Physiological and biochemical responses of *Scytonema javanicum* (cyanobacterium) to salt stress

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Abstract

*Scytonema javanicum* (Kutz.) Born et Flah (cyanobacterium) is one of the species distributed widely in the crust of desert soils regularly subjected to severe water stress. To investigate the response of the species to salt stress, many physiological and biochemical parameters, including growth rate, ratio of variable fluorescence to maximum fluorescence ($F_{v}/F_{m}$), reactive oxidative species (ROS), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD), were determined in culture. The results showed that 50 mM NaCl inhibited growth and $F_{v}/F_{m}$ in the medium BG-110, and that the inhibition was maximum after 1–2 days' exposure to salt stress; 50 mM NaCl also increased the contents of ROS and MDA in treated cells, which suggests that salt stress may lead to oxidative damage and lipid peroxidation in the alga. Further, changes in the antioxidative enzymes SOD and CAT in the treated alga were consistent with changes in ROS and MDA at certain extent. These observations suggest that oxidative stress resulting from salt stress in *S. javanicum* could result in the production of antioxidative enzymes to counteract the oxidative damage, and the enzymes may contribute to the ability of *S. javanicum* to survive the adverse desert environment.

Keywords: Antioxidative enzymes; Lipid peroxidation; Oxidative damage; Photosynthetic efficiency; Salt stress; *Scytonema javanicum*

1. Introduction

Excessive soil salinity usually results from natural processes or from irrigation with saline water. Salinization is a major cause of soil degradation and affects 19.5% of irrigated agricultural land and 2.1% of dry land worldwide (FAO, 2000). Effects of salinity are more conspicuous in arid and semi-arid regions, which are characterized by limited rainfall, high evapotranspiration, and high temperature. When a plant is exposed to high salinity, its major processes such as photosynthesis, protein synthesis, and energy and lipid metabolism are affected. Crucial changes in ion and water homeostasis caused by high concentrations of salts lead to damage at the molecular level, arrested growth, and even death (Tijen and Ismail, 2006).
Cyanobacteria are photosynthetic oxygen-evolving prokaryotes that have successfully colonized a wide range of biotopes in their long evolutionary history as a result of their remarkable capacity to develop elaborate protective mechanisms and adapt to environmental changes (Whitton and Potts, 2000). In general, cyanobacteria are considerably tolerant to salt stress and, therefore, have been used in reclaiming saline and sodic lands (Singh, 1950; Thomas and Apte, 1984). The extant cyanobacteria are suitable models for studying the physiology of salt tolerance and the molecular mechanism of similar processes in plants (Paula et al., 2005).

Scytonema javanicum (Scytonemataceae) is a filamentous cyanobacterium, which adopts several strategies to survive in adverse biotopes. These strategies include (1) increasing the water-holding capacity (evapotranspiration from the soil surface encrusted with Scytonema was only about 0.05% of that from a surface without such crust (Metting, 1981); (2) forming heterocysts and fixing atmospheric nitrogen, both of which are important in crust formation and maintenance of productivity; and (3) producing scytonemin, a predominantly UV-A-photoprotective pigment (Proteau et al., 1993).

As part of our effort to explore algal crusts as potential tools in combating desertification, we attempted to evaluate the effects of salt stress on the desert-dwelling cyanobacterium S. javanicum. The results might help us to better understand the physiological and biochemical mechanisms of salt tolerance, to learn more about the formation and colonization of natural or man-made microalgal crusts, and also to obtain some leads on ameliorating desert environments.

2. Materials and methods

2.1. Cultures and materials

Scytonema javanicum (Kutz.) Born et Flah FACHB-887 was originally isolated and purified from the desert algal crust in Shapotou, Zhongwei County, Ningxia Autonomous Region, China (37°27′ N, 104°57′ E) by the authors’ research group, and maintained as one of the accessions (No. 887) at the Freshwater Algae Collection of the Institute of Hydrobiology (FACHB), the Chinese Academy of Sciences. The inoculum was gently mixed in a glass homogenizer and grown in BG-110 medium (Rippka et al., 1979) at 25 ± 1°C. The cultures were illuminated with cool white fluorescent light at 40 μmol m⁻² s⁻¹ and aerated with humidified air. All containers were sealed with cotton plugs to prevent contamination. After 12 days, cells were harvested by centrifugation, transferred to a fresh medium, and grown under the same conditions for another 2 days; NaCl was then added to the salt stress group to give a final concentration of 50 mM, and the cultures were sampled every 24 h by removing 45 ml of the culture each time. Each 45 ml sample was divided as follows: 5 ml for chlorophyll a, 2 ml for chlorophyll fluorescence, and the rest for total proteins, reactive oxidative species (ROS), malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD). Each subsample was centrifuged at 8000 rpm for 10 min at 4°C, the pellets were washed three times with sterilized water, and ground to a slurry with a mortar and pestle with 2 ml of phosphate buffer (pH 7.8, containing 4 mmol/L EDTA-Na₂ and 0.4% PVP) at 4°C. The homogenate was centrifuged again at 8000 rpm for 20 min at 4°C and the supernatant stored at 4°C until required. All the experiments were repeated three times.

2.2. Determination of chlorophyll a, protein content, and chlorophyll fluorescence

Chlorophyll a was extracted in 95% ethanol and characterized by the method of Wintermans and de Mots (1965). Protein content was determined by following the method described by Bradford (1976), using bovine serum albumin as a standard. Fluorescence of chlorophyll a was measured in vivo with a Phyto-Pam fluorometer (Heinz Walz, Germany).

2.3. Analysis of reactive oxidative species (ROS) and malondialdehyde (MDA)

Kits for assay of ROS were purchased from Jiancheng Bioengineering Institute, Nanjing, China. Since the Fenton reaction is a common chemical reaction producing OH⁻, the amount of H₂O₂ is directly proportional to the amount of OH⁻ produced. When given an electron acceptor and stained with Gress, production of red
color is proportional to OH\textsuperscript{-}, which (expressed as H\textsubscript{2}O\textsubscript{2}) was employed as an indicator of ROS. One unit of ROS was defined as that which reduced the concentration of H\textsubscript{2}O\textsubscript{2} in the reaction system by 1 mM in 1 min at 25 °C, and the results were expressed as U mg\textsuperscript{-1} protein.

The content of MDA was determined using the thiobarbituric acid reaction, following the method of Madhava Rao and Sresty (2000), and calculated from the absorbance at 532 nm. The measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm, and the results expressed as \(\mu\)mol g\textsuperscript{-1} protein.

2.4. Assay of catalase (CAT) and superoxide dismutase (SOD) activity

Catalase (EC 1.11.1.6) activity was detected by a method that was a slight modification of the method of Cakmak and Marschner (1992). The reaction mixture, in a total volume of 3 mL, contained 1.5 mL of 50 mM sodium phosphate buffer (pH 7.0) and 1 mL of 0.2% H\textsubscript{2}O\textsubscript{2}. The reaction was initiated by adding 0.5 mL of the enzyme extract, and the activity determined by measuring the initial rate of disappearance of H\textsubscript{2}O\textsubscript{2} at 240 nm.

Superoxide dismutase (EC 1.15.1.1) activity was estimated spectrophotometrically as the inhibition of photochemical reduction of NBT at 560 nm, a minor modification of the method of Beauchamp and Fridovich (1971). The reaction mixture consisted of 0.3 mL each of 0.75 mM NBT, 130 mM methionine, 0.1 mM EDTA-Na\textsubscript{2}, 0.02 mM Riboflavin, and sterilized water and 1 mL of 50 mM Na-phosphate buffer (pH 7.8). The reaction was initiated by adding 0.5 mL of the enzyme extract and carried out for 20 min at 25 °C under a light intensity of about 300 \(\mu\)mol m\textsuperscript{-2} s\textsuperscript{-1}. One unit of SOD was defined as that which inhibited photoreduction of NBT to 50%.

The results of CAT and SOD assays were expressed as U mg\textsuperscript{-1} protein.

2.5. Data analysis

All data were evaluated by one-way ANOVA using SPSS 6.0.1 for Windows as well as the least significance difference and Tukey’s honestly significant difference tests.

3. Results

3.1. Effect on growth

Chlorophyll \(a\) content was taken as a measure of the growth of \(S. javanicum\), and Fig. 1 shows the dose-dependent effect of NaCl treatment on that growth. Up to Day 2, 50 mM NaCl had almost no effect; the inhibitory effect started from Day 3. On Day 6, the content in the treated group increased to 2.5 times the initial value, but was only 57% of that in the untreated group. Treatment with 100 mM NaCl inhibited growth markedly from Day 1: chlorophyll \(a\) content had decreased by 13% of the initial value on Day 2, by 47% on Day 4, and by 70% on Day 6. Treatment with 200 mM NaCl killed the alga quickly; transferring it to fresh, normal BG-11\textsubscript{0} medium could not revive it (data not shown). Based on these observations, the subsequent experiments were confined to using 50 mM NaCl as the stress condition.

3.2. Changes in the ratio of variable fluorescence to maximum fluorescence (\(Fv/Fm\))

On Day 1, 50 mM NaCl had no apparent effect on the \(Fv/Fm\) ratio (Fig. 2); however, the ratio began to fall after Day 2 and, by Day 6, had been reduced by 28.3% of that in untreated cells.

3.3. Changes in intracellular reactive oxidative species (ROS) and malondialdehyde (MDA)

In cells treated with 50 mM NaCl, ROS content increased sharply in the first 2 days, but then began to decline slowly: in the untreated group, the content increased sharply on Day 3, only to decrease sharply the next day. Throughout the experiment, ROS contents in the treated cells were significantly higher than those in the untreated group except on Day 3 (Fig. 3).
The level of lipid peroxidation was determined in terms of MDA content. As shown in Fig. 4, MDA content of the treated cells increased linearly in the first 2 days; by Day 6, it was 155% of the initial level. The trend was consistent with that of ROS, especially during the first 3 days. The MDA content of untreated cells increased slowly during the first 3 days and decreased slightly over the next 3 days, the final level being close to the initial value. Throughout the experiment, the MDA content was higher in the treated group (Fig. 4).

### 3.4. Changes in superoxide dismutase (SOD) and catalase (CAT)

In the treated group, SOD activity increased linearly over the first 2 days, only to decrease likewise on the following day: in the untreated group, SOD content changed in a wave-like fashion during the first 3 days and
remained relatively stable later (Fig. 5). On Day 2, the level of SOD in treated cells was more than 4 times that in untreated cells; however, over the last 3 days, the trend and the values in both groups were similar (Fig. 5).

Fig. 6 shows changes in the activity of another antioxidative enzyme, namely CAT. In the treated group, the activity increased during the first 2 days and decreased over the following 2 days: in the untreated group, the activity increased on Day 1 and then decreased, reaching the lowest point on Day 3. Catalase contents in the treated group were significantly higher than those in the untreated group on Day 2, Day 3, and Day 5.

4. Discussion

4.1. Salt stress, growth, and photosynthetic efficiency

Salt stress is known to be one of the most important abiotic stresses and seriously affects crop productivity and survival. The deleterious effects of excessive salinity on plant growth are associated with (1) low osmotic
potential of soil solution (water stress), which reduces the availability of water to plants, (2) nutritional imbalance, (3) effect on specific ions (salt stress), and (4) a combination of all the three factors (Ashraf, 1994; Marschner, 1995). All of these cause adverse pleiotropic effects on plant growth and development at physiological and biochemical levels (Gorham et al., 1985; Levitt, 1980; Munns, 2002). According to Hagemann and Erdmann (1997) and Reed and Stewart (1988), the desert cyanobacterium \textit{S. javanicum} is a species sensitive to NaCl. Fig. 1 shows that 50 mM NaCl inhibited the growth of the alga but the inhibitory effect lasted only 2 days, pointing to a degree of resistance to salt stress in the species—higher NaCl concentrations (>100 mM), however, proved lethal.

The ratio of variable fluorescence to maximum fluorescence ($F_v/F_m$) of the dark-adapted chlorophyll $a$ indicates the extent and functional consequences of changes in the maximum photochemical efficiency of Photosystem II (PS II) reaction centers (Franklin et al., 1992; Krause, 1988) and is used as an index of photosynthetic efficiency. In this work, $F_v/F_m$ ratio of the alga was found to be significantly decreased by salt stress from the second day of the treatment (Fig. 2), a decrease indicative of the decline in photosynthetic efficiency, which partly explains the mechanism of the inhibitory effect of salt stress on the growth of \textit{S. javanicum}. The mechanism by which salt stress reduces photosynthetic efficiency includes the peroxidation of lipids in the thylakoids, the damage to PS II complex (see below), and the decrease in overall activity of the

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**Fig. 5.** Course of SOD activity in \textit{S. javanicum} under salt stress. Data are represented as mean ± SE ($n = 3$). Within 2–3 days, SOD activity in the treated alga was significantly different from that in the untreated group ($p<0.05$).

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**Fig. 6.** Course of CAT activity in \textit{S. javanicum} under salt stress. Data are represented as mean ± SE ($n = 3$). On Day 1, Day 2, Day 3, and Day 5 of exposure, CAT activity in the treated alga was significantly different from that in the untreated group ($p<0.05$).
electron transport chain of PS II (Lu and Vonshak, 1999). Salt stress may damage the PS II electron transport chain as demonstrated in *Spirulina platensis* (cyanobacterium) (Lu and Vonshak, 1999); damage of this kind cannot be restored by diphenyl carbazole, an artificial electron donor to PS II. However, it has also been reported that salt stress does not accelerate damage to PS II directly but inhibits the repair of photo-damaged PS II in the cyanobacterium *Synechocystis* sp. PCC 6803 (Allakhverdiev et al., 2002).

### 4.2. Salt stress and oxidative damage

Salt stress, like many other abiotic stresses, may also lead to oxidative stress through the increase of ROS such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^\cdot$) (Neill et al., 2002). These ROS are highly reactive and can alter normal cellular metabolism through oxidative damage to lipids, proteins, and nucleic acids (Imlay, 2003). Lipids have vital roles in the tolerance to several physiological stressors in a variety of organisms including cyanobacteria (Asish and Anath, 2005). In cyanobacteria, lipids present in the thylakoids contain a high percentage of polyunsaturated fatty acid (PUFA) residues and are thus susceptible to peroxidation (Halliwell and Gutteridge, 1999). The content of MDA, a product of lipid peroxidation, has been considered an indicator of oxidative damage (Meloni et al., 2003; Shalata et al., 2001).

In the present work, the elevated levels of ROS (Fig. 3) and MDA (Fig. 4) in the treated group point to oxidative stress, which might cause cellular damage. Figs. 3 and 4 also show a synchronous trend between changes in ROS and those in MDA, a trend that suggests that oxidative stress is a consequence of the deterioration of lipid peroxidation (indicated by MDA) brought about by ROS. Smirnov (1993) suggested that ROS may directly damage such cellular components as the PS II complex and membrane lipids. The observation that ROS content of untreated cells was as high as that in treated cells on Day 3 may be attributed to the high photosynthetic efficiency in the exponential growth phase: increased photosynthesis leads to high concentration of internal O$_2$, which stimulates the generation of activated oxygen species (Asada and Takahashi, 1987; Steiger et al., 1977).

Besides salt stress, some other stress conditions may also cause oxidative damage in cyanobacteria. For example, it has been reported that UV-B can induce increased levels of ROS and MDA in the cyanobacterium *Anabaena* sp. (He and Hader, 2002). Microgravity has also been reported to induce increased levels of ROS in *Anabaena* sp. PCC 7120 (Li et al., 2004). Similar responses to salt stress have also been observed in higher plants, e.g. maize (André Dias et al., 2005).

### 4.3. Oxidative damage and antioxidative enzyme

To mitigate the oxidative damage caused by ROS, plants have developed a complex antioxidative system, including antioxidants of low molecular mass as well as such antioxidative enzymes as SOD and CAT (Noctor and Foyer, 1998). Superoxide dismutase is a major O$_2^-$ scavenger and its action results in H$_2$O$_2$ and O$_2$; the H$_2$O$_2$ produced is then scavenged by CAT, which splits H$_2$O$_2$ into H$_2$O and O$_2$ (McKersie and Leshem, 1994). In the present study, the changes in SOD contents of both untreated and treated groups were consistent with the changes in MDA and ROS, especially during the first 3 days. This suggests that increased levels of ROS not only result in lipid peroxidation but also stimulate production of the antioxidative enzyme SOD. In the treated group, levels of both SOD and CAT remained high, although the extent of variation in SOD content was more than that in CAT. In untreated group, SOD content was the highest on Day 3 whereas CAT content declined to its lowest point at the same time, suggesting that the two antioxidative enzymes act synergistically to resist oxidative damage induced by salt stress in *S. javanicum*. However, SOD offers a more sensitive and effective intrinsic defense than CAT, which serves as a potential antioxidant. Similar patterns of matching changes in CAT and SOD were also observed in other plant tissues under NaCl stress (Mittal and Dubey, 1991).

*Scytonema javanicum* is one of the dominant species that inhabit the crust of desert soils and plays an important role in the desert ecosystem. Some of the attributes of the species, such as heterocysts (for nitrogen fixation) and scytonemin (for protection from UV), may greatly enhance its significance in desert areas. This work studied the effects of salt stress on the growth of *S. javanicum* and on the relationship of some
physiological and biochemical indexes (ROS, \(Fv/Fm\), MDA, SOD, and CAT), which may throw some light on the mechanism of tolerance to salt stress.

At lower concentration (< 50 mM), NaCl inhibited the growth of \(S. javanicum\); at higher concentrations (> 100 mM), it killed the plant quickly. Salt stress also induced increase in the content of ROS, which resulted in higher levels of MDA, an important index of lipid peroxidation. Damage to lipids, especially those in the thylakoids of cyanobacteria, has a negative influence on photosynthetic activity (Havaux and Niyogi, 1999), leading to decreased \(Fv/Fm\) ratio and slower growth. On the other hand, algae have evolved adaptive ways to combat environmental stresses, adjusting the antioxidative enzymes being one such adaptive mechanism. In this study, high values of ROS, MDA, SOD, and CAT were observed in the treated groups during the first 2 or 3 days: ROS and SOD remained at relatively high levels whereas MDA continued to increase. Throughout the experiment, changes in ROS and SOD were synchronous to some extent, which may imply that SOD plays a more important role than CAT in scavenging ROS in \(S. javanicum\). It is likely that oxidative stress in cells depends mainly on maintaining a balance between ROS content and the antioxidative system—a strategy that may be an adaptive response that protects the alga from more extensive and irreversible oxidative damage.

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