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ARBUSCULAR MYCORRHIZA PROTECTS THE ULTRASTRUCTURE OF MESOPHYLL CELLS AND PHOTOCHEMICAL ACTIVITY OF *LYCIUM BARBARUM* UNDER SALT STRESS

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Abstract

Salt stress is restricting crop productivity worldwide, especially in arid and semiarid areas. The plants in salinized areas have various strategies to adapt to salt stress, including forming mutual symbiosis with arbuscular mycorrhizal fungi (AMF). Due to the ubiquity of AMF in terrestrial ecosystem and their performance in aiding host plant to face to the environmental challenge, they can be a potential facilitator for reclaiming salinized soils. Plants inhabiting in salinized soils suffer from structural damage due to osmotic and oxidative pressure induced by salt stress, however, the role of AMF played in the ultrastructural changes of host plants is less clear. *Lycium barbarum* (Goji) is a tree species with medicinal value which usually suffers from salt stress. Here we investigated the impacts of inoculating *Diversispora versiformis* on the ultrastructure and chlorophyll fluorescence of mesophyll cells of Goji under salt stress. The extent of plasmolysis in mesophyll cells of Goji inoculated with *D. versiformis* was lower compared with control under 200 mmol/L NaCl. The chloroplasts of non-mycorrhizal Goji were swollen, with distorted thylakoids and large gap between chloroplast membrane and plasma membrane under salt stress. However, mycorrhizal Goji had integrated chloroplast and well organized grana, the gap between chloroplast membrane and plasma membrane was smaller than control under salt stress. Concurrently, the chlorophyll fluorescence performance of mycorrhizal Goji suffered less than non-mycorrhizal Goji under 200 mmol/L NaCl. Collectively, our results demonstrate that AMF could confer higher salt tolerance to Goji through protecting the ultrastructure and photochemical activity.

Key words: *Lycium barbarum*, *Diversispora versiformis*, Chloroplast, Plasmolysis, Salt tolerance.

Introduction

Soil salinization is a critical challenge for many regions, constraining the agricultural productivity, especially for the developing countries located in arid and semi-arid areas (Hajri *et al*., 2018). To date, the land area affected by salinization has reached 3.6×10^7 ha in China, whose proportion is 4.88% of the country's total available land (Li *et al.*, 2014). With the intensive evaporation and less rainfall caused by climate changes, the area of salinized soils is increasing rapidly.

More than 90% of the terrestrial plants establish mutualistic symbiosis with arbuscular mycorrhizal fungi (AMF) (Smith & Read, 2008). AMF are able to improve the water status (Wu *et al.*, 2015), regulate hormone balance (Liu *et al.*, 2016), maintain the ionic balance (Wu *et al.*, 2010), augment the photosynthesis rate (Sheng *et al.*, 2008), and stimulate the anti-oxidative system (Wu *et al.*, 2016) of host plants to confer it higher salt tolerance. However, the mechanisms of AMF facilitating plant salt adaptations have not been fully understood. The osmotic stress and oxidative injuries induced by soil salinization may potentially cause the structural damage in mesophyll cells of plants. But the evaluation of AMF influencing the ultrastructure of mesophyll cells of plants under salt stress is insufficient. More evidences, especially for economically important crops, are necessary to reveal the structural mechanisms of AMF enhancing salt tolerance of hosts. The structural disorder of mesophyll cells of plants due to decreased activity of electron transport chain under salt stress, likely induces the inhibition of photochemical activity (Oukarroum *et al.*, 2015). Chlorophyll fluorescence is commonly used to assess the efficiency of photosystem II owing to the advantage of non-destructive detection.

Lycium barbarum (commonly known as Goji) is a tree species of Solanaceae family, whose fruits and leaves are rich in medicinal ingredients, e.g. flavonoids, polysaccharides and amino acids (Liu *et al.*, 2017a). Goji is thus of great medicinal and economic significance due to its health benefits of nurturing kidney and eyes, anticancer, neuroprotective and maintaining vessels health (Chung *et al.*, 2010). Goji is prevalently cultivated in Northwest China where soil salinization is widespread (Liu *et al.*, 2017b). The growth of Goji is restricted by high level salt stress (Wei *et al.*, 2006). The growth, photosynthesis and medicinal composition of Goji were negatively affected by high salt level (Liu *et al.*, 2016; Liu *et al.*, 2017a). Hence, it is urgent to seek for solutions to improve the salt tolerance of Goji, aiming to improve the fruit quality and extend its growing area in marginal land.

Naturally, Goji harbor a diverse AMF community in salinized soil (Liu *et al.*, 2017b). We previously showed that AMF could ameliorate the deleterious effect of salt stress on Goji via hormonal regulation, osmotic equilibrium and secondary metabolism (Liu *et al.*, 2016;

Liu *et al.*, 2017a). However, it is unknown whether AMF exert protective effect on the ultrastructure of Goji mesophyll cells. Hence, our research objective was to investigate the impact of AMF on the ultrastructural changes and chlorophyll performance of Goji suffering from salt stress.

Materials and Methods

AMF inoculant, plants and experimental design: The inoculum *Diversispora versiformis* was purchased from Bank of Glomales in China (BGC, Beijing, China) and was propagated with an open pot culture using maize.

The seeds of Goji cv. Ningqi No. 1 were surface sterilized with 10% hydrogen peroxide for 10 min and washed with distilled water. The sterilized Goji seeds were grown in autoclaved mixed substrate of vermiculite and sand (1:1, v/v) for germination. Uniform seedlings were selected and transplanted into pots after 4 weeks (1 seedling per pot). Prior to seedling transfer, mycorrhizal treatment (M) received 100 g soil based *D. versiformis* inoculum (containing about 527 spores) in half number of the pots. The remaining pots received 100 g autoclaved AMF inoculum as well as 10 ml of inoculum filtrate, as non-mycorrhizal treatment (NM).

The pot experiment consisted of two factors: mycorrhizal treatments: inoculated with *D. versiformis* (M) or not (NM); 2), three salt levels: 0, 100 and 200 mmol/L NaCl. Each treatment had 3 replicates. Thus, the experiment was a 2 × 3 factorial design.

Growth condition and salt treatments: The soil used in this study was obtained from the campus of Northwest A&F University, Yangling, Shaanxi, China. The soil properties were consistent with that in Liu *et al*., (20016). The growth substrate was composed of soil, sand and vermiculite (1:1:1, v:v:v), which were autoclaved at 121ºC for 2 h.

The Goji seedlings were cultivated in a greenhouse of Northwest A&F University with natural solar light. The temperature and humidity were maintained between 22~35ºC and 70%~75% during the experiment, respectively.

Salt stress was implemented on Goji plants after 10-weeks growth. The prescribed NaCl solutions (300 mL per pot) were given to Goji for 10 weeks to impose salt stress. The electrical conductivities of 0, 100 and 200 mmol/L salt treatments were 0.19, 10.52 and 20.81 dS/m, respectively. Goji plants continued to grow for another 5 weeks under these conditions till test.

Determination of AMF colonization rate: The roots of three Goji plants were collected to assess AMF colonization. The Goji roots were stained according to the protocol of Phillips & Hayman (1970). The gridline intersect method was employed to calculate the percentage colonization (Giovannetti & Mosse, 1980).

Ultrastructural observation of mesophyll cells: The protocol of Zhao *et al.*, (2015) was employed with some modifications to observe the ultrastructure of the Goji mesophyll cells (25 weeks old). The third fully expanded leaf of Goji plants from all treatments was collected to check ultramicroscopically the mesophyll cells. The leaves were cut into 1-2 mm segments and subsequently placed in a freshly prepared stationary liquid of 2.5% (w/v) lutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4). The leaf segments were then degassed and fixed under vacuum for 4 hours at room temperature. The segments were washed for 3 times using the same buffer, followed by being post-fixed in 1% (w/v) osmium tetroxide for 1 hour. The fixed samples were rinsed with the same buffer for three times (15 min each time). The processed samples were placed in 50% ethanol for half an hour, followed by gradients in 70%, 90%, 95% and 100% ethanol (30 minutes in each concentration), to be dehydrated rapidly. The low-viscosity epoxy resin was employed to embed the specimens. Ultra-thin sections (80 nm) were picked up on copper grids and stained with uranyl acetate and lead citrate before being examined at 80 kV in electron microscope JEOL JEM-1230. Ninety randomly chosen mesophyll cells from three replicates were analyzed for each treatment.

Determination of chlorophyll fluorescence parameters: The measurements of chlorophyll fluorescence were conducted on the third fully expanded Goji leaf (25 weeks old) with a portable PAM chlorophyll fluorometer (Walz, Germany). Initially, the Goji plants were placed in a dark room for a 30-minutes dark adaptation at room temperature. Then the minimum fluorescence (Fo) and the maximum fluorescence (Fm) were determined. The maximum quantum yield of PSII (Fv/Fm), actual quantum yield of PSII (ΦPSII), non-photochemical quenching yield (qN) and photochemical quenching (qP) were calculated according to the following formula:

Fv/Fm (Fv = Fm-Fo)
ΦPSII = (Fm'-F)/Fm'
qN = 1-(Fm'-Fo')
qP = (Fm'-F)/(Fm'-Fo').

Statistical analysis

The Kolmogorov-Smirnov test and Levene test were conducted to check the normality and homogeneity of the obtained data. Analysis of variance (ANOVA) was used to estimate the influences of AMF and salinity on the chlorophyll fluorescence parameters on SAS 8.01. Duncan's test was employed for post-hoc analysis. Standard error is used in the manuscript.

Result and Discussion

AMF colonization: The colonization rates for M Goji at 0, 100 and 200 mmol/L salt level were 60.91±2.91%, 52.50±3.15% and 57.27±3.90%, respectively. No mycorrhizal structure was observed in the roots of NM Goji plants. The results showed that Goji could form mycorrhizal symbiosis with *D. versiformis* under salt stress. The percent colonization rate of Goji declined after being exposed to salt stress, but not reached to the statistical level (p>0.05).

Ultrastructure of mesophyll cells: The plasma membrane integrity and its close contact with cell walls are critical for perceiving external signals and molecule dialogue between cells. As we can see in Fig. 1, under 0 and 100 mmol/L salt level, the plasma membrane (PM) of both NM and M Goji closely attached to the cell wall (CW) (Fig. 1a-d). However, the vesicules (v) were only observed in the cytoplasm of M Goji, not for NM Goji. Namely, the association between PM and CW was well preserved for both M and NM Goji under low and no salt stress, maintaining the signal sensing of mesophyll cells. The plasma membrane damage has been reported on *Kalidium foliatum* under salt-stressed conditions (Wang & Jia, 2015). In this study, 200 mmol/L salt level induced plasmolysis in the mesophyll cells of NM Goji, leaving large apoplastic space (Fig. 1e, *double arrow*). But the mesophyll cells of Goji plants inoculated with *D. versiformis* showed less extent of plasmolysis (Fig. 1f, *arrow*) in comparison with NM Goji. The result was consistent with mycorrhizal fenugreek in comparison with non-inoculated control, wherein less extent of plasmolysis was attributed to improved osmotic adjustment (Evelin *et al.*, 2013). Similarly, the higher content of soluble sugar and reducing sugar in M Goji leaves possibly contributed to the mitigation of osmotic stress of Goji mesophyll cells (Liu *et al.*, 2016). Hence, the arbuscular mycorrhiza reinforced osmotic regulation efficiency of plants attenuated the physiological drought and avoided cytoplasm shrinkage to maintain the turgor of plant mesophyll cells (Ruiz-Lozano, 2003), thus alleviating the magnitude of plasmolysis.

Ultrastructure of chloroplast: It is essential to keep the structural integrity and orderliness of chloroplast for plants to capture and converse light energy during photosynthesis. Salt stress has been reported to damage photosynthesis apparatus, including swollen grana/stroma lamellae and loose thylakoid membranes (Ibrahim *et al.*, 2015). No significant difference in the chloroplast structure between NM and M Goji was observed under 0 mmol/L NaCl (Fig. 2a-b). To the contrary, under 100 mmol/L NaCl, the chloroplasts detached from plasma membrane in the mesophyll cells of NM Goji (Fig. 2c: *arrow*), whereas M Goji showed less extent of chloroplast separation from plasma membrane (Fig. 2d: *arrow*). More significant difference were captured at 200 mmol/L NaCl, the envelop membrane of chloroplast of NM Goji were drastically ruptured (Fig. 2e), with more oil droplets (o) emerged, and the gap between chloroplast and plasma membrane increased. However, compared with NM Goji, the leaves of M Goji contained well-preserved chloroplast with well-stacked thylakoids and less oil droplets (o) (Fig. 2f). As pointed by arrow in Fig. 2f, smaller gap between chloroplast and plasma membrane was found in the mesophyll cells of M Goji compared with NM Goji. Overall, the chloroplast ultrastructure of NM Goji was drastically damaged at 200 mmol/L NaCl, showing detachment from plasma membrane, envelope membrane injury and increased oil droplet. However, the M Goji plants showed less magnitude of salt injury compared with NM Goji. The ultrastructure of M Goji chloroplasts exerted higher tolerance than control, even in higher salt stress level (200 mmol/L NaCl). We previously showed that the photosynthesis of Goji was negatively influenced by salt stress (Liu *et al.*, 2016), which could be induced by the structural damage in chloroplast. Similar chloroplast structure damage has been suggested on beach plum and fenugreek under salt stress (Zai *et al.*, 2012; Evelin *et al.*, 2013). The protective effect of AMF on the chloroplast structure of host might lie in higher free radical scavenging ability.

Chlorophyll fluorescence parameters: The chlorophyll fluorescence is considered as key indicators to appraise the efficiency of PSII. The present results demonstrated that salt stress notably decreased the quantum efficiency of open photosystem II (Fv/Fm (p<0.05)), efficiency of photosystem II (ΦPSII (p<0.01)), photochemical quenching (qP (p<0.01)) of Goji, but increased non-photochemical quenching coefficient (qN (p<0.01)) (Fig. 3). Under 0 and 100 mmol/L NaCl, the chlorophyll fluorescence parameters (Fv/Fm, ΦPSII and qP) of M Goji were similar with that of NM Goji (Fig. 3). However, at 200 mmol/L NaCl, inoculation with *D. versiformis* significantly increased the ΦPSII, qP and qN by 6.8%, 3.0% and 3.5% (p<0.05) of Goji compared with NM treatment, respectively. Namely, M Goji had higher efficiency of PSII in comparison with NM Goji. Meanwhile, lower qN of M Goji in comparison with NM Goji under 200 mmol/L salt level suggested that less energy was bifurcated to non-photochemical events. As a result, M Goji had higher photochemical and non-photochemical efficiency than NM Goji. This is in accordance with the results of black locust and poplar (Wu *et al.*, 2016; Chen *et al.*, 2017), indicating arbuscular mycorrhizal promotion effect in electron transfer process. This could potentially underlie the protection of AMF on photosynthesis apparatus of host plants from oxidative damage. The enhanced photochemical activity of host plants induced by arbuscular mycorrhiza is of significance for increase the agricultural production and crop quality under stressed conditions.

Conclusions

Taken together, the deleterious effect of salt stress on the ultrastructure of Goji mesophyll cells has been mitigated by AMF, including less extent of plasmolysis and high level of chloroplast integrity. Concurrently, the PSII efficiency of Goji has been strengthened by inoculation of *D. versiformis* under salt stress. This study provides new ultrastructural and photochemical evidence of AMF protecting plants under salt stress.

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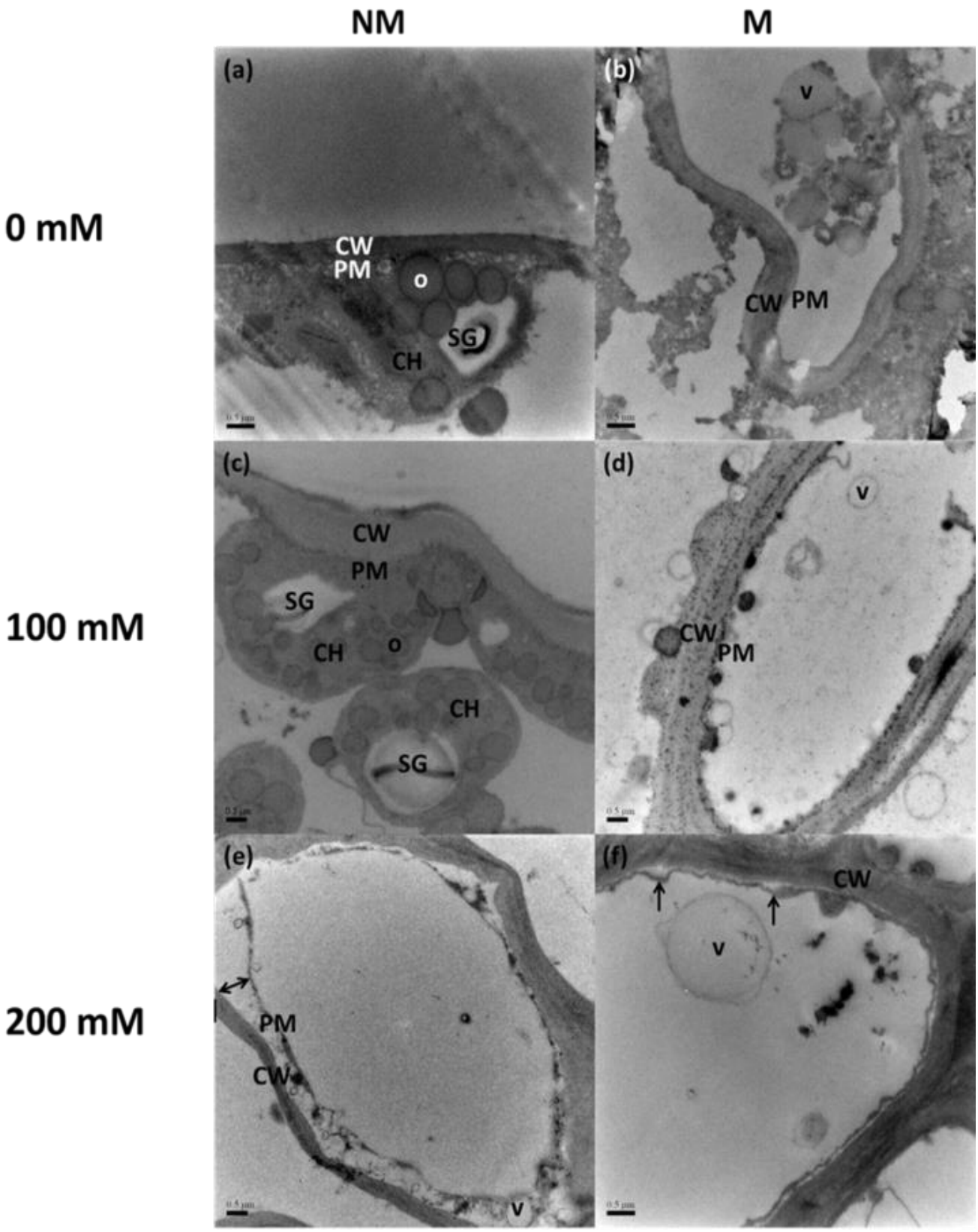

Fig. 1. Transmission electron micrographs of cell wall and plasma membrane in upper mesophyll cells of *Lycium barbarum*: (a) non-mycorrhizal (NM) plants not subjected to NaCl: plasma membrane (PM) closely appressed to cell wall (CW), with oil droplets (o) embracing starch grain (SG) in chloroplast. (b) mycorrhizal (M) plants not subjected to NaCl: well-preserved plasma membrane (PM) closely associated with cell wall (CW), numerous vesicules (v) gathered in cytoplasm. (c) NM plants subjected to 100 mmol/L NaCl: plasma membrane (PM) in close contact with cell wall (CW), plenty of small oil droplets (o) embracing starch grain (SG) in chloroplast (CH). (d) M plants subjected to 100 mmol/L NaCl: plasma membrane (PM) attached to cell wall (CW), small vesicules (v) in cytoplasm. (e) NM plants subjected 200 mmol/L NaCl: tremendous apoplastic space between plasma membrane (PM) and cell wall (CW) (*double arrow*), small vesicules (v) in apoplastic space. (f) M plants subjected to 200 mmol/L NaCl: plasmolysis initiated (*arrow*), large vesicule (v) in cytoplasm. The *values* left denote the concentration of NaCl applied in the soil. *Bar*=0.5 μm.

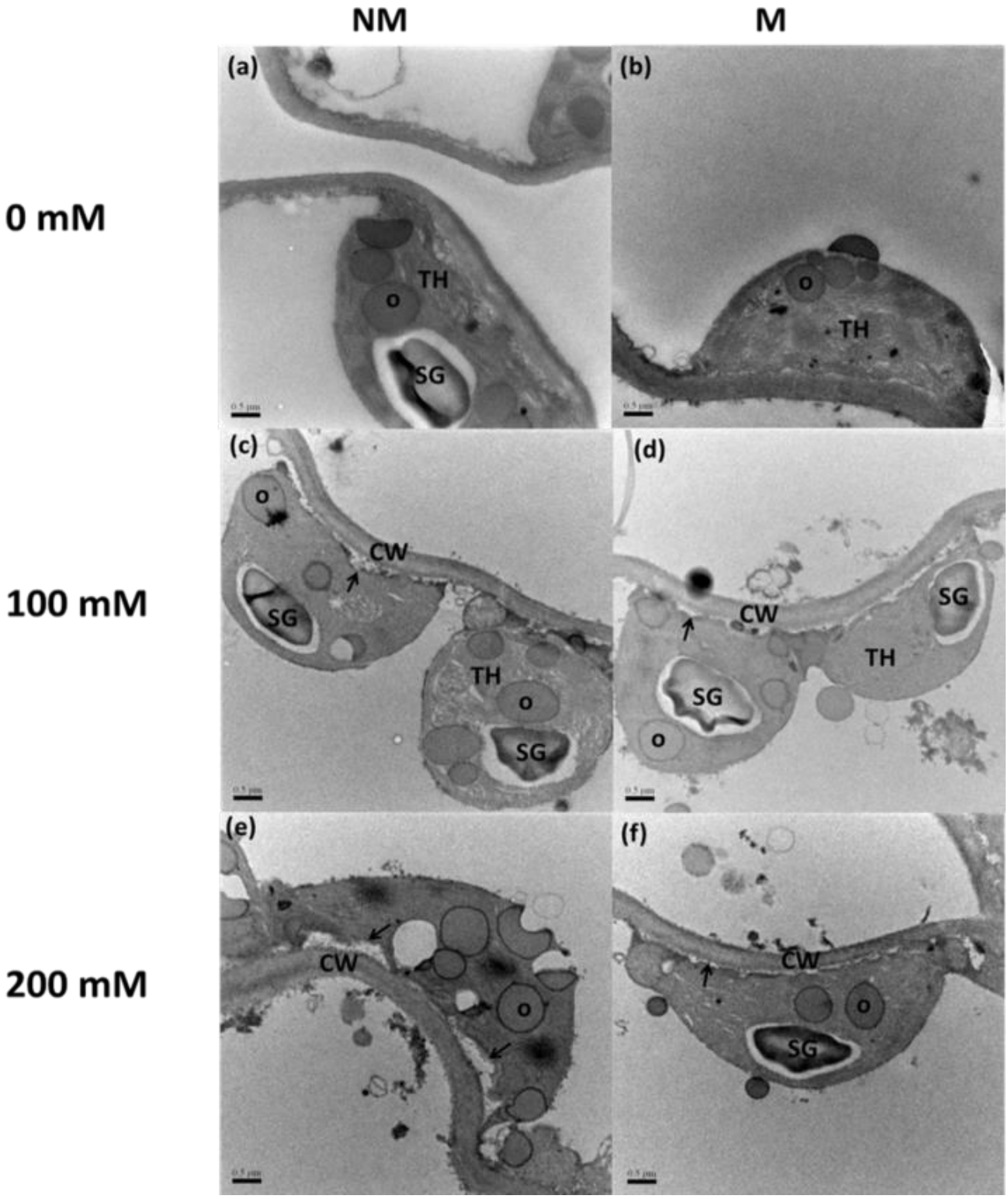

Fig. 2. Transmission electron micrographs of chloroplast in mesophyll cells of *Lycium barbarum*: (a) non-mycorrhizal (NM) plants not subjected to NaCl: well developed with distinct thylakoids (TH), grana and oil droplets (o) close to starch grain (SG) surrounded by a well-defined double-layered membrane, chloroplast closely associated with plasma membrane. (b) mycorrhizal (M) plants not subjected to NaCl: distinct thylakoids (TH) and small oil droplets (o) surrounded by double membrane, elliptical chloroplast press closely to plasma membrane. (c) NM plants subjected to 100 mmol/L NaCl: nearly round chloroplasts with more oil droplets (o) and distorted thylakoids (TH), the gap between chloroplast membrane and plasma membrane observed (*arrow*). (d) M plants subjected to 100 mmol/L NaCl: the chloroplast shows less oil droplets (o) and receding envelope membrane (*arrow*), however, the grana were well organized. (e) NM plants subjected to 200 mmol/L NaCl: double membrane of chloroplast drastically damaged with more oil droplets (o) inside, indistinct thylakoid and grana, increased space between chloroplast and plasma membrane (*arrow*), plastoglobules appeared. (f) M plants subjected to 200 mmol/L NaCl: well preserved chloroplast with less oil droplets (o), well-structured thylakoids and grana, small gap between double membrane and plasma membrane (*arrow*). The *values* left denote the concentration of NaCl applied in the soil. *Bar*=0.5 μm.

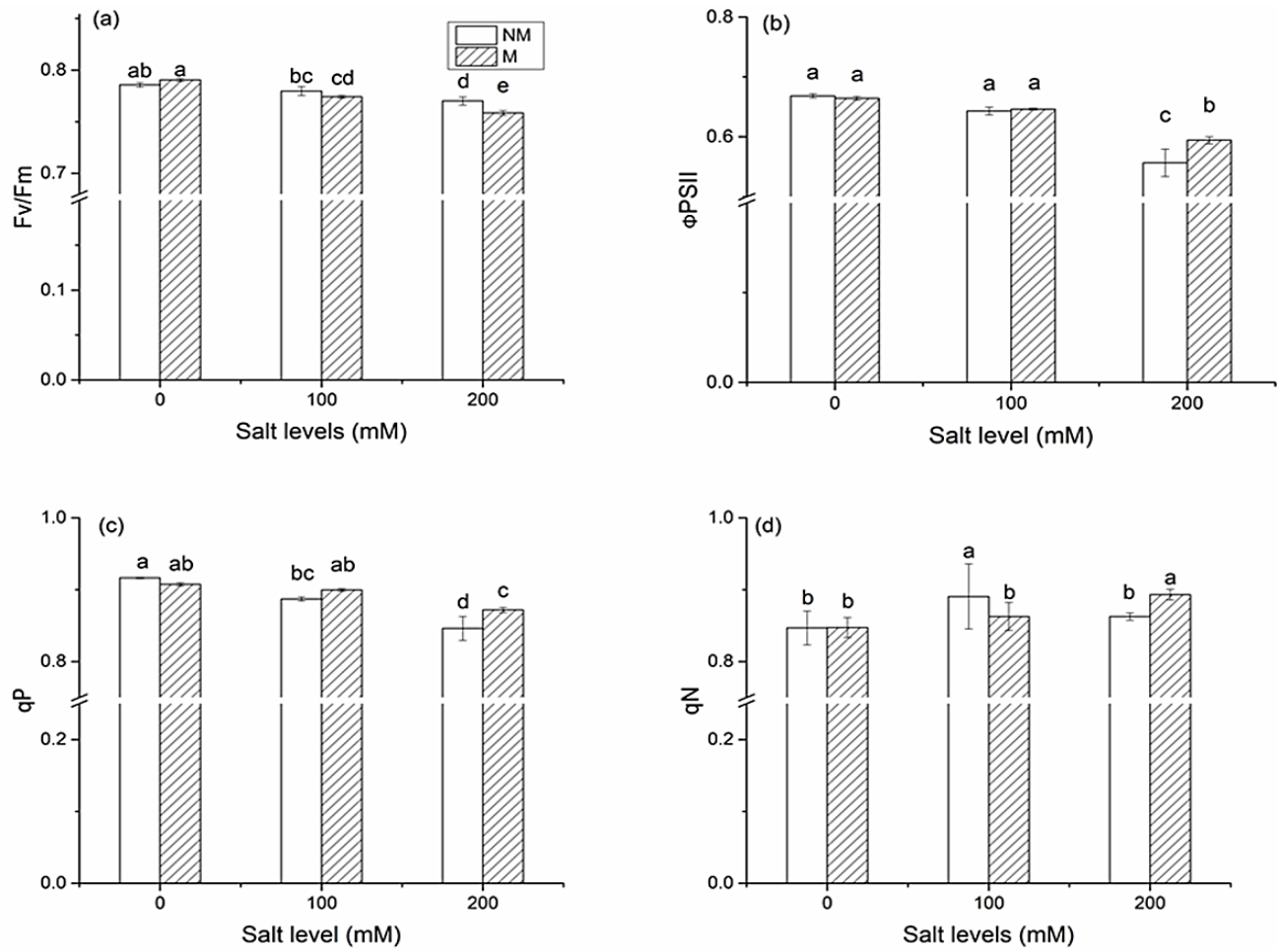

Fig. 3. Effect of AMF and salt stress on the chlorophyll fluorescence parameters of *Lycium barbarum*: (a) the quantum efficiency of open photosystem II (Fv/Fm), (b) the efficiency of photosystem II (ΦPSII), (c) photochemical quenching (qP), (d) non-photochemical quenching coefficient (qN). Data are means±se (n=3). Error bars show the x% confidence interval of the measurement. Bars sharing the same letter mean the values are not significantly different according to Duncan's test at $p<0.05$. *NM* and *M* represents non-mycorrhizal and mycorrhizal plants, respectively.

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