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RESEARCH PAPER

Arbuscular mycorrhizal fungi can ameliorate salt stress in *Elaeagnus angustifolia* by improving leaf photosynthetic function and ultrastructure

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Rhizophagus irregularis; salt stress; oleaster; photosynthetic parameters; photosystem II.

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ABSTRACT

- Arbuscular mycorrhizal fungi (AMF) can form symbiosis with *Elaeagnus angustifolia*, allowing this species to tolerate salt stress. However, the physiological mechanism through which AMF improve *E. angustifolia* tolerance is still unclear.
- In this study, we examined *E. angustifolia* inoculated with AMF *Rhizophagus irregularis* (M) or inactivated inoculum (NM) under 0 and 300 mM NaCl stress for the determination of photosynthetic gas exchange, pigment content, chlorophyll fluorescence, antioxidant capacity and chloroplast ultrastructural in leaves.
- Photosynthetic gas exchange parameters in the leaves of M and NM decreased significantly under salt stress, while the M treatment significantly reduced the effect of salt stress compared with NM. Various chlorophyll components in the M treatment were two- to three-fold higher than in NM, together with a much more complex chloroplast structure and higher number of plastoglobules. The total flavonoid and proline content in leaves of M increased significantly, while the concentration of malondialdehyde (MDA) decreased significantly under salt stress. Chlorophyll fluorescence data also showed good PSII function in the M treatment, together with salt stress reduction of photochemical reactions and sharp enhancements in non-photosynthetic quenching (NPQ). AMF inoculation ameliorated the inhibition on the actual PSII efficiency (ΦPSII) and the photochemical quenching coefficient (q_P) by 10–15%.
- Our results clearly demonstrate that *R. irregularis* can improve the salt tolerance of plants by improving leaf photosynthetic performance, PSII function, antioxidant capacity and leaf chloroplast ultrastructure, and that *E. angustifolia* inoculated with AMF could enhance saline soil rehabilitation.

INTRODUCTION

Soil salinization is a growing problem worldwide. Currently, global salinized land accounts for approximately 10% of the cultivated land area (Porcel *et al.*, 2012; Munns & Gilliham, 2015) and in China covers approximately 100 million ha (Huang, 2018). The negative effect of salt stress on plant physiology is due to the toxic effect of sodium (Na) and chloride (Cl) ions (Silva *et al.*, 2015; Foti *et al.*, 2018), which results in an imbalance in plant nutrients and hinders the absorption of water by roots, leading to destruction of the plant microstructure, malfunctioning of several organs and thus death of the plant (Yang *et al.*, 2011). In recent years, soil salinization and secondary salinization have restricted the sustainable development of agriculture in China (Wang *et al.*, 2016); hence, ameliorating saline-alkali soils has attracted the attention of Chinese policymakers and scientists (Wang *et al.*, 2011).

Soil salinization damages leaf photosynthetic organs, thus maintaining photosynthetic function under salt stress is an important goal for plant salt tolerance. Together with gas exchange characteristics, well-functioning PSII machinery under salt stress treatments, measured as apparent PSII efficiency, F_v/F_m (maximum PSII efficiency), q_P (photosynthetic quenching) and NPQ (non-photosynthetic quenching), is often used in evaluating plant response to different stresses, including saline-alkali stress (Wang *et al.*, 2009; Wang *et al.*, 2013). Salt stress induces changes in many plant structures and functions that help them to adapt to a new environment (Estrada *et al.*, 2013) and affects the ultrastructure of photosynthetic organs (He *et al.*, 2017), *i.e.* chloroplasts and chlorophyll content, which can be used as indicators of adaption to stress.

Elaeagnus angustifolia is a deciduous shrub or small tree that is drought resistant, wind resistant and tolerant of salinity. As a pioneer afforestation tree species, it is mainly found in the north-western provinces of China, western part of Inner

Mongolia and a small area of northern China (Chang *et al.,* 2018). Arbuscular mycorrhizal fungi (AMF) are a group of fungi that can establish symbiotic relationships with plants. AMF colonization of plant root cortical cells accelerates plant nutrient uptake (Zhu *et al.,* 2015), promotes plant growth and development, improves soil physical and chemical properties, and thus can affect ecosystem functioning (Song *et al.,* 2015; Mickan *et al.,* 2016). The symbiosis between AMF and certain plants can alleviate the inhibition of photosynthesis caused by salt stress (Wu *et al.,* 2015; Porcel *et al.,* 2015). In our research group, Chang *et al.* (2018) reported that AMF can significantly increase the biomass of *E. angustifolia* seedlings under salt stress, effectively regulate the antioxidant response and improve ion distribution of the host plants, as well as significantly alleviating the stress caused by salt. Thus, co-utilization of the salt resistance characteristics of *E. angustifolia* and the possible improvement of salt stress resistance after AMF inoculation could benefit afforestation practices in saline-alkali regions, which, in turn, has important practical significance for improvement of saline-alkali degraded land. Therefore, a complete examination of photosynthetic gas exchange, PSII function and chloroplast ultrastructure might clarify the underlying mechanism of plant salt tolerance in plants colonized by AMF.

In this paper, we hypothesized that AMF would ameliorate salt stress of *E. angustifolia* by improving leaf photosynthetic performance, PSII function and leaf chloroplast ultrastructure. The following questions are addressed: (i) does AMF colonization alleviate the damaging effects of salt stress on photosynthetic capacity, PSII function and ultrastructure of leaf chloroplasts of *E. angustifolia* seedlings; and (ii) what is the extent of this amelioration, if present?

MATERIAL AND METHODS

Material

Seeds of *E. angustifolia* L. were provided by Heilongjiang Jinxiu Bioengineering, China. The seeds were cultivated in a mixture of forest soil, vermiculite and sand (5:3:2; v:v:v). The forest soil pH was 7.2, with 1.2% organic matter, 123.4 $mg \cdot kg^{-1}$ effective N, 12.6 $mg \cdot kg^{-1}$ available P, 76.5 $mg \cdot kg^{-1}$ available K, and electrical conductivity of 0.5 $dS \cdot m^{-1}$ (Chang *et al.,* 2018). This medium was autoclaved at 120 °C for 2 h to inactivate AMF then air dried before use for seed cultivation.

The AMF *Rhizophagus irregularis* (RI) inoculum was a mixture obtained from trap cultures (using *Sorghum* sp.) and contained spores, hyphae and mycorrhizal segments, with approximately 25 $spores \cdot g^{-1}$.

Treatments and sampling

The experimental design consisted of two factors: inoculation with AMF and salt stress (0 and 300 mM NaCl). The AMF treatment consisted of inoculation with *R. irregularis* (M) or with the same amount of autoclaved (dead) inoculum (NM). The experiments used a gradient in NaCl concentrations to assess tolerance of *E. angustifolia*, which could not grow normally >300 mM NaCl (Chang *et al.,* 2018), hence, 300 mM NaCl was selected for this research.

Elaeagnus angustifolia seedling cultivation began on 13 May 2017. The seeds were disinfected in potassium permanganate solution, then planted in the medium (12 $seeds \cdot pot^{-1}$). AMF treatments consisted of inoculation with 1% *R. irregularis* (RI) into the soil matrix of 20 pots (30 cm × 15 cm × 15 cm; measured as g $RI \cdot g^{-1}$ soil matrix) per treatment. The NM treatment was inoculated with inactivated RI inoculum, supplemented with a filtrate (<20 μm) of the inoculum to provide the microbial populations accompanying the AMF. All potted plants were placed in the plastic greenhouse of Heilongjiang Botanical Garden, China (45° 42′ 40.09″ N, 126° 38′ 22.23″ W) in a 16-h photoperiod, at 25/18 °C day/night temperature and 60% relative humidity.

In August 2017, *E. angustifolia* seedlings were randomly selected for determination of mycorrhizal colonization. Salt stress treatment was initiated when the mycorrhizal colonization reached >90%. For the M and NM treatments, ten pots of seedlings were randomly selected for the salt stress treatments (0 and 300 mM NaCl) added to the soil substrate from a 2 M stock saline solution based on the amount of substrate in pots. The concentration of NaCl in the soil was increased gradually on alternate days to prevent osmotic shock. It took 6 days to reach the desired concentration of 300 mM NaCl and this was maintained for 21 days. The experiments consisted of four treatments, M, M^{+NaCl}, NM and NM^{+NaCl}, and each treatment had ten replicated pots. Between 09:00–11:00 h on 11 September (NaCl stress 0 day), 17 September (NaCl stress 7 days), 24 September (NaCl stress 14 days) and 1 October (NaCl stress 21 days), three seedlings with six to eight upper leaves each were randomly selected from different pots for photosynthesis measurements. Chlorophyll fluorescence parameters were simultaneously determined at 09:00 h on 1 October (19 °C). On 2 October, the same six to eight leaves were collected from the top of each measured seedling and immediately placed into 2.5% glutaraldehyde solution for subsequent leaf ultrastructure observations under a microscope. The rest of the leaves were stored in liquid nitrogen in a −80 °C freezer until determination of chlorophyll content.

Experimental design

Determination of the mycorrhizal colonization rate of *E. angustifolia* seedlings was performed using the potassium hydroxide decolourizing acid fuchsin staining method (Phillips & Hayman, 1970). From each of the four treatments, root segments were collected from five seedlings, *i.e.* a total of 20 seedlings. The roots were dyed, decolourized, observed under a microscope, and the mycorrhizal colonization rate was calculated as:

$$\text{Mycorrhizal colonization rate} = \left(\frac{\text{number of infected root segments}}{\text{total number of root segments observed under microscope}}\right) \times 100\% \quad (1)$$

Photosynthesis was measured using a CI-340 Handheld Photosynthesis System (CID Bio-science, Camas, WA, USA) at 25 °C with an ambient CO_2 concentration of 400 $\mu mol \cdot mol^{-1}$, relative humidity of 75% and 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity. Measurements included net photosynthesis (P_n), transpiration rate (T_r), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i).

Chlorophyll fluorescence parameters were measured using an Li-6400 (Li-Cor, Lincoln, NB, USA) with leaf chamber 6400-40. Samples were first dark-adapted for 30 min before measurement. Maximum fluorescence (F_m), minimum fluorescence (F_o) and F_v/F_m of leaves were first determined. Then plants were exposed to natural light for at least 1 h. After which maximum fluorescence (F_m'), minimum fluorescence (F_o') and steady-state fluorescence (F_s) of leaves were measured. The following parameters were calculated: ΦPSII, q_P, NPQ and F_v/F_o (Rascher *et al.*, 2000).

Determination of the chlorophyll content was based on the ethanol extraction method (Li *et al.*, 2000). The content of malondialdehyde (MDA) in leaves was measured using the barbituric acid method (Hodges *et al.*, 1999) and proline content with the acid ninhydrin method (Bates *et al.*, 1973). The content of total flavonoids in leaves was determined with the aluminium trichloride method (Basma *et al.*, 2011).

The leaves of *E. angustifolia* were fixed with 2.5% glutaraldehyde, immersed in pH 7.2 phosphate buffer, flushed with 1% osmic acid, subjected to an ethanol dehydration series and propylene oxide transition, embedded using Epon812, sliced, used for transmission electron microscopy (Hitachi H-7650, Tokyo, Japan) and photographed.

Statistical analysis

Together with absolute values for each parameter, relative changes were also used to facilitate comparison. Salt-stress-induced down-regulation of each parameter was calculated as $(P_{NM} - P_{NM+NaCl})/P_{NM} \times 100\%$. Improvement to growth after AMF treatment (M treatment) was calculated as $(P_M - P_{M+NaCl})/P_M \times 100\%$. A negative value for relative changes means that the treatment resulted in a reduction in a parameter compared with no treatment (NM), while a positive value means that the treatment resulted in an increase in growth. Given that salt stress down-regulates photosynthetic functions and that the M treatment up-regulates photosynthesis, the co-treatment amelioration of salt stress can be identified as reductions in percentage down-regulation compared with salt stress treatment. SPSS 16.0 (SPSS, Chicago, IL, USA) and Origin 8.5 (OriginLab, USA) software were used for data processing and statistical analysis. The repeated collections of data during the experiment were analysed using repeated measures ANOVA. The significance of the differences between treatment swas analysed with two-way ANOVA and the S-N-K test.

RESULTS

Rhizophagus irregularis colonization

Growth of *E. angustifolia* seedlings was examined under a microscope on three occasions, 20 August, 30 August and 10 September 2017, using the acid magenta method. On 20 August, mycorrhizal fungi were detected in plants inoculated with RI; the colonization rate of all examined plants reached 100% and percentage of infected roots was approximately 50%. Vesicles and mycelia were found in the root sections during this test (Fig. 1). Root sections examined on 30 August contained more vesicles and mycelia than on 20 August, with an infection percentage of approximately 85%. The inoculated seedlings were tested again on 10 September.

Rhizophagus irregularis growth

There were no significant differences in plant growth between NM and M (Table 1). However, salt stress decreased the total biomass and single leaf area, and also significantly increased leaf abscission ($P < 0.05$) regardless of whether the plant had been inoculated with RI.

Net photosynthetic and other gas exchange parameters

With increasing salt stress duration, the changes in P_n, T_r, g_s and C_i in leaves of *E. angustifolia* seedlings in both NM and M treatments were similar (Fig. 2), *i.e.* they decreased slightly as a whole. Changes of P_n, T_r, g_s and C_i in the leaves of *E. angustifolia* seedlings treated with M^{+NaCl} and NM^{+NaCl} also showed similar patterns, i.e., sharp decreases but higher values in M^{+NaCl} ($P < 0.05$).

The P_n, T_r, g_s and C_i in leaves of *E. angustifolia* in M^{+NaCl} and NM^{+NaCl} decreased more sharply than those of M and NM treatments, showing that salt stress disrupts photosynthetic gas exchange of seedling leaves. Under the same salt stress time, the P_n, T_r, g_s and C_i in leaves of M-treated seedlings were 1.3–1.5-fold higher than those of NM. Similarly, the P_n, T_r, g_s and C_i in leaves of M^{+NaCl}-treated seedlings were significantly higher than those of the NM^{+NaCl} treatment ($P < 0.05$). Table 2 shows that after 21 days of salt addition, the decrease in each parameter in the M^{+NaCl} treatment was less than that of the NM^{+NaCl} treatment, and the difference was significant ($P < 0.05$), while the difference for C_i was even more significant ($P < 0.01$). The results show that inoculation with RI significantly improved the photosynthetic capacity of *E. angustifolia* seedlings and alleviated salt stress. A significant recovery of 2.0% in P_n, 4.8% in T_r and 3.7% in g_s was observed. As shown in C_i values, the salt stress treatments (NM^{+NaCl} and M^{+NaCl}) had much lower internal CO_2 concentrations (100–-150 μmol·mol^{-1}), less than half of the values in the control

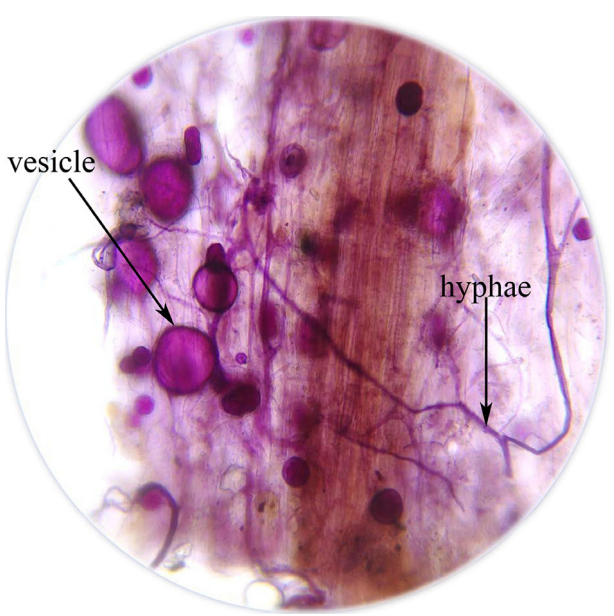

Fig. 1. The hyphae and vesicles of AMF *R. irregularis* inside the roots of *E. angustifolia* (400×).

Table 1. Effects of *R. irregularis* inoculation and salt stress on plant growth.

	Total biomass (g)	Single leaf area (cm^2)	Abscised leaves (%)
NM	1.76 ± 0.09[a]	4.02 ± 0.29[a]	45.36 ± 2.21[b]
M	2.18 ± 0.16[a]	5.14 ± 0.42[a]	38.17 ± 2.91[b]
NM^{+NaCl}	0.44 ± 0.06[b]	0.78 ± 0.21[b]	70.41 ± 1.41[a]
M^{+NaCl}	0.90 ± 0.12[b]	1.86 ± 0.25[b]	61.97 ± 3.41[a]

Values represent mean ± SD, different lowercase letters indicate a significant difference in the same column ($P < 0.05$).

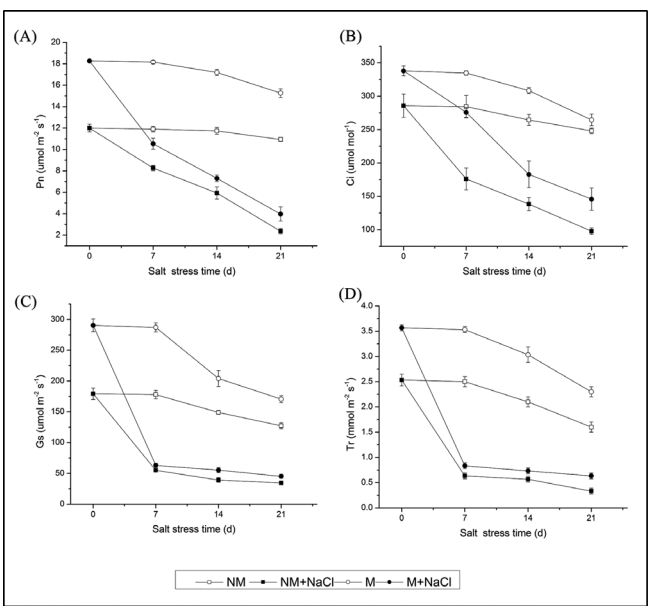

Fig. 2. Effects of *R. irregularis* on (A) net photosynthetic rate (P_n), (B) intercellular CO_2 concentration (C_i), (C) stomatal conductance (g_s) and (D) transpiration (T_r) of *E. angustifolia* under salt stress.

treatment. The significant of 8.9% in C_i showed that the M treatment strongly ameliorated the CO_2 shortage in the intercellular photosynthetic machinery (Table 2).

Chlorophyll content and components

Salt stress significantly decreased leaf chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid content in seedlings, both with and without RI colonization (Table 3). However, the decreases in RI-infected seedlings were much less than without RI colonization. After 3 weeks of salt stress, compared with NM, the leaf chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid content in the NM^{+NaCl} treatment decreased by 59.8%, 69.6%, 60.2% and 71.8%, respectively. However, the corresponding values for the M^{+NaCl} treatment were 32.7%, 50.5%, 34.7% and 48.1%, respectively. Under the same salt stresses, *E. angustifolia* seedlings inoculated with RI had significantly higher leaf chlorophyll content as well as of the measured components. When relative values were used, the percentage amelioration of salt stress by RI colonization ranged from 19.1% to 27.1%, showing the importance of RI in the protection of photosynthetic pigments in leaves.

Table 2. Relative changes in photosynthetic parameters between M^{+NaCl} and NM^{+NaCl} treatment.

	P_n (%)	T_r (%)	g_s (%)	C_i (%)
NM^{+NaCl}	−80.3	−87.1	−84.4	−65.8
M^{+NaCl}	−78.3	−82.3	−80.7	−56.9
Amelioration	2.0*	4.8*	3.7*	8.9**
F value				
S (Salt)	2685.021***	3388.125***	2155.456***	313.321***
M (Mycorrhizal)	290.083***	228.571***	210.134***	30.434***
S × M	102.083***	68.143***	142.904***	0.041^{NS}

Levels of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

Chlorophyll fluorescence parameters

Under salt stress, F_v/F_m, ΦPSII, q_P and F_v/F_o decreased significantly ($P < 0.05$), regardless of whether *E. angustifolia* seedlings had been inoculated with AMF (Table 4). However, the relative decrease in M^{+NaCl} was significantly lower than that in NM^{+NaCl} treatment ($P < 0.05$), indicating that inoculation with RI increased the actual quantum yield of seedlings under salt stress, ensuring more energy entered the photosynthetic cycle. The NPQ of *E. angustifolia* seedling leaves treated with NM^{+NaCl} and M^{+NaCl} was significantly increased compared with corresponding values in NM and M treatments ($P < 0.05$), and the value in NM^{+NaCl} was significantly higher than in the M^{+NaCl} treatment ($P < 0.05$). The decreases in NPQ and F_v/F_o were not significant in M^{+NaCl} and NM^{+NaCl}, while the decreases in F_v/F_m, ΦPSII and q_P were significant in M^{+NaCl} and NM^{+NaCl}.

Malondialdehyde (MDA), proline and total flavonoids

Compared with NM, the M treatment significantly increased the content of proline and total flavonoids and significantly decreased the content of MDA in *E. angustifolia* seedlings without salt stress (Fig. 3). After 3 weeks of NaCl stress in the corresponding M and NM treatments, the total flavonoid content of *E. angustifolia* seedlings treated with M^{+NaCl} or NM^{+NaCl} decreased significantly, and the proline and MDA content increased significantly ($P < 0.05$). However, the content of total flavonoids and proline in the M^{+NaCl} treatment were significantly higher than in the NM^{+NaCl} treatment, while the MDA content in the M^{+NaCl} treatment was significantly lower than in the NM^{+NaCl} treatment ($P < 0.05$). After 3 weeks of salt stress, total flavonoids of seedlings treated with NM^{+NaCl} decreased by 42.53% compared with those treated with NM, while total flavonoids of M^{+NaCl} decreased by 40%. The proline and MDA content of seedlings treated with NM^{+NaCl} increased by 124.25% and 206.51%, respectively, while those of M^{+NaCl} increased by 112.27% and 191.01%, respectively. These results show that the content of total flavonoids and proline in leaves of *E. angustifolia* seedlings significantly increased, and the content of MDA significantly decreased after RI inoculation. The changes in total flavonoids, proline and MDA in mycorrhizal *E. angustifolia* seedlings were also relatively small after salt stress (Table 5).

Table 3. Effects of *R. irregularis* inoculation and salt stress on chlorophyll content.

	Chl *a* (mg·g^{-1} FW)	Chl *b* (mg·g^{-1} FW)	Car (mg·g^{-1} FW)	Chl (mg·g^{-1} FW)
NM	1.64 ± 0.04^{b}	0.79 ± 0.06^{b}	0.85 ± 0.09^{ab}	2.46 ± 0.05^{b}
M	2.51 ± 0.04^{a}	1.01 ± 0.06^{a}	1.04 ± 0.05^{a}	3.66 ± 0.06^{a}
NM^{+NaCl}	0.66 ± 0.08^{c}	0.24 ± 0.03^{d}	0.24 ± 0.01^{d}	0.98 ± 0.03^{c}
M^{+NaCl}	1.69 ± 0.12^{b}	0.50 ± 0.03^{c}	0.54 ± 0.01^{c}	2.39 ± 0.02^{b}
Relative values (%)				
NM^{+NaCl}	−59.8	−69.6	−71.8	−60.2
M^{+NaCl}	−32.7	−50.5	−48.1	−34.7
Amelioration	27.1**	19.1**	23.7**	25.5**
F value				
S (Salt)	340.417***	395.016***	271.122***	1066.253***
M (Mycorrhizal)	378.998***	81.000***	52.834***	960.453***
S × M	359.708***	238.008***	161.978***	1013.353***

Values represent mean ± SD, different lowercase letters indicate a significant difference in the same column ($P < 0.05$). Levels of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

Table 4. Effects of *R. irregularis* inoculation and salt stress on chlorophyll fluorescence parameters.

	F_v/F_m	ΦPS	q_P	NPQ	F_v/F_o
NM	0.701 ± 0.007^{ab}	0.440 ± 0.020^{b}	0.600 ± 0.020^{b}	2.020 ± 0.090^{c}	2.440 ± 0.090^{b}
M	0.733 ± 0.004^{a}	0.550 ± 0.020^{a}	0.780 ± 0.020^{a}	1.870 ± 0.090^{d}	3.290 ± 0.050^{a}
NM^{+NaCl}	0.632 ± 0.003^{c}	0.120 ± 0.010^{c}	0.180 ± 0.020^{d}	3.020 ± 0.070^{a}	1.940 ± 0.040^{c}
M^{+NaCl}	0.672 ± 0.005^{ab}	0.220 ± 0.010^{c}	0.280 ± 0.020^{c}	2.790 ± 0.080^{b}	2.630 ± 0.040^{b}
Relative changes (%)					
NM^{+NaCl}	−9.8	−72.7	−70	49.5	−20.5
M^{+NaCl}	−8.3	−60	−64.1	49.2	−20.1
Amelioration	1.5*	12.7**	5.9**	−0.3^{NS}	0.4^{NS}
F value					
S (Salt)	463.72***	1374.39***	714.15***	416.11***	194.08***
M (Mycorrhizal)	142.244***	143.458***	66.150***	16.299**	342.058***
S × M	302.982***	758.928***	390.150***	216.203***	268.067***

Values represent mean ± SD, different lowercase letters indicate a significant difference in the same column ($P < 0.05$). Levels of significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

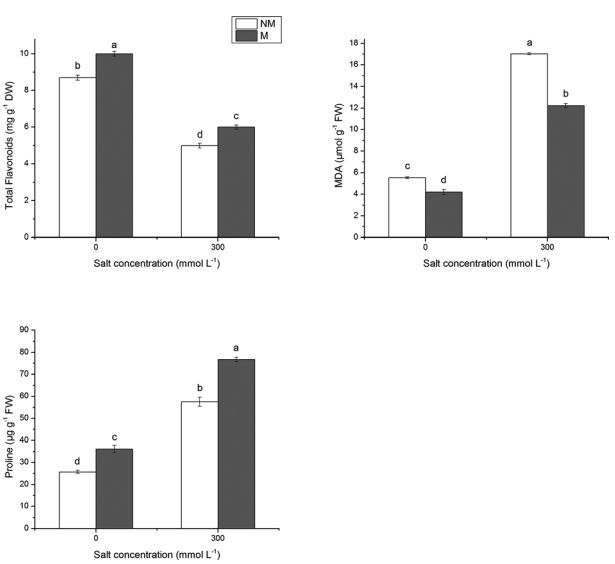

Fig. 3. Effects of *R. irregularis* on total flavonoid, MDA and proline content in leaves of *E. angustifolia* under salt stress.

Ultrastructural changes

Without salt stress (including NM and M treatments), leaf cells remained intact, and there was no separation of cell walls, nucleoli were visible as were chloroplast organelles (Fig. 4). However, in the high (300 mM) salt stress treatment, cell ultrastructure of leaves was seriously damaged, *i.e.* the membrane structure of cells was no longer intact, cell membrane was clearly separated, the plasma membrane was seriously damaged and the number of chloroplasts decreased. After mycorrhizal colonization (M^{+NaCl} treatment), the damage was ameliorated, as shown by the more intact cell membrane, no plasmolysis and more chloroplasts than in the NM^{+NaCl} treatment. The results show that inoculation with RI can alleviate damage caused by salt stress to the cell membrane integrity in *E. angustifolia*.

Without salt stress, there were many starch granules in both NM and M treatments, as well as some plastoglobuli in M-treated leaves (Fig. 5). Chloroplast thylakoid and grana stacking structures were also present in both NM and M treatments. In the leaves of salt-treated *E. angustifolia* (NM^{+NaCl}) the plasmalemma were severely disrupted and there were no starch granules in the chloroplasts; grana lamellae were destroyed,

Table 5. Effects of *R. irregularis* inoculation and salt stress on total flavonoids, MDA and proline.

	MDA (%)	Proline (%)	Total flavonoids (%)
NM^{+NaCl}	206.51	124.25	–42.53
M^{+NaCl}	191.01	112.27	–40.00
Amelioration	15.5**	11.98**	2.53*
F value			
S (Salt stress)	10612.981***	1844.752***	2623.451***
M (Mycorrhizal)	1050.779***	309.793***	234.071***
S × M	329.831***	26.638***	953.835^{NS}

Levels of significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant.

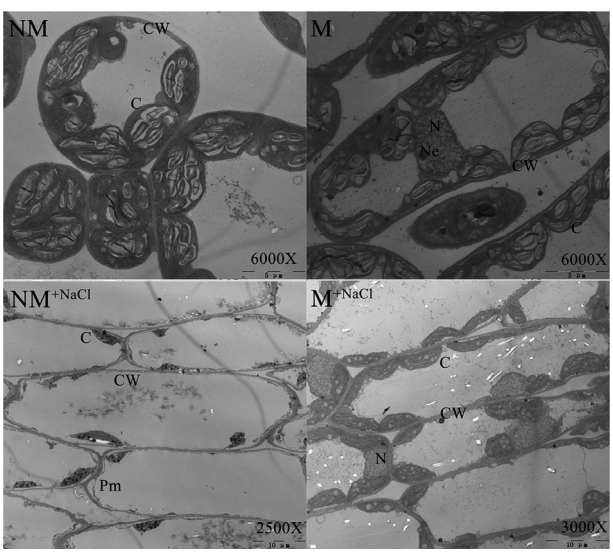

Fig. 4. Ultrastructure of leaf cells of *E. angustifolia* seedlings under different treatments. NM: without *R. irregularis* inoculation; M: *R. irregularis* inoculation; NM^{+NaCl}: without *R. irregularis* inoculation + 300 mM NaCl; M^{+NaCl}: *R. irregularis* inoculation + 300 mM NaCl; CW, cell wall; Pm, plasma membrane; C, Chloroplast; N, nucleus; Ne, nucleolus.

thylakoid membranes disintegrated, and plastid globules were black and abnormally enlarged. In the M^{+NaCl} treatment, cell membrane structure was intact, with non-plastid cell wall separation, a more intact chloroplast structure and obvious lamellar thylakoid structure compared with the NM^{+NaCl}. Compared with the M^{+NaCl} treatment, the chloroplast structure of *E. angustifolia* leaves in the M treatment was intact. After salt treatment, starch granules disappeared completely, and plastoglobules, thylakoids and grana were observed. The number of plastid 'balls' in leaves treated with M^{+NaCl} was significantly higher than in NM^{+NaCl}.

DISCUSSION

Inoculation with AMF improved photosynthetic performance and increased pigment accumulation

The chlorophyll content of woody plant leaves under salt stress is directly related to the photosynthetic process of the plants but is also one of the important physiological indices for salt

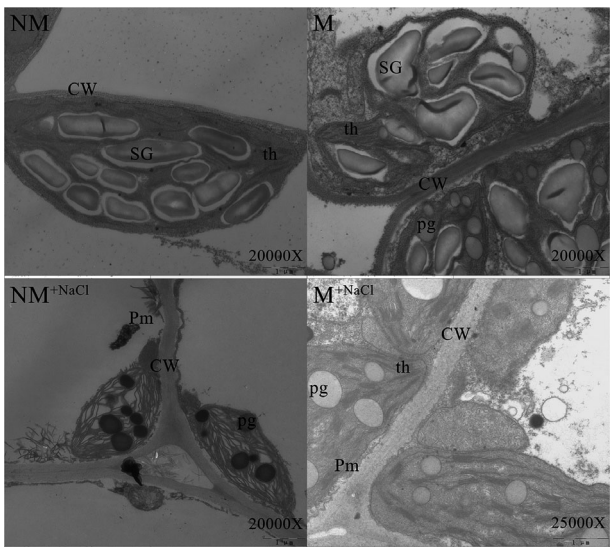

Fig. 5. Chloroplast ultrastructure changes under different treatments. NM: without *R. irregularis* inoculation; M: *R. irregularis* inoculation; NM^{+NaCl}: without *R. irregularis* inoculation + 300 mM NaCl; M^{+NaCl}: *R. irregularis* inoculation + 300 mM NaCl; CW, cell wall; Pm, plasma membrane; pg, plastoglobuli; th, thylakoid; SG, starch granules.

tolerance (Sun *et al.*, 2016). Photosynthetic pigments are located in the mesophyll cells, which makes these pigments more sensitive to salt stress than other intimately protected oxidases (Jacoby *et al.*, 2011). Salt stress inhibits the activity of chlorophyll synthase and increases the activity of the chlorophyll degradation enzyme, resulting in decreased chlorophyll content in plants under salt stress (Zhao *et al.*, 2019). AMF inoculation can maintain the K^+/Na^+ balance and improve plant photosynthetic capacity under salt stress (Liang *et al.*, 2019). Many studies have shown that under salt stress, AMF can improve chlorophyll synthase activity and increase the chlorophyll content of plants, such as Sesbania (*Sesbania sesban*) (Allah *et al.*, 2015), orange (*Citrus reshni* Hort. ex Tan.) (Navarro *et al.*, 2014), tomato (*Solanum lycopersicum* L.) (Xie *et al.*, 2019) and poplar (*Populus* × *canadensis* 'Neva') (Liu *et al.*, 2015). The results of this study also showed that under salt stress, the content of chlorophyll in both NM^{+NaCl} and M^{+NaCl} treatments significantly decreased, while AMF inoculation (M^{+NaCl} treatment) showed a significantly smaller decrease than the NM^{+NaCl} treatment. Statistical analysis showed that in the M treatment group (both M and M^{+NaCl}), the leaf chlorophyll content was two- to three-fold higher than in the corresponding NM treatment group (NM and NM^{+NaCl}) ($P < 0.05$). In particular, there were much larger increases in Chl *a* compared with Chl *b* and carotenoids. Their AMF-induced amelioration was 25%, while the Chl *b*-related percentage was <20%.

Photosynthetic CO_2 fixation is essential for rapid plant growth and is very sensitive to changes in the environments, including salt stress (Wang *et al.*, 2006a,b). Nutrients, such as N, micronutrients and the water supply are mainly obtained from the soil, which can limit photosynthesis substrate supply and stomatal regulation. It is known that salt stress can down-regulate leaf photosynthesis and slow plant growth and development (Duarte *et al.*, 2013; Zhang *et al.*, 2018), and direct

associations have been found in species such as peanut (Zhang *et al.,* 2020), *Robinia pseudoacacia* (Mao *et al.,* 2016) and poplar. Ruiz-Lozano *et al.* (2012) found that AMF–plant symbiosis increased the gas exchange capacity, and thus increased photosynthesis of seedlings. In this study, the amelioration provided by AMF led to a 2% increase in P_n, which was reflected in increases in intercellular CO_2 supply (8.9%) and reduced stomatal limitation (3.7%). Moreover, leaf transpiration and stomatal conductance decreased faster in the first week of salt stress, showing a rapid stomatal response to physiological drying caused by soil salt stress. In another study of *E. angustifolia* seedlings, there was a similar response to salt stresses (Sun *et al.,* 2016).

Higher photosynthetic pigment content is the basis for higher photosynthetic gas exchange, and our results confirmed this. However, much higher AMF-induced pigment amelioration (20–25%; $P < 0.05$) matched lower AMF-induced photosynthetic amelioration (2%; $P < 0.05$). Photosynthetic gas exchange complex and is adjusted in relation to substrate supply (CO_2, light and water), pigment amount and transport of photosynthate from chloroplasts to other organs, and plants under stress maintain water balance through stomatal regulation (Zu *et al.,* 2005). Plants adjust the stomatal aperture to ensure conservation of water for photosynthetic water use efficiency, showing that stomatal function is plastic and can facilitate plant survival in xeric as well as hydric habitats. This could favour plant survival under extreme salt stress (*e.g.* by improving the water balance) and could allow rapid response and increased photosynthesis when the stress is relieved.

Malondialdehyde (MDA), proline and total flavonoids

The degree of damage to plant tissues caused by salt stress can be measured as the permeability of cell membranes. Salt stress disrupts photosynthetic electron transport resulting in excess accumulation of toxic reactive oxygen species (ROS), and ROS at high concentrations damage membranes through lipid peroxidation (Li *et al.,* 2017). As an end product of lipid peroxidation, MDA content increased significantly in *E. angustifolia* under salt stress, leading to damage to the structure of plasma membranes and thus affecting normal physiological metabolism of cells (Yoon *et al.,* 2013; Diao *et al.,* 2014; Li *et al.,* 2017).

In this study, the content of MDA in the M and M^{+NaCl} treatments were significantly lower than in the NM and NM^{+NaCl} treatments ($P < 0.05$), which in accordance with Abdel-Fattah *et al.* (2016). This may be due to a substantial increase in antioxidant activity in mycorrhizal plants leading to less lipid peroxidation, where antioxidants scavenge ROS before they react with the membrane lipids and thus minimize lipid peroxidation (Hashem *et al.,* 2015). In plants, proline synthesis and accumulation regulates osmotic pressure in cells to improve water-holding capacity, thus helping the plants to resist salt and other stress conditions (Reddy *et al.,* 2015). The results showed that the contents of proline in the leaves of *E. angustifolia* treated with M^{+NaCl} and NM^{+NaCl} increased significantly under salt stress, but the content of proline in the M and M^{+NaCl} treatments was significantly higher than in the NM and NM^{+NaCl} treatments ($P < 0.05$). This suggests the beneficial role of AMF in enhancing the stress tolerance by contributing to maintenance of cellular water content (Shekoofeh *et al.,* 2012). Flavonoids, as important antioxidant substances, have various functions in the plant, including resistance to biotic and abiotic stresses (Aseel *et al.,* 2019). Flavonoid content was significantly enhanced after AMF colonization (Nana *et al.,* 2016; Aseel *et al.,* 2019). Under salt stress, the protein content related to flavonoid metabolism in plant leaves changed after AMF inoculation (Jia *et al.,* 2019). In the study, the content of total flavonoids in the M and M^{+NaCl} treatments was significantly higher than in the NM and NM^{+NaCl} treatments ($P < 0.05$). Some studies have shown that under certain salt stress conditions, the content of flavonoids increases significantly to cope with the stress (Jorge *et al.,* 2019). In this study, high salt stress led to a significant decrease in the content of flavonoids. However, AMF significantly increased the flavonoids content, giving the plants increased tolerance to high salt stress.

Function of PSII and chloroplast machinery in salt-stressed AMF plants

Chlorophyll fluorescence reflects the initial photochemical reactions in PSII and changes in the structure and state photosynthetic sites, revealing the adaptability of plants to different habitats and providing tools for selecting salt-tolerant plant varieties. Evelin & Kapoor (2014) found that salt stress interfered with the electron transport chain in chloroplasts of fenugreek, and overproduction of ROS caused oxidative damage to the cell membrane system (Evelin *et al.,* 2013). Under salt stress, F_v/F_m, ΦPSII, q_P and F_v/F_o significantly decreased in M^{+NaCl} and NM^{+NaCl} treatments, together with an increase in NPQ ($P < 0.05$). Compared with the NM treatment, AMF-inoculated seedlings significantly alleviated damage caused to the PSII system. This was because under salt stress, the share of light absorbed by *E. angustifolia* leaves for photochemical electron transport decreased, *i.e.* q_P decreased significantly, while the share of heat dissipated by leaves increased and NPQ increased markedly, while excess light energy was dissipated. F_v/F_m reflects the transformation efficiency of the PS II reaction centre, which is closely related to the ability to tolerate salt and alkaline conditions. F_o increased under high salt stress because it led to the separation of PSII and pigments, while F_m decreased under high salt stress because the stress caused photoinhibition in leaves. The results show that salt stress resulted in significant decreases in both F_v/F_m and *Fv/Fo*, indicating that the activity of the PS II photoreaction centres decreased under salt stress, which hindered the primary photosynthesis process and reduced the conversion efficiency of light energy, resulting in severe photoinhibition and a strong decrease in electron transfer activity, ultimately leading to a reduction in plant carbon assimilation efficiency. The M treatment alleviated the damage caused to the photosynthetic systems of *E. angustifolia* leaves under salt stress to some extent and enhanced activities in F_v/F_m and F_v/F_o, unlike the NM treatment. However, under 300 mM NaCl treatment, the effect of AMF and symbiosis on *E. angustifolia* leaves was weakened, and the fluorescence parameters were reduced due to the effect of AMF and were significantly lower than that in the NM^{+NaCl} treatment. This indicates that mycorrhizal *E. angustifolia* seedlings could protect the photosynthetic system by changing chlorophyll fluorescence, thereby improving salt tolerance of *E. angustifolia*.

The integrity of the plant cell membrane system is a precondition for ensuring normal metabolism, including

photosynthetic reactions. It has been found that lipid peroxidation in halophytes damages the cell membranes when the salt concentration exceeds 300 mM for a week or longer (Ozgur *et al.,* 2013; Nath *et al.,* 2016). Damaged cell membranes lose their biological functions and affect the normal metabolism of plants (Jabeen *et al.,* 2014). Oxidative damage also destroys the membrane system of chloroplasts and inhibits photosynthesis (Shu *et al.,* 2013). Chloroplasts are the cell organelles most sensitive to salt stress. Under high salt stress, the thylakoids and grana begin to disintegrate and disappear due to a change in the cation concentration in chloroplasts, and thylakoid swelling and membrane damage are related to this (Kaya *et al.,* 2009). Compared with the M^{+NaCl} treatment in this study, the leaf cells in the NM^{+NaCl} treatment had more obvious cell wall separation, plasma membrane damage, chloroplast disappearance, and complete thylakoid degradation, indicating that the chloroplast structure had been destroyed. In the M^{+NaCl} treatment, the chloroplast structure was still intact, and many normal plastoglobuli could be seen. The AMF-mediated reduced damage may be due to higher osmolyte (glycine betaine, sugars) and polyamines concentrations, and more and bigger plastoglobuli (higher α-tocopherol concentration) in M^{+NaCl} plants as compared to NM^{+NaCl} plants (Evelin *et al.,* 2013). Plastoglobuli, as the synthesis sites of tocopherol, play a protective role in thylakoid membranes and proteins (Austin *et al.,* 2006). Tocopherol possibly prevents the photooxidation of membrane lipids and protects PSII (Brehelin *et al.,* 2007; Engel *et al.,* 2016). In addition, the result described here under severe salt stress might show that AMF limit plant absorption of sodium ions, thus avoiding plant toxicity (Goussi *et al.* 2018). AMF can also increase antioxidant enzyme activity to enhance the ability of host plants to scavenge free radicals (Garg & Singla, 2015). Under salt stress, the proteins regulating the expression of genes related to antioxidant enzymes in plant leaves changed after AMF inoculation (Jia *et al.,* 2019). While lower Na^+ and Cl^- ions assured less ionic toxicity, higher osmolyte and tocopherol content ensured osmotic adjustment and better capacity to scavenge free radicals generated by salt stress (Evelin *et al.,* 2013). Therefore, AMF-induced functioning of PSII and photosynthetic gas exchange under salt stress are possibly the result of the undamaged photosynthetic machinery, with a complete cell membrane system protected from oxidative damage by the large number of functional plastoglobuli (Smith *et al.,* 2000), unlike these in the NM^{+NaCl} treatment.

In summary, salt stress can lead to adjustment of the stomatal aperture, down-regulate photosynthesis, degrade photosynthetic pigments, produce excessive energy damage to photosynthetic systems, damage the structure of plant cells and chloroplasts, and finally inhibit the functioning of the whole photosynthetic system, from gas exchange to chlorophyll fluorescence. AMF inoculation can promote efficient photosynthetic electron transport, slow damage caused to the reaction centres of PSII, significantly improve net photosynthesis rate of leaves of *E. angustifolia* seedlings and protect membrane structure and integrity of chloroplasts. Thus, the utilization of salt-tolerant plants and AMF symbiosis has potential for the improvement of saline-alkali land.

CONCLUSION

Addition of AMF favours plant growth under various stresses, and this paper confirmed that AMF inoculation improved photosynthetic performance through higher pigment accumulation in leaves under heavy salt stress, and also increased the content of proline and total flavonoids in leaves and decreased the MDA content. Moreover, poor functioning of PSII (as indicated by chlorophyll fluorescence) and the chloroplast machinery (shown in microscopy images) is possibly attributed to salt stress. Our findings indicate that saline-alkali soil rehabilitation can be achieved using AMF inoculation which will favour this rehabilitation process.

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AUTHOR CONTRIBUTIONS

Experiments were designed and investigated by BL and FS. The manuscript was written by BL, and revised by WW, WC, XF, YL, AK and FS.

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