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ORIGINAL PAPER

The differential behavior of arbuscular mycorrhizal fungi in interaction with *Astragalus sinicus* L. under salt stress

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Abstract Three arbuscular mycorrhizal (AM) fungi (*Glomus mosseae*, *Glomus claroideum*, and *Glomus intraradices*) were compared for their root colonizing ability and activity in the root of *Astragalus sinicus* L. under salt-stressed soil conditions. Mycorrhizal formation, activity of fungal succinate dehydrogenase, and alkaline phosphatase, as well as plant biomass, were evaluated after 7 weeks of plant growth. Increasing the concentration of NaCl in soil generally decreased the dry weight of shoots and roots. Inoculation with AM fungi significantly alleviated inhibitory effect of salt stress. *G. intraradices* was the most efficient AM fungus compared with the other two fungi in terms of root colonization and enzyme activity. Nested PCR revealed that in root system of plants inoculated with a mix of the three AM fungi and grown under salt stress, the majority of mycorrhizal root fragments were colonized by one or two AM fungi, and some roots were colonized by all the three. Compared to inoculation alone, the frequency of *G. mosseae* in roots increased in the presence of the other two fungal species and highest level of NaCl, suggesting a synergistic interaction between these fungi under salt stress.

Keywords Alkaline phosphatase · Arbuscular mycorrhizal fungi · Nested PCR · Salt stress · Succinate dehydrogenase

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Abbreviations

ALP	Alkaline phosphatase
AM	Arbuscular mycorrhizal
LSU rDNA	Large subunit ribosomal DNA
PCR	Polymerase chain reaction
SDH	Succinate dehydrogenase

Introduction

Soil salinity is a major and increasing worldwide problem because it affects the growth and the development of plants particularly in arid and semi-arid areas (Triantafilis et al. 2001). About one billion hectares of the global land area is not in use due to salinity stress. In China, nearly one hundred million hectares of cropland have suffered from salinization problems, and the soil salinity is becoming one of the major limiting factors for crop production (Niu and Wang 2002). Excessive salts in soil lower water availability, inhibit metabolic processes, and affect nutrient composition, osmotic balance, and hydraulic conductivity resulting in stunted growth and productivity of plants (Al-Karaki et al. 2001). Thus, approaches to improve the tolerance of plants to salinity and increase the utilization of saline soil is becoming an emerging challenge.

Arbuscular mycorrhizas (AM) are important symbiotic associations of plant root and soil microorganisms. AM fungi are present in most soils including saline soils (Jiang and Huang 2003). Although it has been confirmed that salinity can decrease the colonization of AM fungi, many studies have demonstrated that inoculation with AM fungi improves the growth of plants under a range of salinity stress conditions (Ghazi and Al-Karaki 2000; Porras-Soriano et al. 2009), and with exposure to combined

salinity and drought stress, AM fungi extended the time of the stomates open and promoted the stomatal conductance, but in other instances, the addition of salt tended to nullify an AM-induced change in drought response (Cho et al. 2006). Different AM fungal species can differ in their ability to minimize stress effects and to promote plant growth (Daei et al. 2009). It has also been suggested that establishment of mixed communities by different AM fungal species may be more beneficial to the growth of plants than any of individual species (Alkan et al. 2006; Koide 2000).

Detection of different species in an AM fungal community inside the roots is almost difficult (Reddy et al. 2005). Molecular techniques are currently used to identify glomeromycota fungi in root system (Kjøller and Rosendahl 2000; Renker et al. 2003). Nested PCR using primers with different levels of taxonomic specificity obtained from the LSU rDNA has been shown to be an efficient approach for identifying different AM fungi in trypan blue-stained roots (Dong and Zhao 2006; van Tuinen et al. 1998) and has subsequently been used to monitor AM fungi populations in soils or mycorrhizal roots (Gollotte et al. 2004; Hempel et al. 2007; Jacquot et al. 2000; Li et al. 2009; Rosendahl and Stukenbrock 2004; Stukenbrock and Rosendahl 2005).

The presence of AM fungi in roots is not visible without appropriate staining. Trypan blue staining is a routine method to observe intraradical AM fungal mycelium. However, this traditional estimation of the extent of root colonization does not distinguish between dead and living fungal structures. Succinate dehydrogenase (SDH) activity is used as an indicator of fungal viability in mycorrhiza (Zhao et al. 1997) but does not appear to reflect mycorrhizal efficiency in plant growth enhancement (El-Atrach et al. 1989). Alkaline phosphatase (ALP) activity which is located within the phosphate-accumulating vacuoles of AM fungal hyphae has been proposed as a physiological marker for phosphate metabolism of mycorrhizal fungi (Tisserant et al. 1993). Measurement of the activity of these two enzymes makes it possible to compare the total production of fungal tissue with the proportion that is living or functional (Chen and Zhao 2007; Zhao et al. 1997).

The objective of this study was to investigate the effect of three AM fungi inoculated either individually or as a mixture on the growth of *A. sinicus* plants in salt-amended soil and on fungal colonization patterns in roots. Enzyme activity of SDH and ALP was measured to evaluate the effect of NaCl application on the function of the AM symbiosis. Root colonization by the different AM fungi was monitored in mix-inoculated roots by nested PCR. We expected to find whether there is any difference in competition between the three AM fungi in response to salt stress.

Materials and methods

Soil preparation and treatments

The agricultural soil was sampled from a field near the farm of Huazhong Agricultural University in Wuhan, Central China. The soil was sieved (5 mm) and mixed with river sand (1:1, *v*/*v*), then autoclaved for 1 h on two consecutive days to kill indigenous AM fungi. Finely powdered NaCl was added by mixing with the soil in a large container in each pot at rates of 0, 0.14, 0.53, and 0.96 g NaCl per kilogram soil. The application levels of NaCl were decided based on our preliminary experiment.

Plant material and fungal inoculum

Seeds of *A. sinicus* were surface-sterilized by soaking first in 75% alcohol and then in 3% sodium hypochlorite solution for 10 min. After washing five times with sterile water, the seeds were germinated in sterile Petri dishes at 28°C. Four uniform germinated seeds were sown in each pot. The mycorrhizal fungi were *Glomus mosseae* HAU-B15 (BEG185; isolated originally from a local corn field), *Glomus intraradices* Schenck and Smith (BEG141), and *Glomus claroideum* Schenck and Smith (BEG23). The inoculum consisted of 15 g dry soil mixture of spores and mycelium, and colonized root fragments were placed in a layer beneath the seeds in the pot filled with 300 g soil. For each treatment, the three species of AM fungi were inoculated either individually or as a mixture with equal amounts of inoculum-containing soil. Uninoculated controls received 15 ml of the mixed inoculum washings which was sieved through a filter (Whatman no. 1) twice to ensure similar microbial populations in all treatments. Each treatment comprised three replicates to give a total of 60 pots. A preliminary MPN test (Porter 1979) indicated that there was no significant difference in infective propagules between the three AM fungal inocula.

Experimental conditions and measurements

The experimental design included three salt levels (0, 0.14, 0.53, and 0.69 g NaCl kg^{-1}) and the following mycorrhiza treatments (none, *G. mosseae*, *G. claroideum*, and *G. intraradices* each added alone and all three added together).

Pots were randomized within blocks in a growth chamber. Plants were grown under constant conditions (22/16°C with a 14-h photoperiod at a light intensity of 300 μmol $m^{-2}s^{-1}$) and received 15 ml Hoagland solution weekly with 1/10 of phosphate (Hoagland and Arnon 1950). Plants were harvested 7 weeks after sowing. Shoots were cut just above the soil surface, and roots were carefully washed free of soil with ice-cold water. To assay mycorrhizal development, root

Table 1 PCR primers used in this study

Code	Sequence(5′–3′)	Target organisms	Reference
LR1	GCATATCAATAAGCGGAGGA	*Eucaryotic*	van Tuinen et al. (1998b)
NDL22	TGGTCCGTGTTTCAAGACG	*Eucaryotic*	van Tuinen et al. (1998b)
FLR3	TTGAAAGGGAAACGATTGAAG	*Glomeromycota*	Gollotte et al. (2004)
FLR4	TACGTCAACATCCTTAACGAA	*Glomeromycota*	Gollotte et al. (2004)
B15	AAATCTGTTGGGTTCCACTTA	*G. mosseae*	This study
Gc 2	CAATCCCGACCACCACAC	*G. claroideum*	This study
Gi 2	CGT CCGGTTGATCA TT T G C	*G. intraradices*	This study

systems were cut into 1-cm segments and subsamples stained either with trypan blue (Trouvelot et al. 1986) or for SDH and ALP activity using the modified procedure of Zhao et al. (1997). SDH activity was revealed by a purple-black precipitate, and ALP activity was indicated by a dark brown precipitate in mycorrhizal tissues. Root colonization was estimated by the method described by Trouvelot et al. (1986) using the MYCOCALC program (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). Presence or absence of AM fungi and extent of root cortex colonization was expressed as F% or M%.

Molecular monitoring of the three AM fungi in mix-inoculated root systems

To determine which fungal species was responsible for mycorrhizal formation after mixed inoculation at each salt level, 15 fragments of trypan blue-stained roots (70–90% colonization) were randomly taken for nested PCR. The stained roots were rinsed in sterile H_2O, crushed in 40 μL of TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA), and heated at 95°C for 10 min in the presence of 10 μL of 20% Chelex-100. The crude DNA suspension was separated from cellular fragments by centrifugation at 12,000×*g* for 5 min and diluted ten times that served as the template. The eukaryotic-specific primer pair LR1 and NDL22 was used for the first amplification (van Tuinen et al. 1998). The products, diluted 500–1,000 times, served as templates for the second reaction using taxon-discriminating primers B15, Gc2, and Gi2 (shown in Table 1) in combination with LR1 or NDL22. The PCR was performed as described by Dong and Zhao (2006). Reactions were performed in a final volume of 20 μL containing 2 μL 10× PCR buffer (plus Mg^{2+}), 2 μL 2 mM dNTP, 0.8 μL 10 μM each primer, 0.2 μL 5 U μL^{-1} *Taq* polymerase, 9.2 μL H_2O, and 5 μL template DNA. The amplification was performed in a thermal cycler programmed as follows: initial denaturation at 93°C for 3 min, followed by 34 cycles of denaturation at 93°C for 40 s, annealing at 58°C for 50 s, and extension at 72°C for 50 s. The last circle was followed by a final extension at 72°C for 5 min. The conditions of the second amplification were as above except for an annealing temperature of 61°C.The products were separated by gel electrophoresis on 1.2% agarose gel in TAE buffer, and DNA was visualized after staining with ethidium

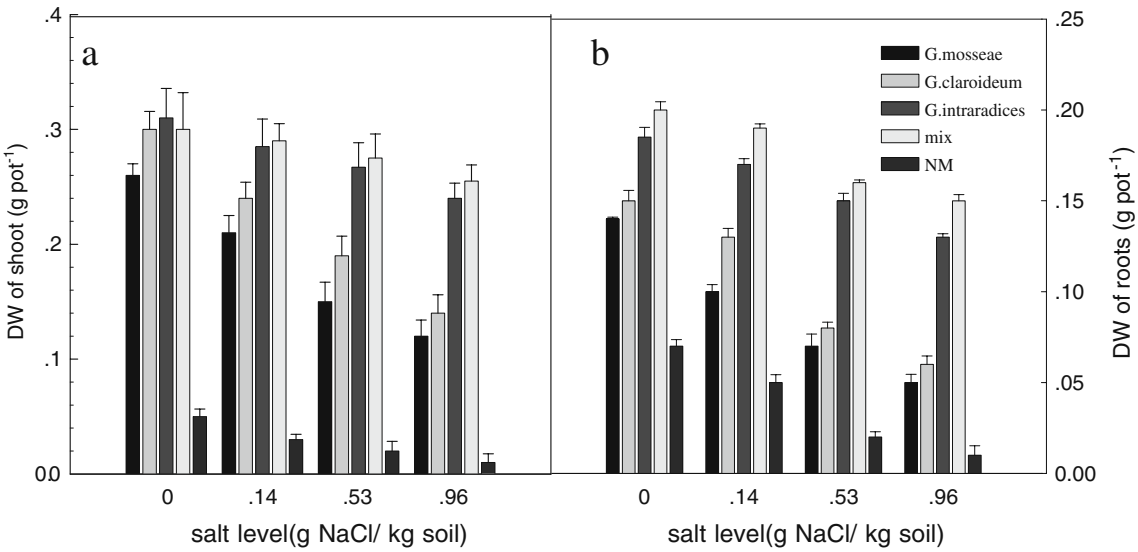

Fig. 1 Biomass of the shoot (**a**) and root (**b**) of *A. sinicus* colonized by three mycorrhizal fungi influenced by four salt levels. *Columns* represent mean±SE, $n=3$

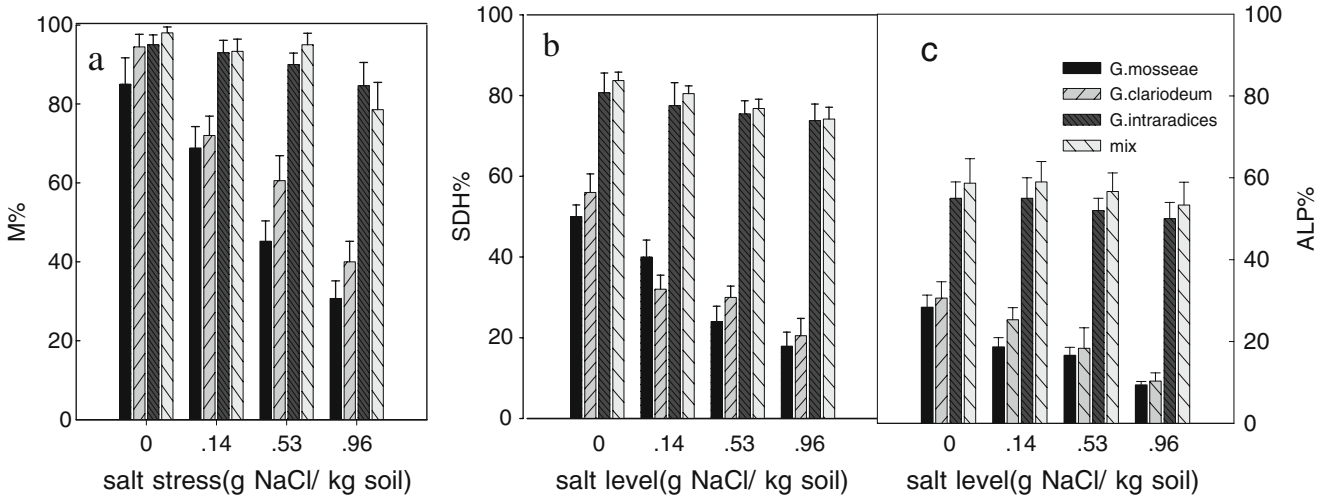

Fig. 2 Intensity of root colonization (*M%*) staining by trypan blue (**a**), succinate dehydrogenase activity (*SDH%*) (**b**), and alkaline phosphatase activity (*ALP%*) (**c**) in the roots of *A. sinicus* inoculated by three mycorrhizal fungi influenced by salt levels. *Columns* represent mean±SE, $n=3$

bromide. The fraction of mycorrhizal fragments (F%) in each fungus was estimated for each salt level.

Statistical analysis

Statistical analysis of data was performed following ANOVA analysis of variance using SAS 8.1. Means and standard errors were calculated for three replicate values. Comparisons between means were carried out using Duncan's multiple range tests at a significance level of $P<0.05$.

Results

Shoot and root biomass

Addition of NaCl in the soil significantly decreased the growth of uninoculated plants and that of plants inoculated with *G. mosseae* and *G. claroideum*. The dry weight of uninoculated plants was seriously limited in the absence and the presence of salt stress conditions (Fig. 1). However, inoculation with AM fungi significantly increased plant biomass in all treatments. Compared with the non-mycorrhizal control plants, inoculation with *G. intraradices* increased shoot dry weight 22-fold and root dry weight 12-fold with NaCl at 0.96 g kg^{-1}. The dry weight of plants inoculated with *G. intraradices* or mixed inoculum was considerably higher than that with *G. mosseae* and *G. claroideum,* especially at higher salt level.

Mycorrhizal colonization

No root colonization was observed in uninoculated plants. NaCl application markedly reduced root colonization by *G. mosseae* and *G. claroideum*. In roots colonized by *G. intraradices* and the mixed inoculum, the intensity of root cortex colonization (M%) was not obviously affected by salinity level and was maintained at 80–95% (Fig. 2a). Vital staining indicated that the proportion of SDH- or ALP-active hyphae paralleled the total mycorrhizal colonization estimated by trypan blue staining. For *G. mosseae* and *G. claroideum* treatments, the percentage of root length with enzyme-active hyphae decreased with increases of NaCl addition (Fig. 2b, c). However, the enzyme activity of intercellular hyphae produced by *G. intraradices* and the mixed inoculum in plant roots was less affected by salt stress. More than 77% hyphae of these two AM fungi inoculants were SDH-active, and up to 50–58% hyphae had ALP activity (Fig. 2b, c).

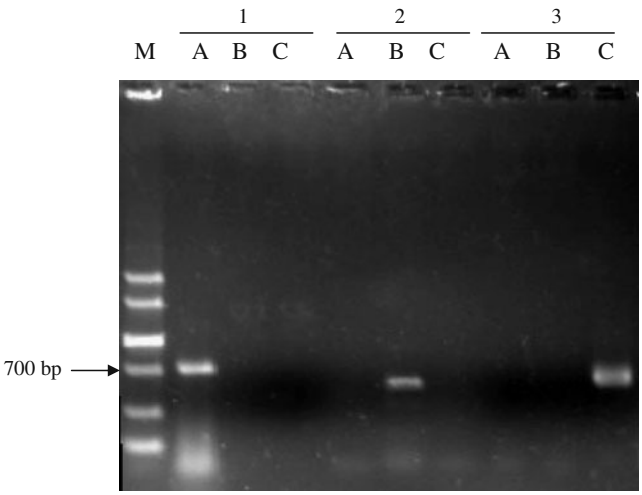

Fig. 3 Amplification products obtained after nested PCR with taxon-discriminating primer pairs B15–NDL22 (*1*), LR1–Gc2 (*2*), and Gi2–NDL22 (*3*) on three single inoculated roots, respectively. Fragment *A*, infected by *G. mosseae*; *B*, infected by *G. claroideum*; *C*, colonized by *G. intraradices*. *M*, marker

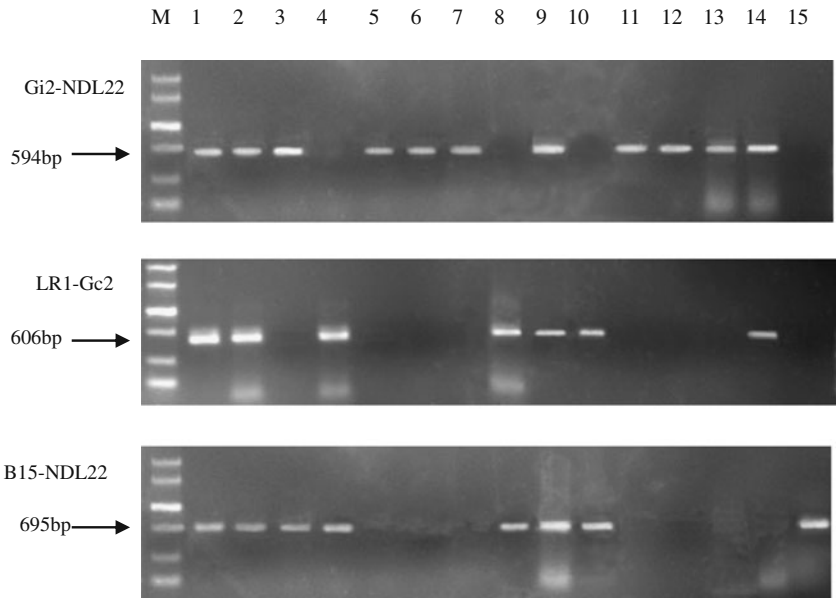

Fig. 4 Amplification products obtained by nested PCR with taxon-discriminating primer pairs Gi2–NDL22, LR1–Gc2, and B15–NDL22 on 15 mix-inoculated roots, respectively. *1–15*, the number of root fragment; *M*, marker

Detection of the three AM fungal species in *A. sinicus* roots

Three taxon-discriminating primers were designed (protocol not shown). The size of the amplification products was obtained by nested PCR using the designed primers. The primer pairs B15–NDL22, LR1–gc2, and gi2–NDL22 were designed for *G. mosseae, G. claroideum*, and *G. intraradices*, respectively, giving products of 695, 606, and 594 bp. There was no cross-amplification between the fungal species (Fig. 3). The primers B15, Gc2, and Gi2, combined with either LR1 or NDL22, were used to detect *G. mosseae*, *G. claroideum*, and *G. intraradices*, respectively, in mix-inoculated root systems (Fig. 4). Fifteen mix-colonized root fragments selected after staining by trypan blue were randomly sampled from the treatments amended with or not with NaCl, and the frequency of each fungus was analyzed. More than one AM fungus was detected in the majority of the root fragments (Fig. 4, Table 2). *G. mosseae* was detected more frequently in saline soil when inoculated in the presence of the other two fungi compared with inoculated alone, especially at the highest salinity level (0.96 g NaCl/kg soil). The colonization frequency by *G. intraradices* or *G. claroideum* was comparable at all salt levels, whether they were inoculated alone or mixed (Table 2).

Discussion

The results of the present study confirmed that AM fungi can differ in their response to salinity. While tolerance of *A.*

Table 2 Frequency of three AM fungi colonized in root systems at four salt levels

Salt level (gkg^{-1})	Strain detected	Frequency (F%)	
		In mix-inoculate roots	In single-inoculate roots
0	*G. mosseae*	80 ab	84 ab
	G. claroideum	93 a	94 a
	G. intraradices	100 a	95 a
0.14	*G. mosseae*	73 b	61 bc
	G. claroideum	66 b	72 b
	G. intraradices	93 a	93 a
0.53	*G. mosseae*	60 bc	31 c
	G. claroideum	66 b	71 b
	G. intraradices	93 a	90 a
0.96	*G. mosseae*	60 b	11 d
	G. claroideum	40 c	40 c
	G. intraradices	73 ab	85 a

The frequency of individual fungus in roots from the mix-inoculated experiment was estimated by nested PCR; the colonization frequency of individual fungus in roots from the experiment with single fungus was determined by TB staining. Different letters indicate significantly different values ($P<0.05$) for each fungus at each salt level

sinicus plants to salinity was enhanced by the three AM fungi with the increasing salt additions, as declared by Al-Karaki et al. (2001), the different fungi varied in their ability to alleviate the inhibitory effect of salt stress. This was reflected by plant growth responses, the intensity of root colonization, and the activity of SDH and ALP in intraradical mycelium. The symbiosis formed by *G. intraradices* exhibited a more stable viability and efficiency compared to that formed by *G. mosseae* or *G. claroideum* with increasing salinity. Analysis by nested PCR of *A. sinicus* roots inoculated with a mixture of *G. intraradices*, *G. mosseae*, and *G. claroideum* indicated that majority of the root fragments were colonized by more than one fungus at all salt levels and that the community was dominated by *G. intraradices*. These results suggested that *G. intraradices* probably had a major influence on the symbiotic performance of *A. sinicus* plants colonized by the mixed inoculum. In addition, root colonization of *G. mosseae* was enhanced in the presence of the other two fungi in mix-inoculated treatments in the saline soil, especially at high salinity levels. We speculate that there was a synergistic interaction between the AM fungi under salt stress. The underlying mechanism may involve a functional complementation with respect to P acquisition between the AM fungal species, as suggested by Koide (2000).

AM symbiosis can increase host resistance to salinity stress. The mechanism remains unclear. Some studies indicated that the enhanced salt tolerance mainly contributes to mycorrhizal-mediated enhancement of host mineral nutrient uptake, especially of immobile soil nutrients (Al-Karaki 2000, 2001).While other mechanisms were put forward, such as greater osmotic adjustment in AM plants, reduced oxidative damage. Porras-Soriano et al. (2009) reported that AM fungi colonization enhanced plant growth and K acquisition under salt stress. Potassium, as the most prominent inorganic solute, plays a key role in the osmoregulation processes. The higher K^+/Na^+ ratio is one of the determinants of plant salt tolerance (Naidoo and Naidoo 2001). He et al. (2007) suggested that the enhanced salt tolerance in AM symbiosis was mainly related with the elevated superoxide-dismutase, peroxidases, and ascorbate peroxidase activity by AM fungi, which degraded more reactive oxygen species and so alleviated the cell membrane damages under salt stress.

The present investigation gives novel information about root colonization and plant growth stimulation by different AM fungi inoculated either alone or as a mixed inoculum under salt-stressed soil conditions. The three AM fungi studied were affected differently by salt additions in terms of root colonization, SDH and ALP activity, as well as their effect on the growth of *A. sinicus*. Under the experimental conditions used, *G. intraradices* appeared the most effective fungus, *G. mosseae* the least, and *G. claroideum* intermediate in response to salinity. Nested PCR analyses of mix-colonized roots revealed *G. intraradices* to be the predominant fungus in plant roots at all salt levels and indicated the existence of a synergistic interaction between the three AM fungi. These data underline the importance of appropriate combination of different AM fungi in potential applications of mycorrhiza for vegetation of saline soils.

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