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Effect of *Glomus mosseae* on chlorophyll content, chlorophyll fluorescence parameters, and chloroplast ultrastructure of beach plum (*Prunus maritima*) under NaCl stress

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Abstract

The present study was undertaken to investigate the effect of *Glomus mosseae* on chlorophyll (Chl) content, Chl fluorescence parameters and chloroplast ultrastructure of beach plum seedlings under 2% NaCl stress. The results showed that compared to control, both Chl *a* and Chl *b* contents of NaCl + *G. mosseae* treatment were significantly lower during the salt stress, while Chl *a/b* ratio increased significantly. The increase of minimal fluorescence of dark-adapted state (F_0), and the decrease of maximal fluorescence of dark-adapted state (F_m) and variable fluorescence (F_v) values were inhibited. The maximum quantum yield of PSII photochemistry (F_v/F_m), the maximum energy transformation potential of PSII photochemistry (F_v/F_0) and the effective quantum yield of PSII photochemistry (Φ_{PSII}) increased significantly, especially the latter two variables. The values of the photochemical quenching coefficient (q_P) and the nonphotochemical quenching (NPQ) were similar between *G. mosseae* inoculation and noninoculation. It could be concluded that *G. mosseae* inoculation could protect the photosystem II (PSII) of beach plum, enhance the efficiency of primary light energy conversion and improve the primitive response of photosynthesis under salinity stress. Meanwhile, *G. mosseae* inoculation was beneficial to maintain the integrity of thylakoid membrane and to protect the structure and function of chloroplast, which suggested that *G. mosseae* can alleviate the damage of NaCl stress to chloroplast.

Additional key words: beach plum; chlorophyll content; chlorophyll fluorescence; chloroplast ultrastructure; *Glomus mosseae*; NaCl stress.

Introduction

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, 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, 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 is now a serious problem that limits widespread cultivation in salinized soil in China.

Growth inhibition of plants is the most sensitive physiological process (Kanazawa *et al*. 2000, Zhang *et al*. 2009) and photosynthesis is an important foundation for

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Abbreviations: AM – arbuscular mycorrhizal; Chl – chlorophyll; F_0 – minimal fluorescence of dark-adapted state; F_m – maximal fluorescence of dark-adapted state; F_v – variable fluorescence; F_t – steady-state fluorescence parameters; $F_{m'}$ – maximum fluorescence yield; q_P – photochemical quenching coefficient; NPQ – nonphotochemical quenching; F_v/F_m – maximum quantum yield of PSII photochemistry; F_v/F_0 – maximum energy transformation potential of PSII photochemistry; PAR – photosynthetically active radiation; PSII – photosystem II; Φ_{PSII} – effective quantum yield of PSII photochemistry.
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growth. The Chl fluorescence signals *in vivo* contain rich photosynthesis information. There have been some reports about the use of Chl fluorescence kinetics characterization in the identification of plant salt tolerance, and the above technology can be used for screening salt-tolerant varieties or genotypes of wheat (Baker and Rosenqvist 2004, Rana *et al*. 2010). However, it has not yet been fully substantiated that whether salt stress affects the activity of PSII and the change in Chl fluorescence parameters of plants.

The introduction of arbuscular mycorrhizal (AM) fungi to sites with saline soil may improve plant tolerance and growth (Al-Karaki 2000b). As the organ of photosynthesis, chloroplast is the place where numerous biochemical reactions occur. Although there are a lot of studies in the influence of salt stress on plant cells and photosynthetic organelle ultrastructure (Aschan *et al*. 2005, Redondo-Gómez *et al*. 2010), the impact of inoculated AM fungi on subcellular ultrastructure has not been reported. It has been discovered that roots of beach plum could form symbiotic associations with AM fungi and *G. mosseae* inoculation could improve seedlings growth under salt stress (Zai *et al*. 2007, 2009). The improvement of growth under salt stress after *G. mosseae* inoculation could be related to the photosynthesis facilitation, however, there are few reports about this field. In this experiment, the content of Chl, the parameters of Chl fluorescence and ultrastructure of chloroplast and their relationship in beach plum under salt stress after *G. mosseae* inoculation are studied.

Materials and methods

Plant and soil treatment: The soil used in this study was collected from the top layer (0–20 cm) of a soil in Nanjing City, Jiangsu Province, China. With 5% (w/w, the same below) peat, 1% sand and 0.5% superphosphate, the collected soil was improved to be used as cultivation substrate after shattering, blending and screening. The basic components of cultivation substrate were as follow: 1.35% organic matter, 0.048‰ available N (w/w, the same below), P 0.025‰, K 0.148‰, pH 7.2.

The new-born healthy semilignified branches of beach plum were selected as the materials, which collected from the agricultural sightseeing garden in Lishui County of Jiangsu in March 2009. Then these materials were propagated in the seedling breeding sand bed at the Mufu Campus of Nanjing Jinling Institute of Technology. And three months later, 5000 healthy beach plums with similar shape were selected and transplanted in the pots filled with above cultivation substrate.

AM inoculum: The cultivation substrate was sterilized in 160°C for 2 h, and then loaded into the 15-cm (diameter) sterile pot with a hole in the floor. A layer of filter paper was put at the bottom of pot and 4 kg cultivation substrate was loaded into the pot.

In the first group (2,500 pots), 25 g inoculated agent (BGCJX01, 1,285 spores/20 ml agent) was applied into each pot. The mycorrhizal propagules were evenly distributed to form a thin layer, which was 10 cm below the surface. In the second group (2,500 pots), the nonmycorrhiza received an equivalent amount of inoculum sterilised twice at 121°C for 30 min, together with a 0.25 μm-filtrate of unsterilised medium to provide similar microflora, but without viable AM fungi.

The mycorrhizal fungi used was *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe, 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 separated from Jiangxi sweet osmanthus tree rhizosphere, and then propagated on maize plants growing in sandy soil for 10 weeks. The original inoculum (BGCJX01) was kindly provided by Prof. Y.S. Wang (Institute of Plant Nutrition and Fertilisers, Chinese Academy of Agriculture).

Experimental design: This experiment was conducted inside a greenhouse under a temperature of 22–30°C, 11–14 h day light, and 70–75% relative humidity, between March and October 2009, at Jinling Institute of Technology, Nanjing City, Jiangsu Province, China. Treatments were factorial combinations of two factors: (*1*) nonmycorrhizal control and *G. mosseae* as a mycorrhizal inoculum`and (*2*) two NaCl levels of 0% and 2%. The experiment is designed into three treatments: (*1*) salt (2%) treatment, and inoculated fungi (recorded as NaCl + *G. mosseae*), (*2*) salt (2%) treatment and noninoculated fungi (recorded as NaCl), (*3*) nonsalt (0%) treatment, non-inoculated fungi (recorded as control).

After 1-month growth, 300 beach plums pots with beach plums of similar size (height from 59 cm to 61 cm, number of leaves from 64 to 66) were selected as test materials of an experiment (100 inoculated pots and 100 noninoculated pots for salt-stress treatment, another 100 noninoculated pots with same tap water for control). The experiment was repeated ten times. The salinity of the soil 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 7^{th} d to 21^{th} d after salt treatment, pouring occasional leakage back to the trays after 1 h. The pots were watered every 5–7 d in order to maintain the soil humidity within 75–80%. After the salt-stress treatment, each pot was watered with 500 mL of Hoagland nutrient solution every 3 d until the 60^{th} d.

Measurement of Chl content and fluorescence parameters: After 15 d and 45 d, the 3^{rd} to 5^{th} leaves from the end of beach plum were selected to test the Chl content.

The extraction from 50 mg of fresh material was incubated in 5 mL of 80% acetone in the dark at 4°C. After incubation, the extract was read at 645 and 663 nm in an *Uvikon 940* spectrophotometer with spectral slit-width 1.8 nm. The following parameters were caculated: Chl *a* = $12.7A_{663} - 2.69A_{645}$, Chl *b* = $22.9A_{645} - 4.68A_{663}$.

Further, the same leaves were used to measure the parameters of Chl fluorescence, according to the methodology of Chen (2008). The *MINI-PAM (MINI-PAM, Waltz*, Germany) was used to measure fluorescence induction curve and the rapid light response curve of beach plum leaves under salt stress, and in each case 4 duplicates of measurement were used. Fluorescence induction curve methods: Firstly, plants were kept in dark for 30 min before measuring. Secondly, the measuring light was opened (wavelength 650 nm, modulation frequency of 0.6 kHz, photosynthetically active radiation (PAR) less than 0.15 μmol m^{-2} s^{-1}, and the modulation frequency automatically switched to 20 kHz when the saturation pulse light or actinic light opened). Finally, F_0 was measured. F_m was measured by the saturation pulse light (continued 0.8 s, PAR greater than 8,000 μmol m^{-2} s^{-1}). After 40 s, the actinic light should be kept (PAR of about 1,000 μmol m^{-2} s^{-1}) on and the saturation pulses light should be opened every 30 s. The steady-state fluorescence parameters (F_t) and PAR of light-adapted sample could be measured when the sample achieved stable status, and the maximum fluorescence yield ($F_{m'}$) could be measured under saturation pulse light. The calculated parameters were listed as follow: $F_v/F_m = (F_m - F_0)/F_m$, $F_v/F_0 = (F_m - F_0)/F_0$, $\Phi_{PSII} = (F_m' - F_t)/F_m'$, $q_P = (F_m' - F_t)/(F_m' - F_0)$, $NPQ = (F_m - F_m')/F_m'$.

Chloroplast ultrastructure's electron microscopical sample preparation and observation: After 45 d, the third leaf from the end was taken and repaired [≤ (0.1 cm × 0.5 cm)]. Then the leaf was immediately put into 4% glutaraldehyde for 24 h at above 4°C, with 2% glutaraldehyde 2 h to be fixed, acetone to be dehydrated gradually, and Epon 812 to be embeded. The slicing was made by *LKV-V* ultrathin slicer, stained by uranium-lead double staining and observed by the electron microscopec (Wang and Li 2007). Meanwhile, the third healthy leaf from the end of beach plum was taken as control under nonsalt stress.

Statistical analysis: Data of physiological variables were analysed by *ANOVA* using *PC SAS* version *8.2* (*SAS Institute*, Cary, NC, USA). For multiple comparisons of means, *Duncan*'s New Multiple Range Test was employed and the significant levels were chosen to be 0.05.

Results

Effect of *G. mosseae* on Chl content and Chl *a*/*b* ratio of beach plum under NaCl stress: After 15 d of NaCl and NaCl + *G. mosseae* treatments, both Chl *a* and Chl *b* content had no significant differences with control (Table 1). On 45th d, both Chl *a* and Chl *b* content of NaCl + *G. mosseae* treatment were significantly lower than those of control, but substantially higher than NaCl treatment significantly (Table 1). More specifically, Chl *a* content of NaCl + *G. mosseae* treatment was 9.1% lower than that of control and 25.0% higher than that under NaCl treatment on 45th d (Table 1). Chl *a/b* ratio of NaCl + *G. mosseae* treatment was significantly higher than that of both control and NaCl treatments on 15th d. On 45th d, Chl *a/b* ratio varied among the three treatments: the ratio of NaCl treatment was the largest, followed by that of NaCl + *G. mosseae*, with the Chl *a/b* ratio of control being the smallest value (Table 1).

Effect of *G. mosseae* on F_0, F_m, F_v, F_v/F_m, F_v/F_0, Φ_{PSII}, q_P and NPQ of beach plum under NaCl stress: Compared to control, F_0 of NaCl and NaCl + *G. mosseae* treatments increased both 15th d and 45th d (Table 2). F_0 of NaCl + *G. mosseae* treatment was similar to that of control, and significantly lower than NaCl treatment on 45th d. There were no significant differences in F_m among the three treatments on 15th d. F_m of NaCl + *G. mosseae* treatment was similar to that of control, but significantly higher than that of NaCl treatment on 45th d. F_v of NaCl treatment changed greatly during the salt stress, which increased rapidly in the early stage and decreased quickly during the late stage. However, F_v of NaCl + *G. mosseae* treatment was stable, which was not significantly different compared to control on both 15th d and 45th d (Table 2).

As shown in Table 2, F_v/F_m of NaCl treatment was significantly lower than that of control on both 15th d and 45th d, while there were no significant differences between NaCl + *G. mosseae* treatment and control. F_v/F_0 and F_v/F_m of NaCl treatment changed greatly on both 15th d and 45th d, which were markedly lower than that of control. However, F_v/F_0 under NaCl + *G. mosseae* treatment was higher than that under NaCl treatment, but was significantly lower than that of control on 45th d. Φ_{PSII} changes were similar to F_v/F_0. The q_P and NPQ of *G. mosseae* inoculation and noninoculation had the similar trends on both 15th d and 45th d. (Table 2).

Effect of *G. mosseae* on the chloroplast ultrastructure of beach plum under NaCl stress: Chloroplasts were obvious organelles with approximate shape of oval, clear internal structure of grana lamella and stroma lamella under control (Fig. 1*A,B*). Under NaCl stress, *G. mosseae* inoculation played a key role in chloroplast mitigation effect (Fig. 1*C,D,E,F*). As shown in Fig. 1*C,D*, the chloroplast was swelling, disordered and disintegrable, with loose structure, ruptured thylakoid membrane, disappeared stroma lamella, and increased number of

Table 1. Effect of *G. mosseae* on the content of chlorophyll (Chl) and the ratio of Chl *a*/*b* in leaf of beach plum (mean ± SE for 10 replicates of samples) under 2% NaCl stress. *Lowercase letters* indicate the groups differentiated by *Duncan* tests at p=0.05.

Item	Treatment group	Treatment time [d]	
		15	45
Chl *a* [mg g^{-1}(FM)]	Control	0.21 ± 0.01^{a}	0.22 ± 0.01^{a}
	NaCl	0.21 ± 0.03^{a}	0.16 ± 0.02^{c}
	NaCl + *G. mosseae*	0.22 ± 0.03^{a}	0.20 ± 0.02^{b}
Chl *b* [mg g^{-1}(FM)]	Control	0.30 ± 0.02^{a}	0.35 ± 0.03^{a}
	NaCl	0.31 ± 0.04^{a}	0.22 ± 0.02^{c}
	NaCl + *G. mosseae*	0.31 ± 0.03^{a}	0.29 ± 0.01^{b}
Chl *a/b*	Control	0.70 ± 0.06^{b}	0.63 ± 0.05^{c}
	NaCl	0.68 ± 0.08^{b}	0.72 ± 0.04^{a}
	NaCl + *G. mosseae*	0.71 ± 0.04^{a}	0.69 ± 0.04^{b}

chloroplast internal lipid ball on 45th d under 2% NaCl stress. (Fig. 1*A,B,C,D*). NaCl + *G. mosseae* treatment slightly increased the diameter of chloroplast, inflated the shape and increased the number of lipid balls, but the overall lamellar structure of chloroplast was much better, with clearly views of grana lamella and intact thylakoid membrane (Fig. 1*E,F*). The changing trends of mitochondria and nucleus structure were similar to chloroplast (Fig. 1*A–F*).

Discussion

Mycorrhizal symbiosis is a key component in helping plants survive under adverse environmental conditions (Sawers *et al.* 2008). Our results showed that beach plum inoculated with *G. mosseae* had higher Chl content and Chl *a/b* ratio, F_m, F_v and F_v/F_m, both F_v/F_0 and Φ_{PSII} increased significantly than nonmycorrhizal plants under salt stress. Meanwhile, *G. mosseae* inoculation inhibited the increase in F_0 to a certain degree, and alleviate the damage of salt stress to chloroplast. This is in agreement with many greenhouse studies on tomato (Al-Karaki 2000b), barley (Mohammad *et al.* 2003), and maize (Sheng *et al.* 2008).

The Chl concentration in leaves is an important physiological index representing the rate of photosynthesis in plants. Mycorrhizal fungi enhanced both Chl content and Chl *a/b* ratio in beach plum leaves, a result in congruence with other studies (Sannazzaro *et al.* 2006, Colla *et al.* 2008, Sheng *et al.* 2008). Mycorrhizal inoculation enhances phosphorus and magnesium uptake and reduces sodium concentrations in the plant; this in turn helps in increasing both Chl content and Chl *a/b* ratio, and improves the overall performance of mycorrhizal plants (Giri and Mukerji 2004).

The Chl fluorescence analysis has become one of the most powerful and widely used techniques in studying plant photosynthesis. In the brief history of Chl fluorescence analysis, a large number of different coefficients have been calculated to quantify photochemical and nonphotochemical quenching. F_v/F_m, F_v/F_0, q_P, and Φ_{PSII} are a measure of the capacity of the primary photochemistry of PSII, which themselves are particularly sensitive to a variety of environmental stress-inducing factors (Huang and Zhang 2004, Baker and Rosenqvist 2004, Chen *et al.* 2008, Henriques 2009). The results of this study pointed out that F_v/F_0 and Φ_{PSII} in the leaves of mycorrhizal plants were much more higher than that in nonmycorrhizal plants. This, together with higher F_v/F_m in the leaves of mycorrhizal plants, imply that the efficiency of PSII photochemistry of mycorrhizal plants is higher than that of nonmycorrhizal plants. q_P reflects the share of light energy absorbed by PSII antenna pigments which are used for photochemical electron transfer. NPQ reflects the part of light energy absorbed by PSII antenna pigments which can not be used for photosynthetic electron transport and dissipated in the form of heat (Chen *et al*. 2006). In this experiment, the q_P and NPQ of AM fungal inoculation and noninoculation had the similar trends, which was in agreement with the results of Zhang (2008). It also indicated that in terms of beach plum, the q_P and NPQ had little to do with salt tolerance.

In addition, our data pointed out the rising trend in F_0 in the leaves of both mycorrhizal and nonmycorrhizal plants during salt stress, but F_0 was lower in the leaves of mycorrhizal plants than that in nonmycorrhizal plants at 2% NaCl levels. An increase in F_0 due to stressful conditions indicates the destruction or malfunction of PSII reaction center, or disruption of electron transport for excitation of reaction centers (Araus *et al.* 1998, Hu *et al*. 2008, Qin *et al*. 2011). Hence, our results suggested that salt stress could destroy PSII reaction center or disrupt electron transport in photosynthetic apparatus in both mycorrhizal and nonmycorrhizal plants, whereas the toxic influence of salinity on PSII reaction center could be mitigated by AM symbiosis.

Many stressful conditions, such as aridity, salinity,

Table 2. Effect of *Glomus mosseae* on the parameters of chlorophyll fluorescence in darkness of beach plum (mean ± SE for 10 replicates of samples) under 2% NaCl stress. *Lowercase letters* indicate the groups differentiated by *Duncan* tests at p=0.05. F_0 – minimal fluorescence of dark-adapted state; F_m – maximal fluorescence of dark-adapted state; F_v – variable fluorescence; q_P – photochemical quenching coefficient; NPQ – nonphotochemical quenching; F_v/F_m – maximum quantum yield of PSII photochemistry; F_v/F_0 – maximum energy transformation potential of PSII photochemistry; Φ_{PSII} – effective quantum yield of PSII photochemistry.

Item	Treatment group	Treatment time [d] 15	45
F_0	Control	710 ± 29^{b}	700 ± 35^{b}
	NaCl	854 ± 22^{a}	895 ± 42^{a}
	NaCl + *G. mosseae*	717 ± 28^{b}	738 ± 29^{b}
F_m	Control	2820 ± 147^{b}	2770 ± 138^{a}
	NaCl	3294 ± 183^{a}	2530 ± 142^{b}
	NaCl + *G. mosseae*	2720 ± 165^{b}	2642 ± 121^{ab}
F_v	Control	2220 ± 174^{ab}	2152 ± 153^{a}
	NaCl	2413 ± 163^{a}	1792 ± 149^{b}
	NaCl + *G.mosseae*	2274 ± 170^{ab}	2112 ± 162^{a}
F_v/F_m	Control	0.79 ± 0.01^{ab}	0.78 ± 0.02^{a}
	NaCl	0.73 ± 0.03^{b}	0.71 ± 0.03^{b}
	NaCl + *G. mosseae*	0.84 ± 0.02^{a}	0.80 ± 0.01^{a}
F_v/F_0	Control	3.13 ± 0.13^{a}	3.07 ± 0.19^{a}
	NaCl	2.83 ± 0.11^{b}	2.00 ± 0.18^{c}
	NaCl + *G. mosseae*	3.17 ± 0.20^{a}	2.86 ± 0.16^{ab}
Φ_{PSII}	Control	0.23 ± 0.03^{a}	0.19 ± 0.02^{a}
	NaCl	0.16 ± 0.02^{b}	0.09 ± 0.01^{c}
	NaCl + *G. mosseae*	0.19 ± 0.02^{ab}	0.14 ± 0.03^{b}
q_P	Control	0.40 ± 0.03^{a}	0.19 ± 0.02^{a}
	NaCl	0.41 ± 0.05^{a}	0.16 ± 0.01^{ab}
	NaCl + *G. mosseae*	0.42 ± 0.02^{a}	0.18 ± 0.02^{a}
NPQ	Control	1.96 ± 0.04^{a}	0.87 ± 0.04^{a}
	NaCl	2.02 ± 0.06^{a}	0.82 ± 0.05^{a}
	NaCl + *G. mosseae*	2.11 ± 0.03^{a}	0.85 ± 0.02^{a}

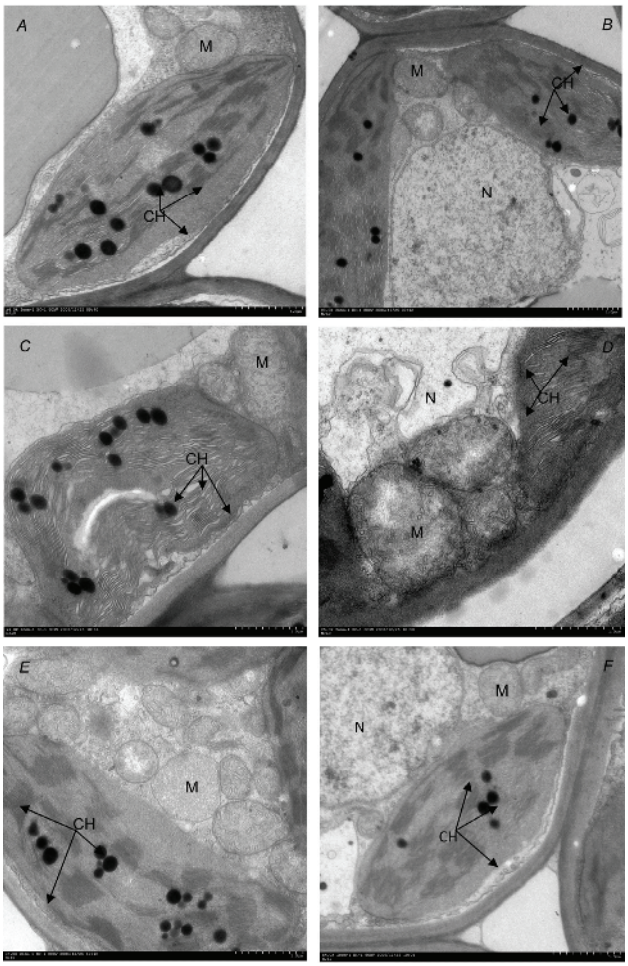

Fig. 1. Effects of *G. mosseae* on the ultrastructure of chloroplast (*arrow*) in chlorenchyma cells of beach plum under 2% NaCl stress for 45 d, in which *A* (4,000 ×): control (+ 0% NaCl – *G. mosseae*); *B* (1,000 ×) control: (+ 0% NaCl – *G.mosseae*); *C* (4,000 ×): NaCl (+ 2% NaCl – *G. mosseae*); *D* (5,000 ×): NaCl (+ 2% NaCl – *G. mosseae*) ; *E* (4,000 ×): NaCl (+ 2% NaCl + *G. mosseae*); *F* (4,000 ×): NaCl (+ 2% NaCl + *G. mosseae*); CH: chloroplast; M: mitochondrion; N: cell nucleus.

and high or low temperature, could disrupt components of plant's photosynthetic apparatus, such as membrane integrity, and further decrease photosynthetic capacity (Demmig-Adams and Adams 1992, Aschan *et al*. 2005). Similar changes took place in chloroplast ultrastructure of beach plum under salt stress. Under 2% NaCl stress, the overall lamellar structure of chloroplast was disrupted obviously. *G. mosseae* inoculation could lighten the damage to grana ultrastructure, improve the integrity of chloroplasts membrane structure. The relative stability of photosynthetic membrane structure was helpful to the chlorophyll synthesis and the normal metabolism of PSII, energy under salt stress. The results of Chl contents and fluorescence parameters also proved it. Nonetheless, the ensuring the conversion efficiency of primary light protection mechanism of *G. mosseae* inoculation for chloroplast ultrastructure still needs further research.

In conclusion, *G. mosseae* can protect beach plum from salt stress. However, it is not very clear how the AM symbiosis affects Chl, chloroplast ultrastructure, and Chl fluorescence in plant leaves. Thus, it is necessary to conduct further studies on the mechanism by which the AM symbiosis influences Chl concentration, chloroplast ultrastructure, and Chl fluorescence of plant leaves.

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