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Arbuscular mycorrhizal fungi (AMF) and phosphate-solubilizing fungus (PSF) on tolerance of beach plum (*Prunus maritima*) under salt stress

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Abstract

The synergic effect of an arbuscular mycorrhizal fungus (AMF) and a phosphate-solubilizing fungus (PSF) on the salt-tolerance of beach plum (*Prunus maritima*) grown in pots was explored. Pot experiments in greenhouse were carried out in one year beach plum inoculated with AMF (*Funneliformis mosseae*) or/and PSF (*Mortierella* sp. SM-1) under 1% NaCl stress. Salinity dramatically increased Na^+ concentrations, but decreased K^+ contents and K^+/ Na^+ in shoot and root significantly. And meanwhile, salinization reduced growth (root and shoot dry weight, total phosphate contents, chlorophyll contents) and biological soil quality (soil available phosphate contents and phosphatase enzyme activities). Plants inoculated with AMF or/and PSF counteracted both Na^+, K^+, K^+/ Na^+ changes and the plant growth parameters. Dual inoculation of AMF and PSF showed significant higher effects on variation of the ion concentration (Na^+, K^+) and plant growth indexes, available phosphate contents, phosphatase enzyme activities and the reduction of pH than those of single inoculated with AMF or PSF. Under NaCl stress, the percentages root colonization of plants co-inoculated with AMF and PSF were significantly higher than those of plants inoculated with AMF alone. It is concluded that AMF inoculation with PSF application could synergistically enhance salt-tolerance of plants.

Keywords: arbuscular mycorrhizal fungi, phosphate-solubilizing fungi, beach plum, NaCl stress.
Abbreviations: AMF_Arbuscular Mycorrhizal Fungi; AM_Arbuscular Mycorrhiza; PSF_Phosphate-solubilizing Fungi; Fm_*Funneliformis mosseae*; Mo_*Mortierella* sp. SM-1.

Introduction

Salt stress is one of the most serious agricultural problems in arid and semiarid regions, where salt accumulates on the soil surface and make it unproductive. Globally, almost 1,000 million ha (7% of all land area) are affected by soil salinity (Giri et al., 2007). There is a huge area of alkali-saline soil in China, which is about 27 million hm^2 (Zhang et al., 2011). Exploitation of these soils by cultivating crops or planting fruit trees adaptive to saline soils has a promising future. But the situation is complicated due to the fact that the soils in these regions generally contain little organic matter and bioavailable mineral nutrients. Thus, the establishment of salt tolerant crops or fruit trees is difficult without the use of fertilizers which are usually expensive to farmers of low incomes. In this respect, biological processes such as mycorrhizal application and phosphate-solubilizing microorganisms to alleviate salt stress are better options (Shi et al., 2013). Arbuscular mycorrhiza (AM) is a symbiosis between soil fungi and plants and occurs naturally in saline soils. Salinity affects the formation and function of AM symbiosis, and AM symbiosis could improve plant growth and productivity under salt stress (Jahromi et al., 2008; Zai et al., 2012). Thus, AM fungi (AMF) under salt stress conditions have been considered as bioameliorators of saline soils (Shi et al., 2013). Meanwhile, the improvement in the plant phosphorus (P) status has been recommended as the most important strategy of salinity stress tolerance in AMF colonized plants (Evelin et al., 2009; Manchanda and Garg, 2011). The inoculation of P-solubilizing microorganisms is also a promising technique because it can increase phosphorus availability in soils fertilized with rock phosphates (Vassilev et al., 2012). P-solubilizing fungi (PSF) have been reported to possess greater ability to solubilize insoluble phosphate than bacteria (Whitelaw et al., 1997). Some PSF can solubilize P precipitated with K^+, Ca^{2+} and other ions in salt-stressed soil (Zhang et al. 2013b) and so enhance the amount of available phosphate in saline alkaline soils (Zhang et al. 2011, 2013a). According to Manchanda and Garg (2007), plant roots are exposed to a range of soil microorganisms with which they have a variety of interactions. However, the effects of combined inoculation with AMF and PSM on salinity stress tolerance in colonized plants under saline conditions remain unclear. Beach plum (*Prunus maritima*) is a tall (3–4 m) shrub that colonizes relatively early successional sand dunes along the North Atlantic coast of America, where soils are often characterized by infertile and high salinity. Beach plum is not limited to sandy soil and it may be planted in any fertile and well drained soil. It enjoys high popularity for both ornamental and utilitarian values, with its profuse white bloom in spring and evergreen period lasting to late autumn, as well as its rich and edible fruit (Yan et al., 2009). Due to its strong adaptation to arid soils and its potential as an economic plant, beach plum was first introduced into China by Nanjing University in 2001. However, poor growth and low survival

of transplanted seedlings are now a serious problems that limits widespread cultivation in salinized soil in China. It has been discovered that roots of beach plum could form symbiotic associations with AMF and *Funneliformis mosseae* (Fm) inoculation could improve seedlings growth under salt stress (Zai et al., 2012). Nevertheless, no information is available on the effects of PSF, especially the dual inoculation of PSF with AMF on the growth responses of beach plum in saline soil. Therefore, the aims of the present study were to evaluate the effects of an AMF (*Funneliformis mosseae*), and a PSM (*Mortierella* sp. SM-1) which was isolated from salt-affected coastal soil samples collected from seashore of Jiangsu province by our laboratory (Zhang et al., 2011), on tolerance of beach plum (*P. maritima)* seedlings under 1% NaCl stress.

Results

Funneliformis mosseae and *Mortierella* sp. SM-1 *on contents of* Na^+, K^+ *in beach plum under NaCl stress*

Salinity increased Na^+ concentrations (mg g^{-1} dry weight) in the shoot (from 0.013% to 0.049%) and Na^+ concentrations in the roots (from 0.014% to 0.046%). In the shoots, K^+ contents decreased from 2.757% to 1.501% and in the roots, K^+ contents (mg g^{-1} dry weight) decreased from 1.312% to 0.722% in response to salinity. Meanwhile K^+/ Na^+ decreased significantly in both shoots and roots in response to salinity ($p \leq 0.05$, Table 1). In treatments with NaCl + Fm, NaCl + Mo and NaCl + Fm + Mo, the accumulations of Na^+ significantly decreased compared with the treatment of NaCl in the shoots and roots. Among the above three treatments, the treatment of NaCl + Fm + Mo was the most effective ($p \leq 0.05$, Table 1).

Funneliformis mosseae and *Mortierella* sp. SM-1 *on growth of beach plum under NaCl stress*

Salinization induced a significant reduction of the plant height, root length, shoot dry weight, root dry weight, chlorophyll and total phosphate contents in plants, while Fm, Mo, or Fm + Mo inoculation counteracted such reductions significantly. Among the three inoculations used, dual inoculation of Fm and Mo was the most effective. Dual inoculation of Fm and Mo showed significantly higher effects on plant growth parameters than those of individual inoculation with Fm or Mo ($p \leq 0.05$, Table 2). Structures characteristic of Fm were not observed in roots of controls and plants inoculated with Mo alone (data not shown). Under NaCl stress, the mycorrhizal colonizations of both Fm and Fm + Mo inoculation were decreased significantly, and the percentages root colonization of plants co-inoculated with Fm and Mo were significantly higher than those of plants inoculated with Fm alone ($p \leq 0.05$, Table 2).

Funneliformis mosseae and *Mortierella* sp. SM-1 *on available phosphate concentration, phosphatase enzyme activities and pH in the rhizosphere of beach plum under NaCl stress*

Salinization induced a significant reduction of the available soil phosphate concentration and phosphatase enzyme activities, while Fm, Mo, or Fm + Mo inoculation counteracted such reductions significantly. Among the three inoculations used, dual inoculation of Fm and Mo was the most effective for both available soil phosphate concentration and acid phosphatase enzyme activities ($p \leq 0.05$, Table 3). Fm, Mo, or Fm + Mo inoculation induced a significant reduction of the pH in rhizosphere soil of beach plum, while dual inoculation of Fm and Mo was the most effective ($p \leq 0.05$, Table 3).

Discussion

The deleterious effects of salinity on plant growth are associated with osmotic stress, ion toxicity or indirect effects of saline ions that cause soil/plant imbalance (Ashraf and Harris, 2004; Zhang et al., 2013). High concentrations of Na^+ can disrupt various enzymatic processes in the cytoplasm (Zhang et al., 2013a). AMF may diminish such deleterious effects of osmotic stress, ion toxicity and enzymatic activities (Guo et al., 2010; Zai et al., 2012; Estrada et al., 2013). Higher K^+ accumulation by mycorrhizal plants under salt stress conditions may help in maintaining a high K^+/Na^+ ratio, thus preventing the disruption of various enzymatic process, inhibition of protein synthesis and beneficial in influencing the ionic balance of the cytoplasm (Giri et al., 2003; Zhang et al., 2013a). This study showed the beneficial effects of inoculation with either Fm or Mo, especially the dual inoculation of Fm and Mo, resulted in enhanced higher K^+ accumulation and K^+/Na^+ ratio of beach plum. Increased phosphorus may result in decreased Na, which is indirectly related to K uptake (Allen and Cunningham, 1983; Giri et al., 2003). In the group of NaCl + Fm + Mo, a synergism of Fm and Mo in combination with NaCl led to the highest content of phosphorus in beach plum compared with the other groups (Table 1, Table 2) may be part of mechanism of NaCl stress alleviation of beach plum. Mycorrhizal symbiosis is a key component in helping plants to survive under adverse environmental conditions (Estrada et al., 2013). Our results showed that plant height, root length, shoot and root dry weights and total phosphate contents of beach plum of the treatments of NaCl + Fm, NaCl + Mo and NaCl + Fm + Mo were significantly higher than those of NaCl group in this study. Particularly, the dual inoculation with Fm and Mo had synergic effects on plant height, root length, shoot and root dry weights. Similar effects were found in *Leucaena leucoce*phala (Osorio and Habte, 2001), clover (Souchie et al., 2006), *Kostelelzkya virginica* (Zhang et al., 2011), etc. PSF contribution on growth promotion was probably due to an effective phosphorus solubilization ability (Souchie et al., 2005) and/or by phytohormone production (Barea et al., 2002). In this paper, Mo contribution on growth promotion may be related to the improvement of phosphorus solubilization ability (Table 1, Table 3). Mycorrhizal fungi enhanced chlorophyll content in beach plum leaves, a result in congruence with other studies (Sannazzaro et al., 2006; Sheng et al., 2008). Mycorrhizal inoculation enhances phosphorus and magnesium uptake and reduces sodium concentrations in the plant; this in turn helps in increasing chlorophyll content, and improves the overall performance of mycorrhizal plants (Giri and Mukerji, 2004). This study showed that combined inoculation of Fm and Mo under NaCl stress could promote the chlorophyll content in beach plum leaves synergistically. This may be due to saline soil inoculated with Mo increased the content of available phosphorus in soil (Table 3), and stimulating the production of plant hormones (Jacobsen et al., 1992). It was clearly demonstrated in this study that salt stress significantly inhibited mycorrhizal colonization. In the presence of NaCl, the germination of spores of AMF tested was delayed, and the specific rate of hyphal extension of AM fungi was reduced, with a subsequent decrease in the spread of mycorrhizal colonization (Juniper and Abbott, 2006). Results

Table 1. Effects of *Funneliformis mosseae* and *Mortierella* sp. SM-1 on contents of Na^+, K^+ in beach plum under stress.

Treatments	Shoots			Roots		
	Na^+ mg g^{-1}	K^+ mg g^{-1}	K^+/ Na^+	Na^+ mg g^{-1}	K^+ mg g^{-1}	K^+/ Na^+
CK	0.13 d	27.57 a	212.07 b	0.14 c	13.12 b	93.71 b
Fm	0.09 d	21.32 b	236.89 a	0.13 c	14.04 a	108.00 a
Mo	0.10 d	21.16 b	211.60 b	0.14 c	13.61 b	97.21 b
NaCl	0.49 a	15.01 d	30.63 e	0.46 a	7.22 d	15.70 e
NaCl + Fm	0.31 b	16.57 c	53.45 d	0.28 b	11.10 c	39.64 d
NaCl + Mo	0.35 b	17.41 c	49.94 d	0.35 b	12.29 c	35.11 d
NaCl + Fm + Mo	0.24 c	17.53 c	73.04 c	0.27 b	12.55 c	45.37 c

Mean separation with each column was by Duncan's New Multiple Range Test ($p \leq 0.05$). Values in each column followed by the same lower-case letter are not significantly different.

showed that combined inoculation of Fm and Mo could promote mycorrhizal root colonization under salt stress synergistically. Beyond the phosphate solubilization, many P-solubilizing microorganisms increase the mycorrhizal root colonization by production of specific metabolites as vitamins, amino acids and hormones (Barea et al., 2002). The effects of AMF on acid phosphatase activities were stimulated by Mo inoculation in rhizosphere soil, but alkaline and neutral phosphatase activities were little changed, and showed no difference among different treatments. This is in agreement with Zhang et al. (2011) studies on phosphatase activities *of Kostelelzkya virginica*. The difference illustrates the fact that different enzymes had different responses to combine inoculation of PSF and AMF in rhizosphere soils of this study. Osorio and Habte (2001) reported that acid production was the major mechanism in the solubilization of rock phosphate by the *Mortierella* sp.. The pH of the growth medium decreased as a result of acid production by PSM. Soil enzymatic activities are usually significantly positive, negative or not correlated to soil pH (Kang and Freeman, 1999; Acosta-Martinez and Tabatabai, 2000). In this study, plants co-inoculated with AMF and Mo manifested positive effects on soil enzymatic activities in rhizosphere soils. In conclusion, all of the above results indicate that co-inoculation with an AMF and PSM increase tolerance of beach plum and growth under 1% NaCl. In non-inoculated plants, 1% NaCl induced lower K^+/Na^+ ratios in the roots and shoots, available phosphate contents and phosphatase enzyme activities in rhizosphere soils, resulting in an important growth reduction in the roots and shoots. The combined inoculation of AMF and PSM alleviated the deleterious effects of 1% NaCl on beach plum and stimulated plant growth principally by increasing K^+ accumulation and maintaining higher K^+/Na^+ ratios in root and shoot tissue and phosphatase enzyme activities in rhizosphere soils. It is concluded that AMF inoculation with PSF application could synergistically enhance salt-tolerance of plants.

Materials and Methods

Plant materials

New healthy semi-lignified branches of beach plum were collected from the agricultural sightseeing garden in Lishui County of Jiangsu in March 2011. The branches were propagated in the seedling breeding sand bed at the Mufu Campus of Nanjing Jinling Institute of Technology. After three months, 5000 healthy beach plums cuttings with similar shape were selected and transplanted in the pots filled with above cultivation substrate. The substrate used in this study was purchased from Red Sun Group, Nanjing, P.R. China. The basic components of cultivation substrate were as follow: 1.35% organic matter, 0.0048% available N (w/w, the same below), P 0.0025%, K 0.0148%, pH 7.2.

Fungal inocula

The mycorrhizal fungus was *Funneliformis mosseae*, in the form of sandy soils containing AM fungal spores and maize root fragments. The strain number of original inoculum (BGCJX01) was a microbial fertilizer of Chinese Academy of Agriculture, which was separated from *Osmanthus fragrans* tree rhizosphere in Jiangxi Province of China, and then propagated on maize plants growing in sandy soil for 10 weeks. The original inoculum (BGCJX01) was obtained from the Institute of Plant Nutrition and Fertilisers, Chinese Academy of Agriculture. The inoculum of *Mortierella* sp. SM-1was prepared using the method of Zhang et al. (2011). To prepare liquid inoculum of *Mortierella* sp., the first step was to activate strains on slants. The fungus was inoculated on solid Martin culture medium (K_2HPO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 11.5 g, peptone 5 g, glucose10 g, gelose10 g, 1/30,000 Bengal red water solution 100 ml, and demineralized water 900 ml), which had been autoclaved for 30 min at 121°C and then incubated in the dark at 28°C for 4 days. After activation, 3 ml sterile water was added to test tube, and then the mixture was poured into 50 ml Martin broth (MB) which was added to 1.15% NaCl; *Mortierella sp.* was grown on a rotating shaker at 180 rpm for 48 h, and this was the starter culture. It was added (5% of volume) to MB and then we added 1.15% NaCl, and the MB was cultured on a shaker for 96 h at 180 rpm. At the end it contained 2.3×10^5 colony forming units mL^{-1} and the solution was stored at 4°C until use.

Experimental design and biological treatments

To study the effects of AMF and PSF on beach plum under NaCl stress, we used a full factorial experimental design, with seven treatments (inoculated with *Funneliformis mosseae* (Fm) 10 g; inoculated with *Mortierella* sp. SM-1 (Mo) 10 ml; inoculated with *G. mosseae* 10 g and 1% NaCl (Fm + NaCl); inoculated with *Mortierella* sp. SM-1 10 ml and 1% NaCl (Mo + NaCl); inoculated with *Funneliformis mosseae* 10 g and *Mortierella* sp. SM-1 10 ml and 1% NaCl (Fm + Mo + NaCl); no inoculated and 0% NaCl control (CK); 1% NaCl (NaCl). Each treatment was replicated three times in a randomized block design and each treatment was comprised of 30 pots (3 plants per pot), making 630 pots in total. On June 18, 2011, healthy beach plums with similar size which were transplanted in the pots (diameter 30 cm, height 20 cm) filled with 900 g cultivation substrate were selected as the experimental treatment. According to the design, per relevant pot received 10 g Fm inoculums (283

Table 2. Effects of *Funneliformis mosseae* and *Mortierella* sp. SM-1 on growth of beach plum under NaCl stress.

Treatments	Plant height (cm)	Root length (cm)	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Mycorrhizal colonization (%)	Chlorophyll content (g plant^{-1})	Total phosphate (mg kg^{-1})
CK	47.9 ab	20.1 ab	5.63 b	0.37 b	0 d	4.23 b	0.227 b
Fm	50.2 a	22.4 a	6.31 a	0.40 a	49.5 a	4.58 a	0.248 a
Mo	49.6 a	21.3 a	6.17 a	0.36 b	0 d	4.31 b	0.235 a
NaCl	34.5 d	11.8 e	3.76 e	0.15 e	0 d	1.73 e	0.094 f
NaCl + Fm	39.7 c	16.4 d	4.19 d	0.23 d	25.7 c	3.24 d	0.176 e
NaCl + Mo	39.2 c	16.6 d	3.99 d	0.21 d	0 d	3.31 d	0.181 d
NaCl + Fm + Mo	43.3 b	18.9 c	4.87 c	0.29 c	36.7 b	3.57 c	0.203 c

Mean separation with each column was by Duncan's New Multiple Range Test ($p \leq 0.05$). Values in each column followed by the same lower-case letter are not significantly different.

Table 3. Effects of *Funneliformis mosseae* and *Mortierella* sp. SM-1 on available phosphate contents, phosphatase enzyme activities and pH in the rhizosphere of beach plum under NaCl stress.

Treatments	Available phosphate (mg kg^{-1})	Phosphatase enzyme activities (mg Phenol g^{-1} d^{-1})			pH
		Alkaline	Neutral	Acid	
CK	7.64 b	1.754 b	1.195 b	0.391 b	7.03 a
Fm	9.32 a	1.932 a	1.387 a	0.487 a	6.61 b
Mo	9.29 a	1.899 a	1.391 a	0.483 a	6.43 b
NaCl	4.77 e	1.511 c	0.845 d	0.166 e	7.05 a
NaCl + Fm	5.13 d	1.702 b	1.084 c	0.277 d	6.47 b
NaCl + Mo	5.08 d	1.684 b	1.055 c	0.263 d	6.34 b
NaCl + Fm + Mo	5.99 c	1.712 b	1.063 c	0.372 c	6.01 c

Mean separation with each column was by Duncan's New Multiple Range Test (P = 0.05).
Values in each column followed by the same lower-case letter are not significantly different.

spores) or/and 10 ml Mo by placing inoculum in soil below the beach plum prior to planting. Each control pot received 5 ml of 10 g inoculums filtrate that was sieved through a 25 mm filter in an attempt to provide similar microbial populations (excluding AMF) in all treatments. Meanwhile, 10 g mycorrhizal inoculums and 10 ml *Mortierella* sp. inoculums which had been autoclaved at 121 °C for 90 min three times were added to control pots. Each pot was placed on a 2-cm-deep plate and displaced in greenhouse under controlled conditions (16 h of 220 μmol $m^{-2}s^{-1}$ daylight intensity at 28 °C, 8 h of night at 18 °C, relative humidity kept at 65–85 %) on June 20, 2011. Plants were irrigated with water and established for 4 weeks. Then, pots were watered by a modified (all nutrient solutions without P) solution of Hoagland and Arnon (1950). The salinity of the substrate used was tested by dry mass of medium, and the process was as follows: dissolving the corresponding amount of NaCl in 3 L of water, pouring the NaCl solution into the pots evenly for 3 times from 7th d to 21th d after salt treatment, giving final NaCl concentration of 1%, and pouring occasional leakage back to the trays after 1 h. The pH of the substrate for all treatments was 7.2. The pots were watered every 5–7 d in order to maintain the soil moisture within 75–80%. After the saltstress treatment, each pot was watered with 200 ml of Hoagland nutrient solution every 3 d until the 90th d.

Soil samples collection

After 90 days, according to Riley and Barber (1969, 1970), the whole plants were removed from pots. The soil obtained by gently shaking roots and collected in sterilized culture dish was considered "bulk soil." The rest soil that adhered to roots was then collected in another sterile culture dish and termed "rhizosphere soil." Soil samples were divided into 2 parts respectively. One part was restored at 4 °C for biological and biochemical analyses and the other was air-dried at room temperature for physical-chemical analysis.

Plant analyses

Root and shoot tissues were analyzed for K and Na content. Dried shoot tissues were digested in a Kjeldahl flask with 1 mL (9.2 M) $HClO_4$, 5 mL (14.3 M) HNO_3 and 0.5 mL (17.8 M) H_2SO_4. P concentrations of the root and shoot tissues were determined by an ammonium molybdate blue method (Jones et al. 1991), and those of K^+ and Na^+ by flame photometry (Sengupta and Chaudhuri, 2002). The 3rd to 5th leaves from the end of beach plum were selected to test the Chlorophyll content (Zai et al. 2012). Three plants from three pots per treatment were selected at random and the number of the root length, the dry weights of roots and shoots and plant height of each beach plum were recorded. All shoots and roots tissues were dried in a forced-air oven at 80 °C for 72 h for dry weight determination. Shoot dry weight was the sum of leaves and stems. The fresh root mass of three plants were used for determining AMF colonization. To assess AMF colonization, roots of three plants were cleared with 10% KOH and stained with 0.05% trypan blue (Phillips and Hayman 1970). The percentage of root length colonized by AMF was estimated according to McGonigle et al. (1990). The roots were cut into 1-cm long bits, and 30 bits were examined per sample for their AMF status under a compound microscope (100X magnification). Positive counts for AMF colonization included the presence of vesicles or arbuscules or typical mycelium within the roots. The percentage of AMF colonization was calculated from the following equation:
Percentage of AMF colonization =Root length colonized/Root length observed ×100%.

Soil chemical analysis and enzymatic activity determination

The available phosphorus concentrations of soils were determined using sodium bicarbonate-extractable phosphorus colorimetric method (Olsen et al. 1954). Meanwhile, the activities of soil enzymes were determined

spectrophotometrically: phosphatase (E.C. 3.1.3.2; Kandeler et al. 1999). Phosphatase activity was determined according to the improved method of Hoffman (Kandeler et al. 1999), and phosphatase was represented to a phenol number of milligrams per gram of soil. Twenty grams of dry soil from each sample were diluted with deionized water (1:5 soil–water, w/v), and measured by pH meter (PHS-P) for pH.

Statistical analysis

All data were statistically analyzed by analysis of variance (ANOVA) using the SPSS software package (SPSS 10 for Windows 2001). Duncan's multiple-range test was performed at $p \leq 0.05$ on each of the significant variables measured.

Conclusion

In conclusion, all of the above results indicate that co-inoculation with an AMF and PSM increase tolerance of beach plum and growth under 1% NaCl. In non-inoculated plants, 1% NaCl induced lower K^+/Na^+ ratios in the roots and shoots, available phosphate contents and phosphatase enzyme activities in rhizosphere soils, resulting in an important growth reduction in the roots and shoots. The combined inoculation of AMF and PSM alleviated the deleterious effects of 1% NaCl on beach plum and stimulated plant growth principally by increasing K^+ accumulation and maintaining higher K^+/Na^+ ratios in root and shoot tissue and phosphatase enzyme activities in rhizosphere soils. It is concluded that AMF inoculation with PSF application could synergistically enhance salt-tolerance of plants.

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