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Effects of catalase on chloroplast arrangement in *Opuntia streptacantha* chlorenchyma cells under salt stress

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In arid and semiarid regions, low precipitation rates lead to soil salinity problems, which may limit plant establishment, growth, and survival. Herein, we investigated the NaCl stress effect on chlorophyll fluorescence, photosynthetic-pigments, movement and chloroplasts ultrastructure in chlorenchyma cells of *Opuntia streptacantha* cladodes. Cladodes segments were exposed to salt stress at 0, 100, 200, and 300 mM NaCl for 8, 16, and 24 h. The results showed that salt stress reduced chlorophyll content, F_v/F_m, ΦPSII, and *qP* values. Under the highest salt stress treatments, the chloroplasts were densely clumped toward the cell center and thylakoid membranes were notably affected. We analyzed the effect of exogenous catalase in salt-stressed cladode segments during 8, 16, and 24 h. The catalase application to salt-stressed cladodes counteracted the NaCl adverse effects, increasing the chlorophyll fluorescence parameters, photosynthetic-pigments, and avoided chloroplast clustering. Our results indicate that salt stress triggered the chloroplast clumping and affected the photosynthesis in *O. streptacantha* chlorenchyma cells. The exogenous catalase reverted the H_2O_2 accumulation and clustering of chloroplast, which led to an improvement of the photosynthetic efficiency. These data suggest that H_2O_2 detoxification by catalase is important to protect the chloroplast, thus conserving the photosynthetic activity in *O. streptacantha* under stress.

To ensure their survival, plants have evolved to optimize the capture of energy and nutrients[1]. Previous studies have shown that organelles, such as mitochondria, peroxisome, and chloroplast are constantly moving within the cells taking specific positions to maximize their metabolic activities during changing environmental conditions[2–4]. In particular, the chloroplast movement in response to light (photo-relocation) is essential for the survival of plants under extreme light conditions[5,6]. Additionally, chloroplast movement has a protective role in response to other abiotic stresses, such as drought and low temperatures[7–9]. Chloroplast movement has been extensively studied in the model plant *Arabidopsis thaliana*, operating with C_3 type photosynthesis[5]. However, only a few studies have been undertaken about chloroplast movement in C_4 and CAM (Crassulacean Acid Metabolism) plants[7,9–11]. Previously, our research group reported that the *Opuntia streptacantha* chloroplasts were grouped together within the cells under combined light and water stress, which probably maintain its photosynthetic process active in CAM plants[9].

Particularly, the high plasticity in the CAM photosynthetic pathway is one of the most successful physiological strategies for plant acclimation and adaptation to water shortage[12]. The water loss is minimized because during the daytime CAM plants photosynthesize with closed stomata, using the CO_2 that was stored in the vacuole in form of organic acids, mainly malic acid, during night[12]. Thus, CAM plants are able to survive under extreme abiotic stress conditions, predominantly scarcity of water and extreme temperatures, in contrast to C_3 and C_4 plants[13].

Opuntia streptacantha is an endemic cactus from Mexico, which is distributed along the southern Chihuahuan Desert[14]. It performs a CAM type photosynthesis, which permits a successful establishment of intracellular levels

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of the plant in arid and semiarid regions of many countries[12,14,15]. Despite the ability of CAM plants to overcome water stress, not all CAM plants are successful to cope salinity[12].

It is known that salinity affects the photosynthesis process in plants[16]. An early effect of salt stress is stomatal closure, which leads to a restriction of CO_2 diffusion into the cells and chloroplasts[17]. Subsequently, the increase of reactive oxygen species (ROS) levels, can seriously affect the plant's photosynthetic machinery[18,19]. However, ROS are also important signaling molecules involved in the control of plant growth, development, photosynthetic functions, and responses to biotic and abiotic stress[19–21]. In particular, the hydrogen peroxide (H_2O_2) is the most significant non-radical ROS[22]. H_2O_2 is produced predominantly during the photosynthesis and photorespiration process in the apoplast, chloroplast, peroxisome, and mitochondria[20,23]. Plants possess efficient scavenging systems for ROS, which protect them from destructive oxidative reactions. The principal H_2O_2 scavenging enzyme in plants is catalase (CAT), which directly converts H_2O_2 into H_2O and O_2[24]. Likewise, ascorbate peroxidase (APX) and glutathione peroxidase (GPX) are important enzymes essentials for the elimination of H_2O_2 levels in the cell[25]. The balance between ROS generation and ROS-scavenging during exposure to stressful environments is essential to regulate the mechanisms of ROS signaling in plants[26,27].

The negative effects on growth and CO_2 uptake in *Opuntia* plants under salt stress have been previously reported[28–30]; however, little information about other physiological data related to salinity has been provided. In this study, our main objective was to examine the effect of NaCl treatments on chloroplast movement in chlorenchyma cells of *O. streptacantha*. Through optical microscopy, we observed the chloroplast arrangement under different treatments with NaCl (0, 100, 200, and 300 mM). Additionally, we measured diverse physiological parameters, such as the chlorophyll fluorescence, photosynthetic pigments content, and ultrastructure of chloroplasts to determine the effect caused by NaCl treatments. We found that the H_2O_2 accumulation, clustering of the chloroplasts, and a decline in photosynthetic activity were consequences of salinity in *O. streptacantha*. Finally, exogenous application of CAT enzyme was used to counteract the effects produced by salt treatment. Our data suggest that H_2O_2 accumulation is relevant for chloroplast clustering under salinity in *O. streptacantha*.

Results

The photosynthetic capacity of *O. streptacantha* cladodes decreases under salt treatments.

We performed chlorophyll fluorescence measurements in *O. streptacantha* to evaluate the effect of salinity on photosynthesis, by using cladode segments exposed to salt treatments at 0, 100, 200, and 300 mM NaCl for 8, 16, and 24 h. The values of the maximum quantum yield of photosystem II (F_v/F_m), the effective photochemical quantum yield of PSII (ΦPSII), and the photochemical quenching (*qP*) showed a decreasing trend related to NaCl concentration and treatment duration (Fig. 1A–C). The excitation pressure (1-*qP*) and the non-photochemical fluorescence quenching (NPQ) values were significantly increased (Fig. 1D and E). At 100 mM NaCl for 8, 16, and 24 h, the values of F_v/F_m, ΦPSII, *qP*, 1-*qP*, and NPQ did not give statistically significant differences, whereas, in the treatment with the highest salt concentrations, the F_v/F_m, ΦPSII, and *qP* values were significantly reduced at the three time periods analyzed compared to the control. Meanwhile, the 1-*qP* and NPQ values were significantly increased. We also found that the photosynthetic electron transport rate (ETR) was significantly reduced at 200 and 300 mM NaCl compared to the control (Supplementary Fig. S1). These results showed that the NaCl concentration and exposure time affected negatively the photosynthetic capacity of *O. streptacantha* cladodes.

Salt treatments effect on photosynthetic pigments content in *O. streptacantha* cladodes. In order to determine the effect of salt stress on the photosynthetic pigments content in *O. streptacantha*, we quantified the chlorophyll *a*, *b*, total ($a+b$), and carotenoids ($x+c$) content in cladodes segments exposed to salt treatments at 0, 100, 200, and 300 mM NaCl during 8, 16, and 24 h. We observed that the chlorophyll *a*, and total ($a+b$) contents tends to decline along with the exposure time to NaCl concentrations (Fig. 2). At 8 h of salt treatment, were observed a decrease in chlorophyll *a*, and total ($a+b$) levels at 100 and 200 mM NaCl concentrations compared to the control. However, no changes were observed in the Chl *a*, and total ($a+b$) levels at 16 h under the salt treatments evaluated. After 24 h of the salt treatments, we found a decrement of chlorophyll *a*, and total ($a+b$) levels in all the salt concentrations tested (Fig. 2A and B). On the other hand, we did not observe a significant difference in the ratio of Chl *a* to Chl *b* (Chl *a*/*b*) at 8 h of salt treatments. Meanwhile, the Chl *a*/*b* ratio was significantly reduced at 16 and 24 h for all the salt treatments (Fig. 2C). The ratio of Chl *a* and Chl *b* to total carotenoids ($a+b$)/($x+c$) was significantly reduced only at 24 h of salt treatments (Fig. 2D). Therefore, salt treatments negatively affected the chlorophyll and carotenoids content in *O. streptacantha* cladodes.

Salt treatments triggers chloroplasts clumping in *O. streptacantha* chlorenchyma cells. In order to analyze the chloroplast arrangement in response to salt treatments, *O. streptacantha* cladode segments were incubated with 0, 100, 200, and 300 mM NaCl under continuous light conditions for 8, 16, and 24 h (Fig. 3). Under the control conditions, the chloroplasts were always dispersed in the cytosol of the cells. However, we observed chloroplast clumping in chlorenchyma cells when the salt concentration was increased. At 100 mM NaCl for 24 h the chloroplasts were redistributed. Furthermore, it was observed that in some cells chloroplasts began to cluster. Additionally, we detected aggregation of chloroplasts at 200 and 300 mM NaCl during the period analyzed. We also observed that NaCl-induced chloroplast clumping at 200 mM for 8 h was reversible when the salt stressed cladodes segments were washed and then incubated in distilled water (Supplementary Fig. S2). These results evidence that salt treatment triggers chloroplasts clumping in *O. streptacantha* chlorenchyma cells.

Salt treatment induced ultrastructural changes in thylakoid membranes of *O. streptacantha* chlorenchyma cells. To analyze if the ultrastructure of chloroplasts gets affected by exposure to salt treatment, cladode segments of *O. streptacantha* were incubated with 200 mM NaCl during 8, 16, and 24 h. Subsequently, the chloroplast membranes were analyzed by a transmission electron microscope (TEM). At the

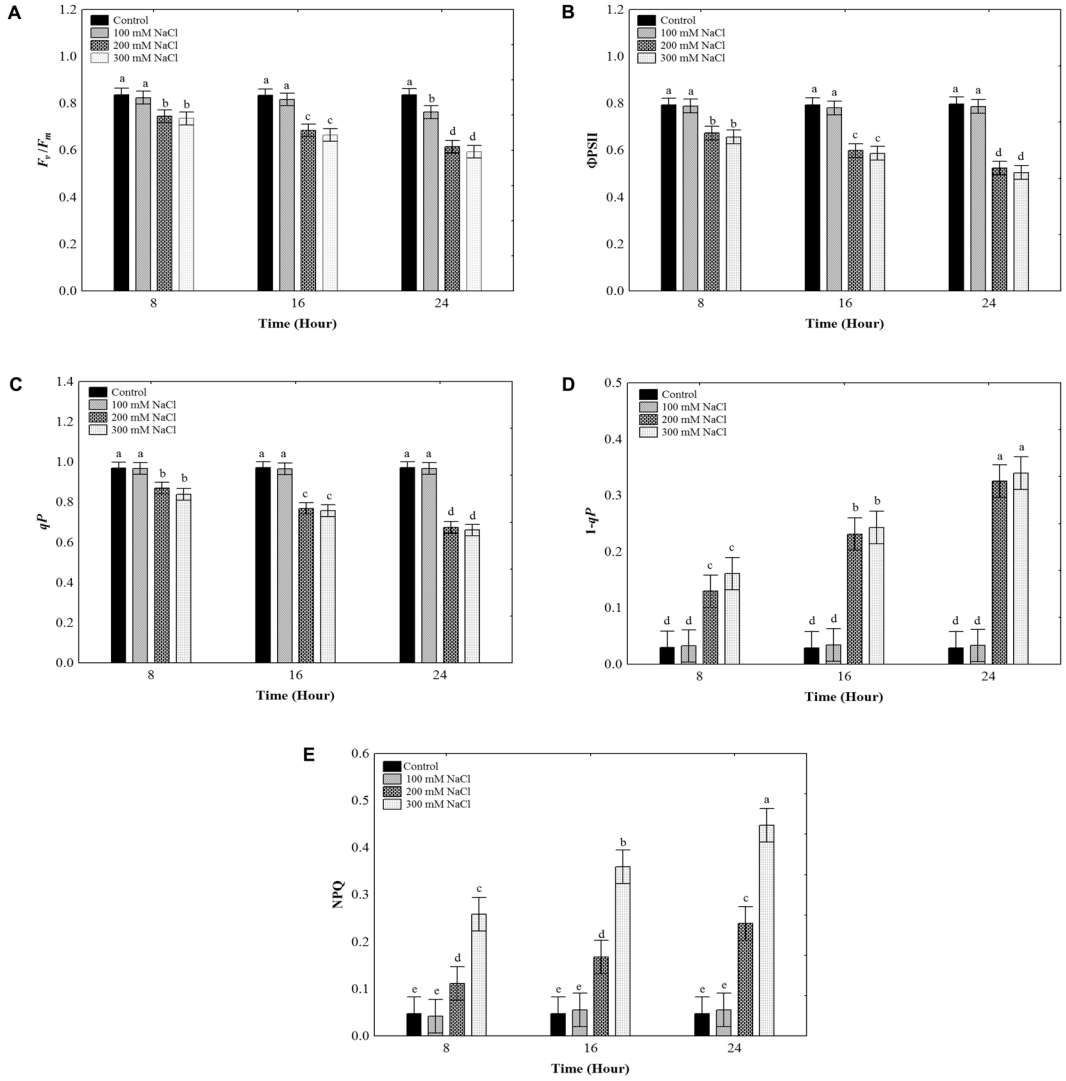

Figure 1. Chlorophyll fluorescence parameters in *Opuntia streptacantha* cladodes under salt treatments. (**A**) The maximum quantum yield of PSII (F_V/F_m). (**B**) The effective photochemical quantum yield of PSII (ΦPSII). (**C**) The photochemical quenching (qP). (**D**) The excitation pressure ($1–qP$). (**E**) The non-photochemical fluorescence quenching (NPQ). Values are means and bars indicate ± SD, ($n = 9$). Different letters indicate significant difference between treatments and time (hours) according to Duncan's multiple range tests at $P < 0.05$.

ultra-structural level, chloroplast distortion was observed in the cladode segments exposed to salt treatment for 8, 16, and 24 h compared to the control (Fig. 4A). Initially, we observed a stacking in the thylakoid membranes at 8 and 16 while at 24 h, a distortion of chloroplasts was highly visible where the thylakoid membranes were fragmented. These results show that the thylakoid membranes were notably affected by the salt treatment. In order to confirm the stacking of thylakoid membranes, measurements of lumen thickness were made on grana lamellae (GL) in separate micrographs (Fig. 4B). Electron microscopy data showed that the width of the thylakoid lumen was significantly diminished by the exposure to 200 mM NaCl during 8 and 16 h compared to the control. The lumen thickness at 24 h under salinity was not examined because the thylakoid membranes were severely distorted. These results indicate that salt treatment induced thylakoid membranes stacking in *O. streptacantha*.

Catalase treatment avoid the clustering of chloroplasts under salt treatment. To determine if the exogenous catalase (CAT) application may counteract the aggregative effect of chloroplasts under salt stress, we incubated cladode segments in solutions with 200 mM NaCl supplemented with 100, 200, and 300 UmL^{-1} CAT during 8 h (Fig. 5). Under the application of 100 UmL^{-1} CAT, we observed that chloroplast grouping was diminished in comparison with the treatment of 200 mM NaCl without CAT. Conversely, we detected that the chloroplasts were completely dispersed throughout the cell when the highest concentrations of CAT were applied (200 and 300 UmL^{-1}). In this regard, our data indicate that the application of CAT may inhibit the clustering of chloroplasts during salt stress in *O. streptacantha* chlorenchyma cells.

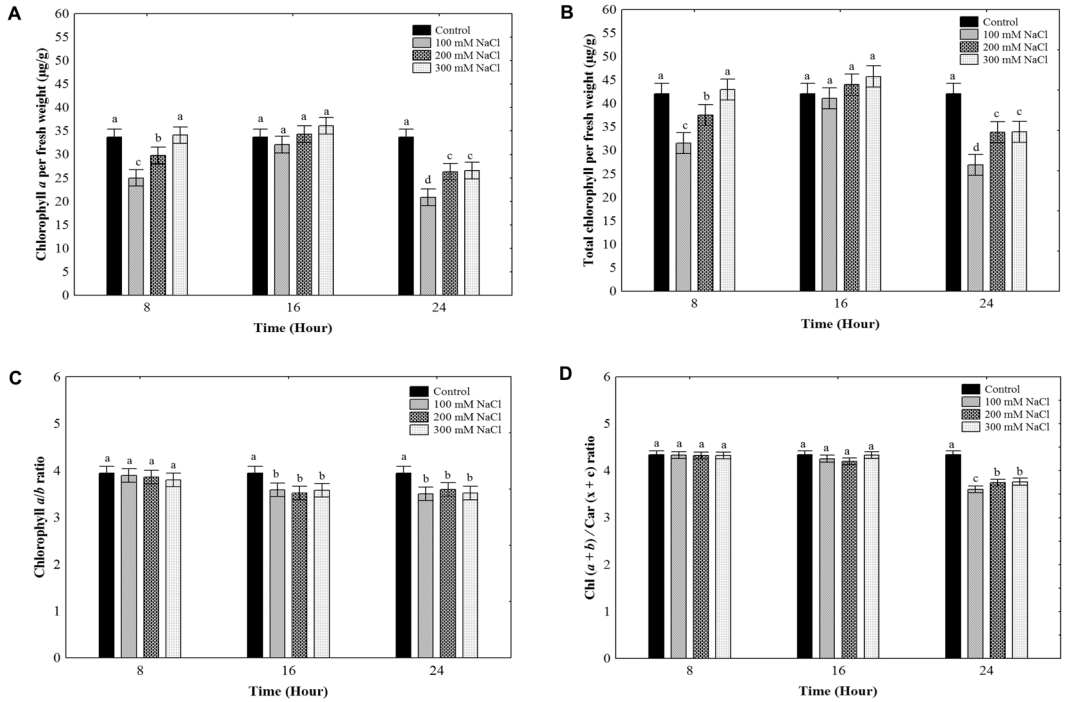

Figure 2. Chlorophyll and carotenoids content in *Opuntia streptacantha* cladodes under salt treatments. (**A**) Chlorophyll *a*. (**B**) Total chlorophyll ($a+b$). (**C**) Chlorophyll *a*/*b* ratio. (**D**) Chl ($a+b$)/Car (x + c) ratio. Values are means and bars indicate ± SD, ($n=9$). Different letters indicate significant difference between treatments and time (hours) according to Duncan's multiple range tests at $P<0.05$.

Catalase alleviated the H_2O_2 accumulation caused by salt stress. In order to determine if the exogenous CAT application reduces the oxidative stress by H_2O_2 scavenging activity under salt treatment, we monitored the H_2O_2 using the fluorescent signals of specific fluorescent probes (DCF-DA) on *O. streptacantha* cladode cells exposed to 200 mM NaCl + 300 UmL^{-1} CAT during 8 h (Fig. 6). We found a marked increase of H_2O_2 production in cells under 200 mM NaCl compared to the control treatment, whereas, accumulation of H_2O_2 was avoided under salt treatment by the application of 300 U mL^{-1} CAT. Thus, our results indicate that the H_2O_2 accumulation in *O. streptacantha* cells induced by salt stress can be scavenged *in vitro* by CAT activity.

Catalase confers protection to the photosynthesis process under salt treatment. To define if the exogenous CAT application confers protection to the photosynthetic efficiency under salt treatment, we performed chlorophyll fluorescence measurements on *O. streptacantha* cladode cells exposed to 200 mM NaCl supplemented with 300 UmL^{-1} CAT during 8, 16, and 24 h. The values of F_v/F_m, ϕPSII, *qP*, (1-*qP*), and NPQ remained close to the control without salt stress (Fig. 7A–E), accomplishing that CAT mitigated the negative effects observed at 200 mM NaCl (Fig. 1). In addition, we observed that the CAT application increased the ETR values in cladodes under salt stress (Supplementary Fig. S3) compared to those cladodes treated only with 200 mM NaCl (Supplementary Fig. S1). Our results show that exogenous catalase allows the operation of the photosynthetic machinery without signs of inhibition in *O. streptacantha* cladodes under that salt treatment.

Catalase prevented photosynthetic pigments degradation. To assess whether the exogenous application of CAT prevents the pigments degradation, we quantified the content of chlorophylls and carotenoids in *O. streptacantha* cladode segments exposed to 200 mM NaCl supplemented with 300 UmL^{-1} CAT for 8, 16, and 24 h.

As we previously described in Fig. 2, the treatment of 200 mM NaCl showed a decrement of chlorophyll content. However, CAT exogenous application reversed the negative effect of 200 mM NaCl on chlorophyll levels, reaching values similar to the control without salt stress (Fig. 8). These results support the notion that exogenous CAT prevents photosynthetic pigments degradation induced by salt stress in *O. streptacantha* cladodes.

Discussion

Abiotic stress affects the development, establishment and survival of wild-plants and crops. Photosynthesis is one of the most important physiological processes through which plants produce the essential energy for their growth and development[16]. Chloroplasts are specialized organs that capture sunlight required to perform photosynthesis[1]. However, when plants are exposed to various environmental stresses, such as salinity[10,17], drought[18], or high light conditions[31], plant's chloroplasts can get damaged, leading to photosynthesis inhibition.

In this study, we analyzed the effect of salt stress on the movement of chloroplasts in the chlorenchyma cells of *O. streptacantha*. Notably, we observed that the chloroplasts were densely clumped towards the center of the cell in response to NaCl treatments. Similar results were reported by Yamada *et al.*[10], who showed that salinity

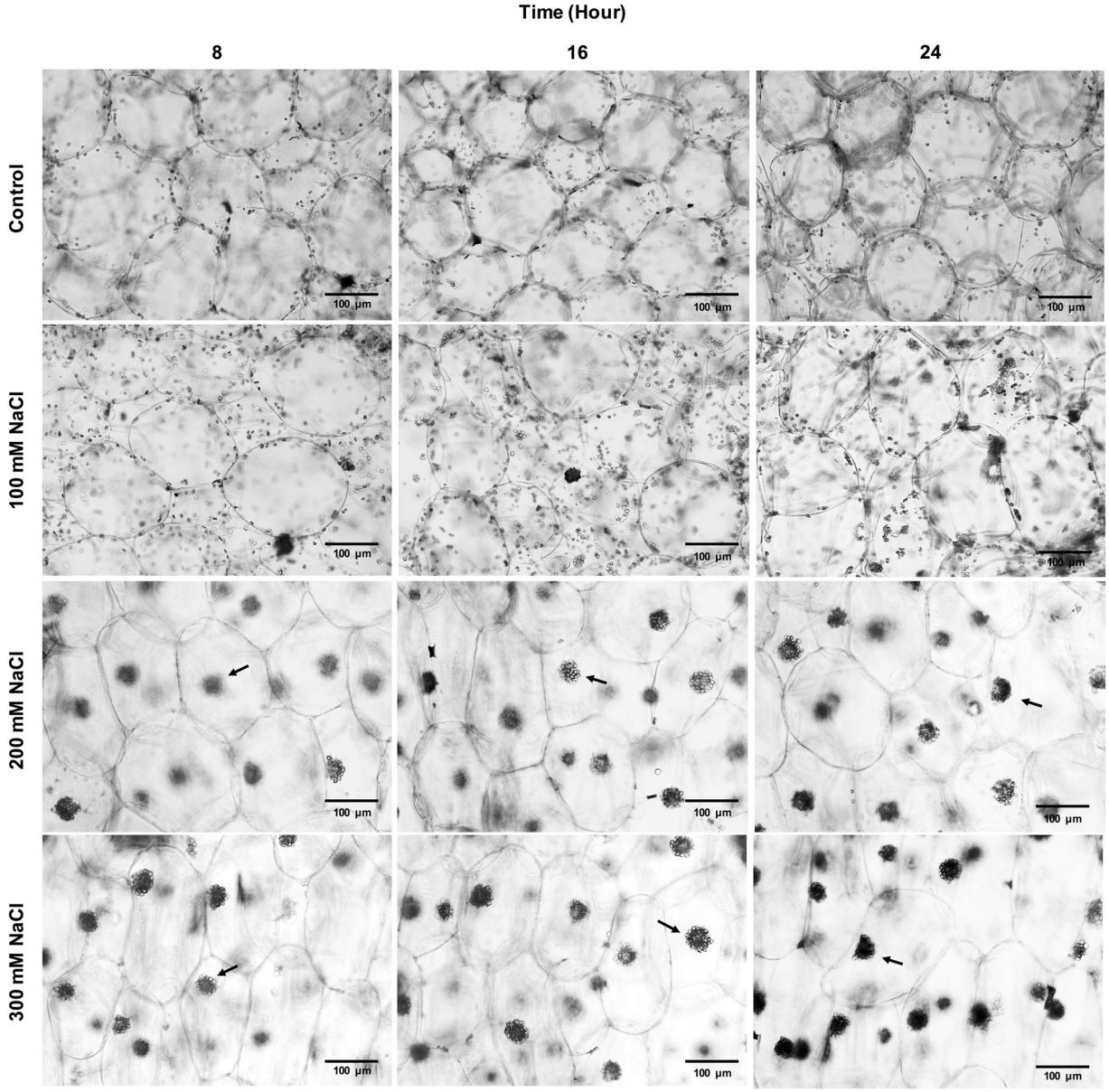

Figure 3. The chloroplast arrangement in chlorenchyma cells from *Opuntia streptacantha* under salt treatments. Representative images of *O. streptacantha* cells and their chloroplast are shown in each panel. Cladode segments were cut, and after they were incubated in 0, 100, 200, and 300 mM NaCl under continuous white light (300 µmol $m^{-2} s^{-1}$) for 8, 16, and 24 h. Transverse sections from cladode were observed with a light microscope. The black arrowheads indicate chloroplast clusters. Scale bar corresponds to 100 µm.

stress induced aggregative movement of chloroplasts in *Eleusine coracana* cells under normal intensity light. The increase of salt causes adverse effects on the functions and structure of the photosynthetic apparatus, leading to a decline in the F_v/F_m, ΦPSII, and *qP* parameters. These changes indicate that the reaction centers (RCs) got photochemically inactive, which reduced electron transport capacity in PSII and that decreased the photosynthetic capacity of *O. streptacantha* cladodes under salt stress.

In addition, the effect of NaCl treatments on the photosynthetic pigment content in chlorenchyma cells of *O. streptacantha* cladodes was analyzed. Our data showed that the decrease in the content of chlorophylls at 8 and 24 h was more pronounced at a low than at a higher salt concentration. One possible explanation could be that *O. streptacantha* cells, when sensing high salt concentrations activate several mechanisms to induce *de novo* chlorophyll synthesis, and thus counteract osmotic and ionic shock. This behavior has been previously described in cell lines of *Medicago sativa* and *Nicotiana tabacum*, which showed a greater accumulation of chlorophylls under salt stress[32,33]. These authors postulate that these changes may be an indicative of physiological processes activation in chloroplasts against saline stress. Therefore, *O. streptacantha* cells could activate mechanisms such as chloroplast biogenesis, osmoprotectants synthesis pathways, detoxification, heat shock protein and late embryogenesis abundant proteins involved in chloroplast protection. Moreover, no significant changes in the chlorophyll content at

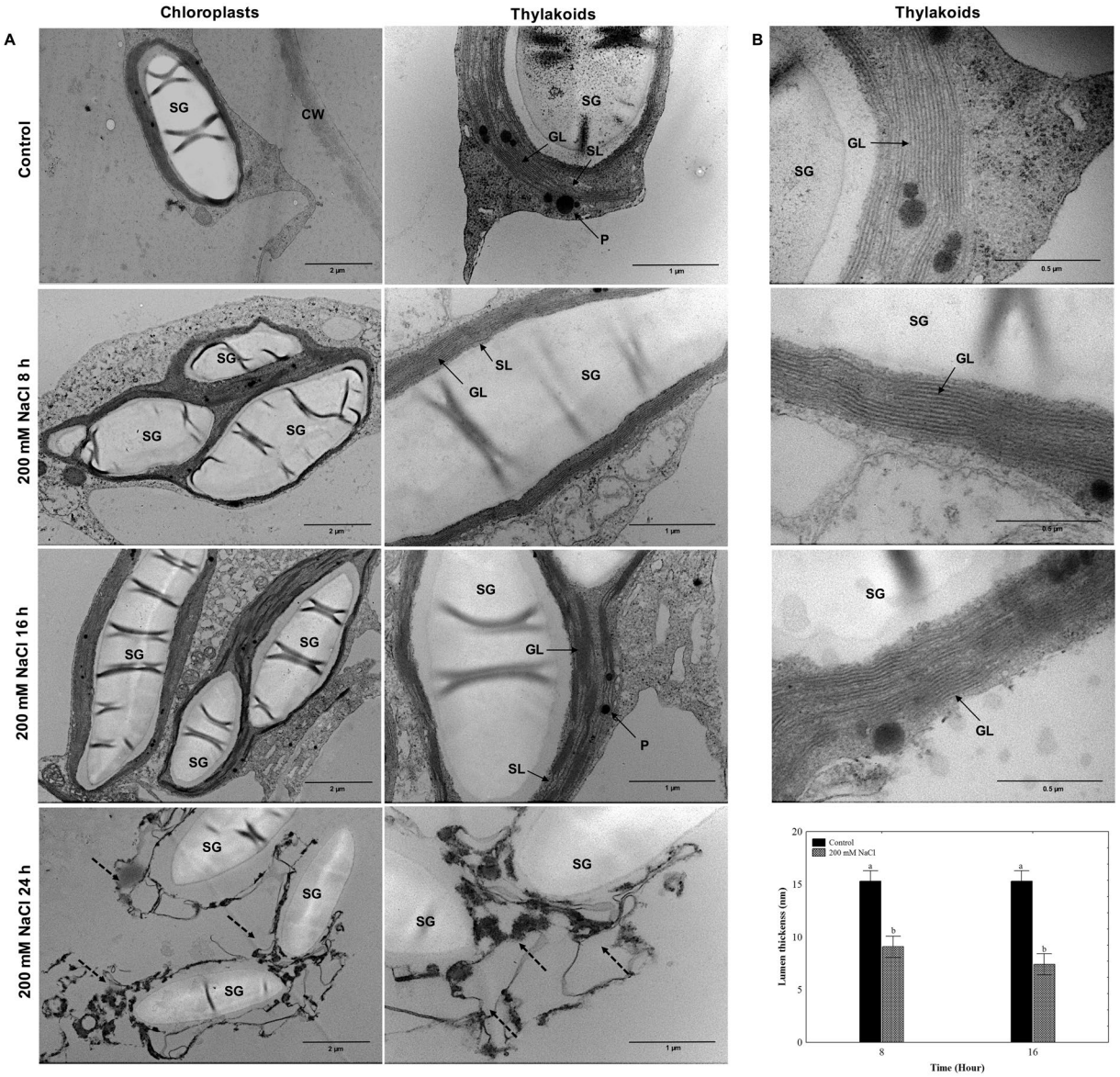

Figure 4. Ultrastructure of chloroplasts and thylakoid membranes of *Opuntia streptacantha* chlorenchyma cells under salt treatment. Representative TEM images of *O. streptacantha* chloroplasts are shown in each panel. (**A**) Cladode segments from *O. streptacantha* were incubated in 200 mM NaCl at 8, 16, and 24 h under white continuous light (300 μmol m^{-2} s^{-1}). The left panel displays individual chloroplast micrographs; the scale bar corresponds to 2 μm. In the right panel thylakoid membranes are shown; the scale bar corresponds to 1 μm. The black arrow dotted indicates the fragmentation of thylakoid membranes. (**B**) Ultrastructural analysis of thylakoid membranes of *O. streptacantha* chloroplasts under 200 mM NaCl at 8 and 16 h. The scale bar corresponds to 0.5 μm. The graph shows analysis of lumen thickness of thylakoid grana membranes within intact chloroplasts. Different letters indicate significant difference between treatments and time (hours) according to Duncan's multiple range tests at $P < 0.05$. SL, stroma lamella; GL, grana lamellae; SG, starch grain; P, plastoglobule; CW, cell wall.

16 h of NaCl treatments was observed, which could be a feature of acclimation by the re-establishing cellular ion homeostasis in cladode segments under salt stress.

We showed that salt stress causes photosynthesis damage, which in turn would provoke the clustering of chloroplasts like a mechanism of protection to prevent light to penetrate to deeper layers and then reduce the photo-oxidation. However, plants have additional mechanisms to protect themselves against damage by an excess of energy[34]. The reduction in the chlorophyll *a*/*b* ratio in cladode segments under the salt treatments showed in *O. streptacantha* can be interpreted as an enlargement in the light-harvesting complex II (LHCII) antenna of PSII. Thus, we suggest that the increase in antenna size could reduce the excitation energy from the antenna to RCs of PSII, or as a direct response of photosynthetic apparatus to salt stress. Similar results were presented by

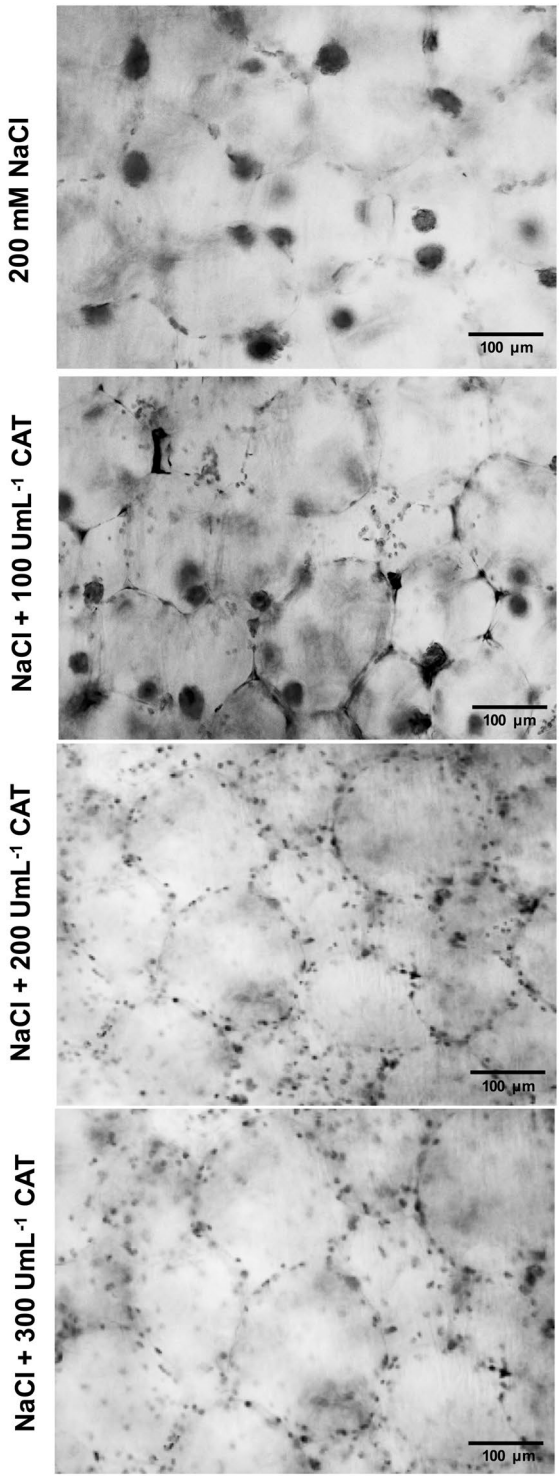

Figure 5. Chloroplast arrangement in chlorenchyma cells from *Opuntia streptacantha* under NaCl treatment supplemented with CAT. Representative images of *O. streptacantha* cells and their chloroplasts are shown in each panel. The cladode segments from *O. streptacantha* were incubated in 200 mM NaCl, 200 mM NaCl + 100 UmL^{-1} CAT, 200 mM NaCl + 200 UmL^{-1} CAT, and 200 mM NaCl + 300 UmL^{-1} CAT under white light conditions (300 µmol m^{-2} s^{-1}) for 8 h. Transverse sections from cladode were observed with a light microscope. Scale bar corresponds to 100 µm.

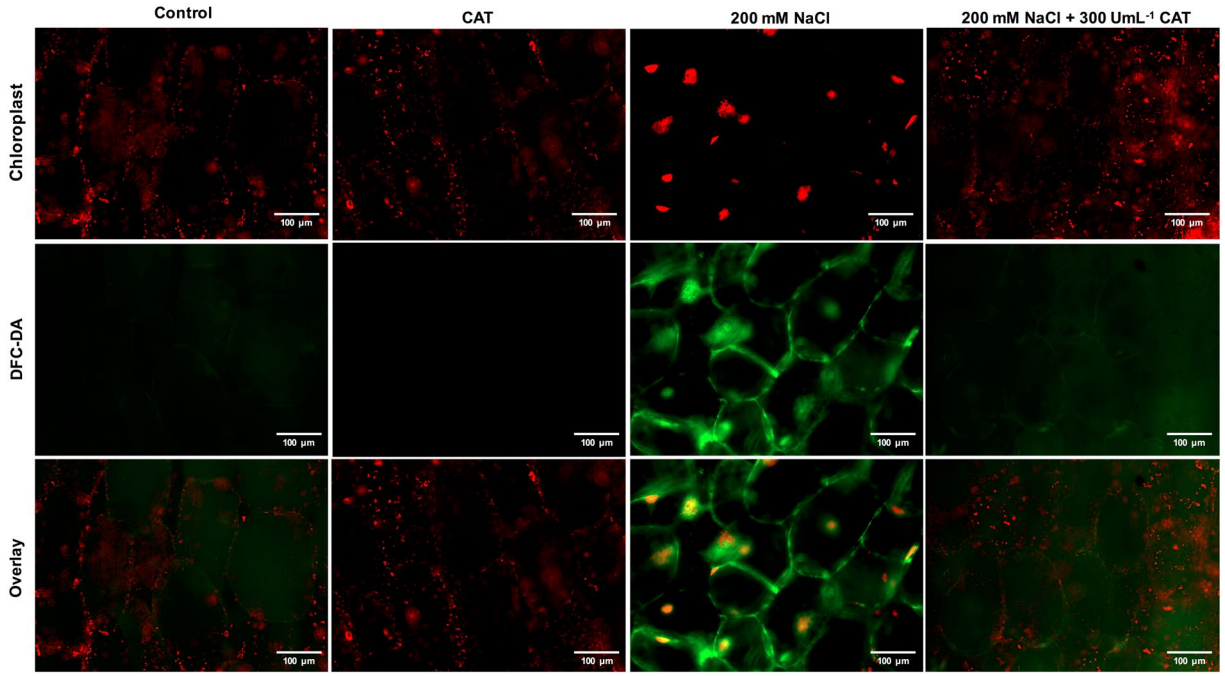

Figure 6. Effects of exogenous CAT application on H_2O_2 in the *Opuntia streptacantha* cells exposed to 200 mM NaCl. Representative images of *O. streptacantha* cells and their chloroplasts are shown in each panel. Segments of cladode were exposed to 200 mM NaCl, 200 mM NaCl + 300 UmL^{-1} CAT and as control 300 UmL^{-1} CAT under white light conditions (300 µmol $m^{-2} s^{-1}$) for 8 h. Consequently, all treatments were incubated with 25 µM 2′, 7′–dichlorofluorescein diacetate (DCF-DA). Changes of fluorescence intensity in cells was observed using an Epi-fluorescence microscope. Scale bar corresponds to 100 µm.

Demetriou *et al.*[35] who reported that salt stress triggered an increase in the photosynthetic effective antenna size in *Scenedesmus obliquus* under low light conditions.

Moreover, it has been demonstrated that a re-arrangement on antenna leads to the stacking of thylakoid membranes[36]. The ultrastructural analysis in *O. streptacantha* chloroplast exhibited that salt treatment induced thylakoid membranes stacking at 8 and 16 h. Dynamics in the stacking of the thylakoid membranes are essential for regulatory processes of the photosynthesis under different abiotic stress conditions[34]. The ability to control the lateral separation of PSI from PSII is considered a functional consequence of grana stacking to minimize the formation of ROS in the PSI through of the decrease of electron transport between photosystems[37,38]. Thus, our results suggest that changes of thylakoid membranes permit a balance in the excitation energy between the two photosystems[37]. These features can therefore be considered as a response of *O. streptacantha* cladodes to salt stress.

Instead, the ratio of chlorophyll to carotenoids at 24 h for the NaCl treatments was low, which indicate damage to the photosynthetic machinery. This result was consistent with the breakdown of chlorophyll caused by chloroplast damage with 200 mM NaCl at 24 h. Several studies have reported that salt stress induce the degradation of photosynthetic pigments in plants by the accumulation of toxic ions and ROS, changing the ultrastructure of the photosynthetic apparatus[16,39–43].

Although the chloroplasts could experience damage under salt stress, our results suggest that the clumping of chloroplasts plays an important role to minimize the excitation pressure (*1-qP*) on the photosynthetic machinery in *O. streptacantha* cladodes. The aggregative arrangement of chloroplasts in *O. streptacantha* seedlings also occurs under drought and high sunlight conditions[9]. Our research group has proposed that chloroplasts move towards the vacuole facilitating malate transportation; thus maintaining the photosynthetic activity under water deficit. The chloroplast-clumping phenomenon is a typical mechanism that has been observed in plants as a response to high solar radiation[11], salinity[10,39], drought[7,9,44], and low temperatures[8]. It has been proposed that the aggregation of chloroplasts may provide protection against photodamage and help to maintain the photosynthetic activity under stressful conditions[10,44,45]. Therefore, this phenomenon could be a common adaptive strategy used by plants for their survival under abiotic stress.

We also examined the effect of exogenous catalase (CAT) on chloroplasts clumping of *O. streptacantha* cladodes under NaCl treatments. Our data showed that CAT avoided the chloroplast clumping in chlorenchyma cells. Therefore, CAT generated an increase in the F_v/F_m, ΦPSII, *qP* values, and photosynthetic pigment levels in cladodes under salt treatments. Also, the cladode segments treated with CAT experienced less photo-inhibition under salt treatment than those without CAT. These results showed that the CAT application improved the photosynthesis under salt stress, counteracting for the clustering of chloroplasts. Similar photosynthesis protective mechanisms in plants under salt stress have been showed with exogenous application of non-enzymic antioxidants such as ascorbate (AsA)[46,47], glutathione (GSH)[48,49], and ∝-tocopherols[50]. It is known that enzymatic antioxidants such

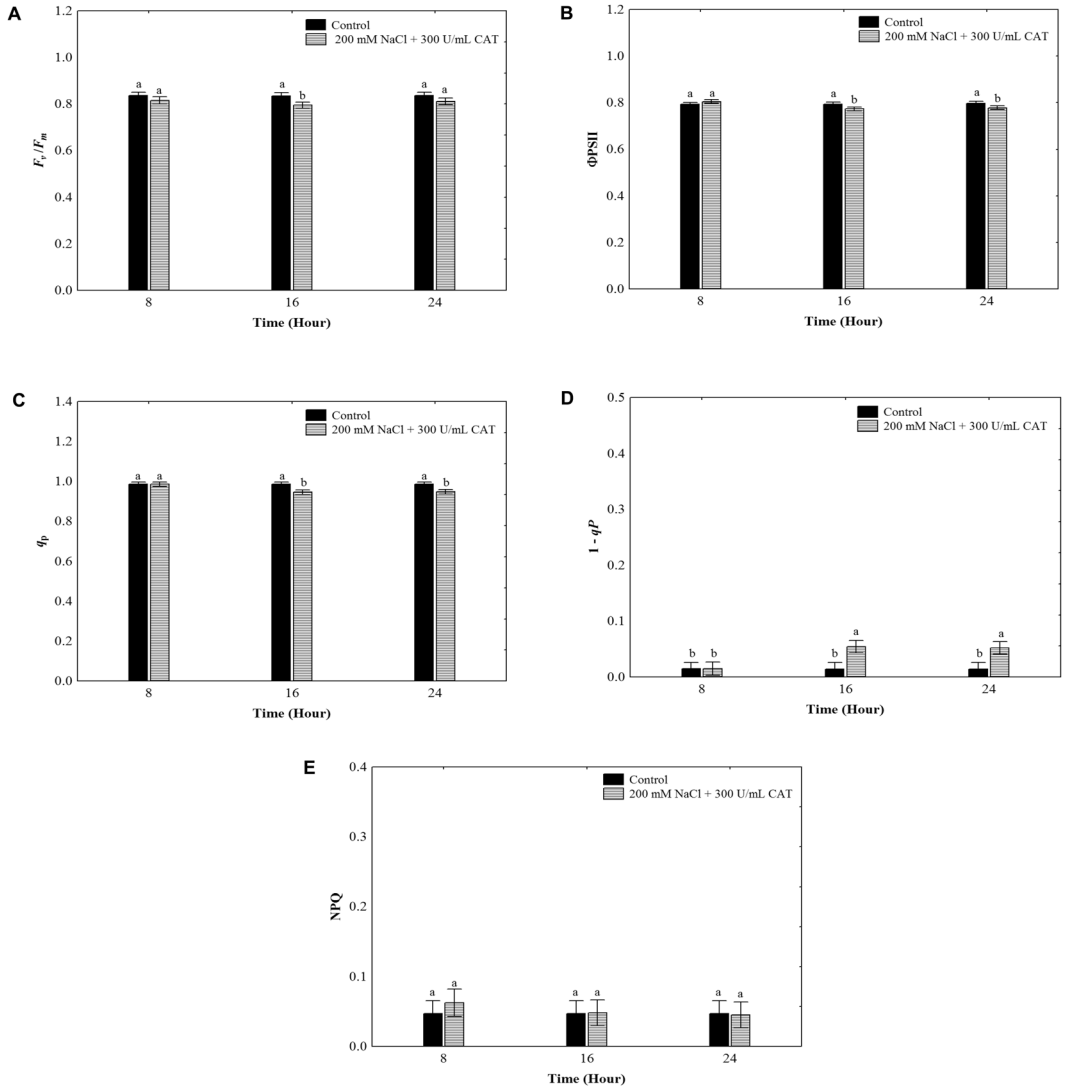

Figure 7. Effects of exogenous CAT application on chlorophyll fluorescence parameters in *Opuntia streptacantha* cladodes exposed to 200 mM NaCl. (**A**) The maximum quantum yield of PSII (F_V/F_m). (**B**) The effective photochemical quantum yield of PSII (ΦPSII). (**C**) The photochemical quenching (*qP*). (**D**) The excitation pressure (1–*qP*). (**E**) The non-photochemical fluorescence quenching (NPQ). Values are means and bars indicate ± SD, ($n = 9$). Different letters indicate significant difference between treatments and time (hours) according to Duncan's multiple range tests at $P < 0.05$.

as CAT provides a very energy-efficient mechanism to remove H_2O_2[51]. A high level of endogenous CAT is essential to maintain the antioxidant system that protects plants from oxidative damage due to various environmental stresses[52]. Thus, the exogenous CAT applied to cladode segments of *O. streptacantha* produced a protection against oxidative damage by H_2O_2 scavenging activity.

The apoplast is an important site for H_2O_2 production in acclimation response of plants to salinity[19,53,54]. It has been proposed that H_2O_2 accumulation in the apoplast could activate a signal for the chloroplast due to their location close to the plasma membrane. Then, the chloroplast may transmit the ROS signaling to the nucleus for the photosynthesis acclimation through nuclear gene expression[20]. Moreover, Wen and Zhang[55] reported that high blue light exposure can induce H_2O_2 generation in the plasma membrane, and that H_2O_2 is involved in chloroplast movements in *Arabidopsis thaliana*. Thus, we proposed that H_2O_2 generated by salt stress could be acting as a signaling molecule which promotes clumping of chloroplasts, particularly as an acclimation mechanism for mitigation of photo-inhibition in salt-stressed cladodes of *O. streptacantha*. However, the role of H_2O_2 in chloroplast movement is still poorly understood.

In higher plants, ROS and cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), are largely recognized to be important signaling messengers of many biological responses[56]. It is known that high NaCl concentrations, particularly under excess of chloride (Cl^-) ions in the cytoplasm, leads to an increase of $[Ca^{2+}]_{cyt}$ concentrations, which initiates the stress signal transduction pathways in plants under salt stress[57–59]. Furthermore, studies in Arabidopsis leaves reported that calcium is involved in the signal transduction for the movement of chloroplast in response to blue light[5,60–62].

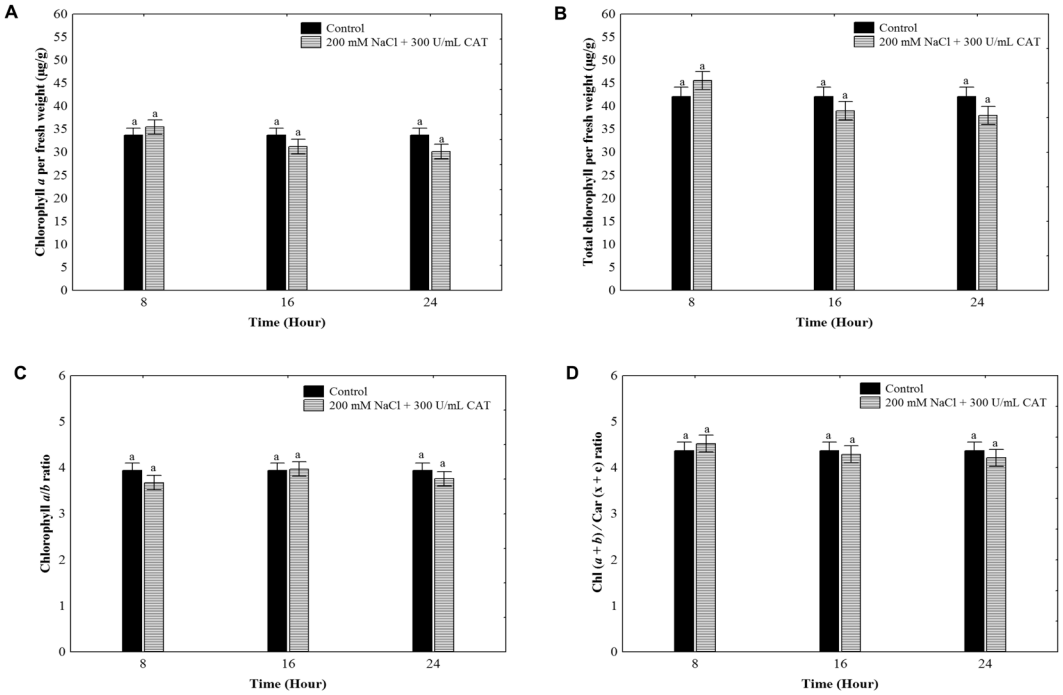

Figure 8. Effects of exogenous CAT application on chlorophyll and carotenoids content in *Opuntia streptacantha* cladodes exposed to 200 mM NaCl. (**A**) Chlorophyll *a*. (**B**) Total chlorophyll ($a+b$). (**C**) Chlorophyll a/b ratio. (**D**) Chl ($a+b$)/Car (x + c) ratio. Values are means and bars indicate ± SD, ($n=9$). Different letters indicate significant difference between treatments and time (hours) according to Duncan's multiple range tests at $P<0.05$.

The authors work shows that the stress by high blue light can increase $[Ca^{2+}]_{cyt}$, which may trigger the activity of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) to generate H_2O_2. In turn; the H_2O_2 generated may promote the chloroplast movements. Thus, it is possible that the clustering of chloroplast in cladodes segments can be regulated by internal Ca^{2+} stores, produced by salt stress. However, the cross-talk between Ca^{2+} and H_2O_2 in the regulation of chloroplast movements in *O. streptacantha* cells is an open question arising from this work that demands future research efforts. Finally, the physiological responses of CAM cacti to salinity are complex and the mechanisms underlying this phenomenon have not been completely elucidated, opening a variety of exciting new questions.

Conclusions

The chloroplasts are usually more sensitive to salinity than other organelles. Our findings show that salt stress causes photosynthesis damage and accumulation of H_2O_2 in the *O. streptacantha* cells. We suggest that H_2O_2 acts as a messenger molecule for the clustering of chloroplasts. The exogenous application of CAT alleviates salt-induced oxidative stress in *O. streptacantha* cladodes most likely through H_2O_2 scavenging activity. CAT activity avoids chloroplasts clustering and protects photosynthetic machinery function in salt-stressed *O. streptacantha*. In this regard, further studies about the effect of H_2O_2 in chloroplast clustering will help to understand the role of this phenomenon during stress.

Materials and Methods

Plant material. *Opuntia streptacantha* seeds were collected in 2007 by Delgado-Sánchez *et al.*[9] from Mexquitic de Carmona municipality San Luis Potosi, Mexico, (22°16′N, 101°07′W at 2,020 m asl). Seeds were sowed in LM-1 substrate (Lambert Professional Peat-Based. Québec, Canada) and watered daily until germination. After one month, the seedlings were transplanted in pots containing peat moss substrate (sunshine Mix # 3, Sungro Horticulture Canada Ltd. Agawam, USA). Pots were maintained under greenhouse conditions at $1059 \pm 5.5\,\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD) with 23 °C average air temperature, and watered every other day, during six months. Then, the plants were transferred to a growth chamber for one month at 25 °C under 16 h light ($300\,\mu mol\ m^{-2}\ s^{-1}$ PPFD) and 8 h dark cycle.

NaCl and Catalase treatments. Segments of cladode (5 × 8 × 0.3 mm) were hand-cut in transverse sections from *O. streptacantha* plants. For salt treatments, the cladode segments were completely immersed in NaCl solutions (NaCl dissolved in distilled water at final concentrations of 100, 200, and 300 mM) or on distilled water as control for 8, 16, and 24 h. NaCl concentrations were chosen based on previous studies performed in *Opuntia* species[28,30,63,64]. The assays were performed using three different cladodes of *O. streptacantha* plants (biological

replicates) and we take three segments of cladode per plant (technical replicates) for each treatment, under continuous white light (300 μmol $m^{-2} s^{-1}$) at 25 °C.

For CAT mixed with NaCl solution treatments, the CAT stock solution was prepared by pre-dissolving 1 mg of CAT (Sigma-Aldrich Ref: C1345) in 1 mL 50 mM potassium phosphate buffer pH 7.0. The CAT solutions at final concentrations of 100, 200, and 300 UmL^{-1} respectively, were added to 200 mM NaCl solution. Subsequently, the cladode segments were completely immersed in these mixtures (NaCl + CAT) for 8, 16, and 24 h. As control, we used distilled water without NaCl or CAT. The CAT concentration was selected according to Aroca[65]. The assays were performed using three different cladodes of *O. streptacantha* plants (biological replicates) and we take three segments of cladode per plant (technical replicates) for each treatment, under continuous white light (300 μmol $m^{-2} s^{-1}$) at 25 °C.

Chlorophyll fluorescence measurements. The chlorophyll fluorescence analysis is a powerful technique that allows to obtain detailed information about of the process of photosynthesis in plants[66] and can also be applied to know how plants respond to abiotic stress factors[67]. Cladode segments of 10 mm diameter were removed from *O. streptacantha* plants using forceps and scalp. Subsequently, these were subjected to the aforementioned treatments. Chlorophyll fluorescence was measured using a MINI-PAM II fluorometer (H. Walz, Effeltrich, Germany) following the manufacturer's instructions. The chlorophyll fluorescence measurements were realized using a pulse the actinic light with an intensity of 820 μmol $m^{-2} s^{-1}$. The maximum quantum yield of photosystem II (F_v/F_m) was determined after dark adaptation of cladodes segments for 30 min. The F_v/F_m values were calculated as described by Kitajima and Butler[68]. On the other hand, light-adapted cladodes segments were used to measure the fluorescence parameters as follows: the effective photochemical quantum yield of PSII was calculated using the equation: $\Phi PSII = (F_m' - F)/F_m' = \Delta F/F_m'$ presented by Genty *et al.*[69]. The photochemical quenching was calculated as $qP = (F_m' - F)/(F_m' - F_O')$ and used to determine the fraction of closed (reduced) PSII reaction centers, also known as excitation pressure, and calculated as $1–qP$. The non-photochemical fluorescence quenching, $NPQ = (F_m - F_m')/F_m'$ was determined according to Bilger and Björkman[70]. The photosynthetic electron transport rate (ETR) was estimated with the following equation $ETR = \Phi PSII \times PPFD \times 0.5 \times 0.84$, where PPFD is photosynthetic photon flux density, the factor 0.5 assumes that photosystems II and I are similarly excited by the irradiance. The factor 0.84 considers that only 84% incident irradiance will be absorbed by the two photosystems[71]. The assays were performed using three different cladodes of *O. streptacantha* plants (biological replicates) and we take three segments of cladode per plant (technical replicates) for each treatment ($n = 9$).

Determination of chlorophylls and carotenoids content. The chlorophyll (*a* and *b*) and carotenoids extraction was performed according to the methodology reported by Lichtenthaler[72,73]. The cladode chlorenchyma fresh segments were homogenized in 80% acetone and incubated in dark at 4 °C for 5 min. Subsequently, it was centrifuged at 13,000 rpm at 4 °C for 5 min. The chlorophyll and carotenoids contents were measured in 200 μL supernatant using a microplate reader (Epoch 2, Biotek, Winooski, VT, United States of America) at 663 (chlorophyll *a*), 646 (chlorophyll *b*), and 470 (carotenoids) nm wavelengths. The assays were performed using three different cladodes of *O. streptacantha* plants (biological replicates) and we take three segments of cladode per plant (technical replicates) for each treatment ($n = 9$). The pigments content, the ratio of chlorophyll *a* (Chl *a*) to chlorophyll *b* (Chl *b*) (Chl *a*/*b*), and Chl *a* and Chl *b* to total carotenoids $(a + b)/(x + c)$ ratio were estimated using the equations proposed by Lichtenthaler[73]:

$$\begin{aligned} Chl\ a &= (12.25 \times A_{663}) - (2.79 \times A_{646}) \\ Chl\ b &= (21.50 \times A_{646}) - (5.10 \times A_{663}) \\ Chl\ a + Chl\ b &= (7.15 \times A_{663}) + (18.71 \times A_{646}) \\ Carotenoids &= \frac{(1000 \times A_{470}) - (1.82 \times chl\ a) - (85.02 \times chl\ b)}{198} \end{aligned}$$

Microscope analyses. To analyze the chloroplast arrangement on *O. streptacantha* cells, segments of cladode (5 × 8 × 0.3 mm) subjected to the treatments described below were observed without fixation under a light microscope Leica DM2000 (Leica, Wetzlar, Germany). The photographs were obtained and digitized with LAS Imaging Software (Leica). For the transmission electron microscope (TEM), the segments of cladodes were treated with a fixative solution (10% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4) during overnight at 4 °C. Then, they were washed using Sorensen's phosphate buffer and dehydrated with ethanol; subsequently, the samples were polymerized in a fresh resin at 60 °C as described by Cocoletzi *et al.*[74]. Ultrathin sections were contrasted using aqueous uranyl acetate (2% w/v) and aqueous lead citrate (2% w/v). Samples were examined with TEM JEOL 200CX (JEOL, Welwyn Garden City, UK) using a 100 kV acceleration voltage.

The ultrastructural analysis of chloroplasts to determine the thickness of the thylakoid lumen was realized using the methodology reported by Kirchhoff *et al.*[38]. The distances of stacking repeat unit (*R*), which includes the two thylakoid membrane bilayers (*M*) and the widths of one partition gap (*P*) were realized with Image J software[75]. Subsequent, the thickness of the thylakoid lumen (*L*) were estimated using the equations proposed by Kirchhoff *et al.*[38]: $L = R - M - P$. The measurements of lumen thickness were made on grana lamellae (GL) using three separate micrographs and we take three grana lamellae per micrographs for each treatment ($n = 9$).

Epi-fluorescence microscope for H_2O_2 images. For observer H_2O_2 signals, segments of cladode (5 × 8 × 0.3 mm) subjected to the treatments described below were transferred to 25 μM 2′, 7′–dichlorofluorescein diacetate (DCF-DA) (Sigma-Aldrich Ref: C1345) dissolved in 10 mM Tris-HCl, 50 mM KCl (pH 7.2) and incubated in dark for 30 min at 30 °C. Subsequent, the segments were washed twice with 10 mM Tris-HCl, 50 mM

KCl (pH 6.1) for 30 min, images were visualized using an Epi-fluorescence microscope (Axio Imager M2; Carl Zeiss Microscopy, LLC, USA). The assays were performed using three different cladodes of *O. streptacantha* plants (biological replicates) and we take three segments of cladode per plant (technical replicates) for each treatment ($n = 9$).

Data analysis. All data obtained from chlorophyll fluorescence parameters and pigments content were statistically analyzed with STATISTICA version 7 software[76], using Duncan's multiple range test at the $P < 0.05$ level of significance between treatments and time (hours). The results are expressed as mean values ± SD (standard deviation) ($n = 9$).

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Author Contributions

D.M.A.M., J.F.J.B., I.M.L. and P.D.S. conceived and designed research. P.D.S. and J.F.J.B. contributed with reagents, materials, and analysis tools. D.M.A.M. and I.M.L. conducted the experiments and analyzed the data. D.M.A.M., J.F.J.B., I.M.L. and P.D.S. contributed to the data interpretation and manuscript preparation. All authors read and approved the manuscript.

Additional Information

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