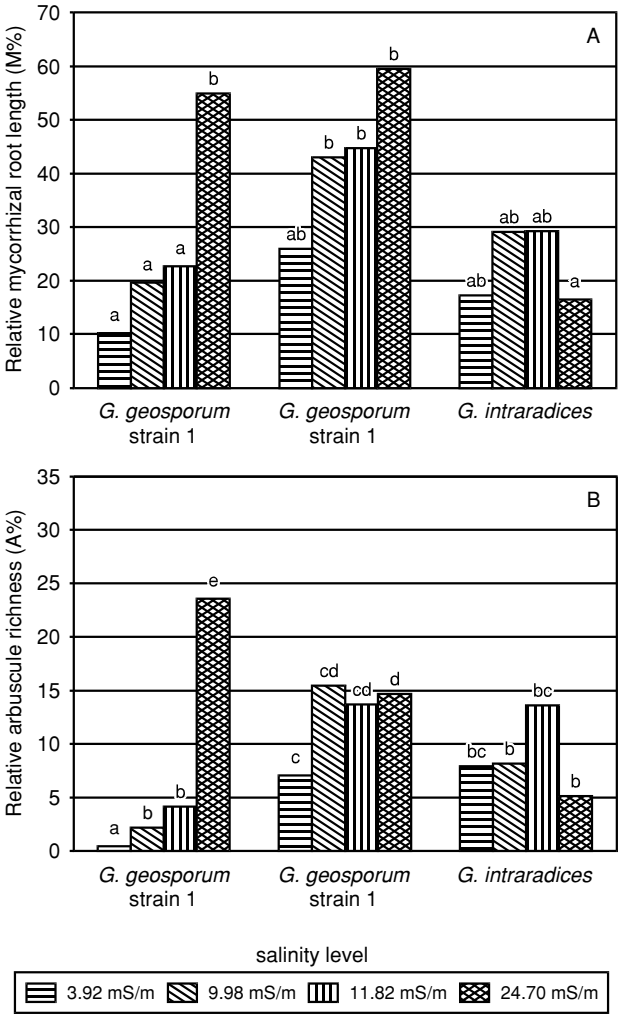

Fig. 4. Mycorrhizal parameters of *Plantago lanceolata* inoculated by *G. geosporum* strains originating from the salt affected soil and *G. intraradices* from non-affected soils, cultivated in substrata of increasing salt level – data estimated on the basis of alkaline phosphatase activity: A – relative mycorrhizal root length (M%) and B – relative arbuscule richness (A%). Different letters above bars indicate statistically important differences between all bars ($p<0.05$).

fungi have been shown by several workers to occur naturally in saline environments (Khan 1974; Allen and Cunningham 1983; Pond et al. 1984; Rozema et al. 1986; Ho 1987; Błaszkowski 1993; Aliasgharzaden et al. 2001; Bothe et al. 2001; Landwehr et al. 2002). The literature concerning mycorrhizal colonization of halophytes is controversial. Plants of wetland and saline sites were described not to be colonized by AMF (Smith and Read 1997). The halophilous species belonging to the families Chenopodiaceae, Plumbaginaceae, Juncaceae, Brassicaceae etc. were considered to be non-mycorrhizal (Hirrel et al. 1987; Smith and Read 1997) On the contrary, some halophytes have been found to be colonized by AMF (Mason 1928; Khan 1974; Hildebrandt et al. 2001). According to Hildebrandt et al. (2001), *Spergularia salina* (Caryophyllaceae) showed distinct colonization (2-14% of colonization) and the halophytic grass *Puccinellia distans* was characterized by variable pattern of mycorrhizal colonization (0-49%). Both species were examined for mycorrhization during the seed formation period and after the reproductive phase. In the present study plants belonging to the families Brassicaceae, Gramineae, Caryophyllaceae were found to be non-mycorrhizal; however, the presence of mycorrhizal structures was verified during the flowering phase. The ability to form mycorrhiza should be checked, however, during the whole vegetation season, including all development stages of the plant, as the degree of mycorrhizal colonization can vary in individual plants during the vegetation period (Smith and Read 1997) or it could depend on other parameters of the soil. *Atriplex hastatum* var. *salinum* was reported as non-mycorrhizal by Rozema et al. (1986) and these data were confirmed in the present paper.

The present study reports that plants from the families Fabaceae, Asteraceae, Plantaginaceae, Ranunculaceae were found to be arbuscular mycorrhizal in a high degree. Some mycorrhizal species, e.g. *Matricaria chamomilla* and *Tussilago farfara* tended to be expansive in the area, while some of halophytic non-mycorrhizal plants showed an opposite tendency what is an obvious sign of decreasing salt level.

In the present study the isolated and identified AM fungal species were members of the genus *Glomus* with dominating *G. geosporum*. These data are in agreement with

previous results of other workers (Allen and Cunningham 1983; Pond et al. 1984; Ho 1987). In a recent study of salt marshes in Central Europe 80% of isolated AM spores belonged to *G. geosporum* (Bothe et al. 2001; Hildebrandt et al. 2001; Landwehr et al. 2002).

The experiment carried out within this study has shown that AM fungi originating from the salt affected stand more efficiently colonized the roots developing in soils of higher salinity than those from non-affected ones. The differences were evident at the highest salinity of growth substratum. The strains of *G. geosporum* (from saline soils) produced also more abundant arbuscules than *G. intraradices*. This suggests better adaptation of the species/strains from saline areas to salt stress conditions occurring in such places and confirms the usefulness of the strain selection for the use in practical application. In addition, the experiment has shown the increase of mycorrhizal colonization and arbuscule richness by *G. geosporum* with increasing salt level. These data are in accordance with the field observations of Füzy et al. (2001), who found higher mycorrhizal colonization and more abundant arbuscule development in roots of *Matricaria chamomilla* and *Festuca pseudovina* at the more salty area.

In the present study no statistically important differences in growth of *P. lanceolata* inoculated with different strains of AM fungi and cultivated at different salt concentrations were found. This suggests that this particular plant species is relatively tolerant to salt. Higher salt levels could be tested but this would be not comparable to levels usually found at the road sides. The research aiming at the protection of plant diversity should consider a broader range of plant species that can react to salt in various ways. The response of individual plant species to different fungal species could vary as well. As shown by Ruiz-Lozano and Azcon (2000) in the experiment concerning inoculation of *Lactuca sativa* with AMF strains originating from saline soils, these strains formed more abundant mycorrhizal structures and stimulated root development, whereas the strain *G. desertico-la* originating from non-saline soils improved plant nutrition. Both AM fungi, however, protected the host from salinity stress.

Both methods of staining applied in this study were useful to detect differences in fungal colonization; however, in the case of data obtained with the vitality method relying on the estimation of alkaline phosphatase activity the differences were more significant, suggesting higher sensitivity of this method. The same vitality method was also found to be the best method to select the most effective mycorrhizal strain for the colonization of heavy metal polluted soils (Orłowska et al. in press).

The strains isolated from the area near Barycz might be of interest for the practical application of AMF inoculum on roadsides, where soils are treated with salt for deicing during winter time. The levels of salinization may reach similar concentrations as those observed in the studied area. AMF are especially important in such environments, as they are considered as bio-ameliorators of saline soils (Singh et al. 1997). Several studies demonstrated that the inoculation with AMF improves plant growth under salinity stress (see Feng et al. 2002 for references). The attenuation of stress was suggested to be related to the improvement of plant phosphorus status (Hirrel and Gerdemann 1980; Al-Karaki 2000), increased carbon dioxide exchange rate, transpiration, stomatal conductance and water use efficiency (Ruiz-Lozano et al. 1996), or to higher accumulation of soluble sugars in roots (Feng et al. 2002). The selection of the most effective strains for practical application is important as the symbiotic efficiency and mycelial infectivity can vary between AMF strains isolated from saline and non-saline soils (Ruiz-Lozano and Azcon 2000). Future work has to show whether the AMF strains originating from soils near Barycz confer salt tolerance to plants.

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