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PHYSICOCHEMICAL PROPERTIES OF AN ASEXUAL *EPICHLOË* ENDOPHYTE-MODIFIED WILD BARLEY IN THE PRESENCE OF SALT STRESS

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Abstract

Endophyte-infected (E+) and endophyte-free (E–) wild barley (*Hordeum brevisubulatum*) tillers were grown in half-strength Hoagland nutrient solution using a hydroponic method. Two weeks later, salt stress treatment was initiated with 200 mM sodium chloride for six days. Relevant physiological indicators were then determined. The results showed that the levels of both chlorophyll a/b and carotenoids were significantly higher in plants with endophytic fungi than in those without endophytes. Peroxidase, catalase and superoxide dismutase activities in the endophyte-infected plants were significantly higher than those in endophyte-free plants. The E+ wild barley plants had higher relative water content than did the E– wild barley plants. The contents of proline and soluble sugars in E+ wild barley increased significantly. Under salt stress conditions, the endophyte infection significantly alleviated the stress by up-regulating photosynthetic pigments, the relative water content, proline, and soluble sugar contents compared to those of the E– plants. In conclusion, the asexual *Epichloë* endophyte significantly reprogrammed the physicochemical properties of the host plants during treatment with 200 mM NaCl.

Key words: Salt stress, Endophyte, Antioxidative enzymes, Osmotic adjustment.

Introduction

Excessive amounts of salt constitute one of the principal physiological dangers to biological communities and result in significant monetary losses in agrarian societies worldwide (Shrivastava & Kumar, 2015). Large amounts of salt cause hyperosmolarity, ion disequilibrium, nutrient imbalance, and reactive oxygen species (ROS) production (Munns, 2008; Nawaz *et al.,* 2010). ROS, namely, superoxide radicals ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·), cause oxidative damage to membrane lipids, proteins and nucleic acids (Casano *et al.,* 1994; Hagar *et al.,* 1996; Alscher *et al.,* 1997; Imlay, 2003). ROS produced from molecular oxygen can accumulate in the leaves (Ahmed *et al.,* 2009), and their overproduction in plant cells reduces plant growth. Hence, ROS detoxification forms an important defence against abiotic stresses (Amrit *et al.,* 2010). Plants have several antioxidant enzyme systems and non-enzymatic antioxidants against ROS accumulation. The detoxifying enzymes include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) as well as those of the ascorbate-glutathione cycle. SOD catalyses the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen. Hydrogen peroxide can be detoxified by CAT or POD to produce water and molecular oxygen (Zhu *et al.,* 2004). Non-enzymatic antioxidants employed by plants include ascorbate, glutathione, α-tocopherols, flavonoids, carotenoids, etc. (Ahmed *et al.,* 2009).

Osmotic adjustment is a key factor in plant responses to stressful environments. One way plants regulate salt stress is to synthesize and accumulate organic compounds such as glycinebetaine, proline, sugars and polyols (Szabados & Savouré, 2010; Iqbal *et al.,* 2014; Per *et al.,* 2017). Proline is a low-molecular weight cyclic amino acid and one of the solutes that has been shown to provide osmotic adjustments in plants under stressful environments (Agami, 2014; Kaur &Asthir, 2015). Other compatible compounds such as sugars can be released from starch in response to stress. Soluble sugars can also be used as typical osmoprotectants (Okunlola *et al.,* 2016).

To enhance salt resistance, plants have evolved different strategies that enable them to withstand salt intrusion. One of these strategies is improving the host plant's adaptation via symbiotic interactions with microorganisms. It is believed that endophytic fungi play an important role in plant acclimation of environmental stress (Rodriguez *et al.,* 2008). Many grasses are parasitic in the *Epichloë* genus, whose abiogenetic types had been previously known as *Neotyphodium* (Leuchtmann *et al.,* 2014). The studies have demonstrated the ability of fungal endophytes to mitigate plant stress caused by excess salt (Khan *et al.,* 2011; Waqas *et al.,* 2012). The response of plants to salinity has been studied for many years. However, the physicochemical properties that confer sodium chloride stress in symbionts of wild barley (*Hordeum brevisubulatum*) hydroculture are still poorly understood. Thus, the goal of this work was to better understand the physiology and mitigation of NaCl stress within wild barley due to the association of this species with endophytes. The goal of this study was to determine the effects of endophytes on the activity of antioxidant enzymes, the relative water content, osmotic adjustment and the photosynthetic pigment content in wild barley leaves in the presence of 200 mM NaCl.

Materials and Methods

Plant material and experimental design: Endophyte-free (E–) individuals were obtained from genotypes that were originally infected with endophytes (E+) in 2012

after treating the E+ ramets with a fungicide (thiophanate methyl, Lanzhou City, China). The seeds of wild barley were disinfected with 0.3% (w/v) sodium hypochlorite for 5 min. Seed germination was performed as described previously (Song *et al.,* 2010). Seedlings were then transplanted to pots filled with potting mix consisting of vermiculite: perlite (4:1) in a greenhouse at 25 ± 2°C from May to August 2014 at Yuzhong Campus (E103°36, N36°28), Lanzhou University, Lanzhou, China. The plants were then fertilized with Hoagland solution at 5-day intervals. After 6 weeks, each plant was examined for endophytic fungi in accordance with the Bacon and White method (Bacon & White, 1994). The plant tillers were then transplanted to a 1.8 L-plastic tank containing aerated 1/2-strength Hoagland solution (Hoagland & Arnon, 1950), which was renewed every 3 d. After 2 weeks of recuperative development, NaCl was added to the medium in 50 mM treatments at 12-h increments until a concentration of 200 mM was reached. After 6 d of treatment, the blades were cut and removed. The blade samples were immediately frozen in liquid nitrogen and stored at -20°C until the physiological analyses were conducted.

Chlorophyll and carotenoid analyses: The chlorophyll (Chl) and carotenoid (Car) contents were extracted from 0.05 g of fresh leaf tissue with 10 ml of 80% acetone in a stoppered tube for 24 h. The solution was homogenized at least twice during this period. The acetone extract was measured in a spectrophotometer (SP-723; Shanghai, China) at a wavelength of 470 nm for the carotenoids as well as 646 and 663 nm for the chlorophyll. The chlorophyll concentration was calculated from the absorbance data, followed by the pigment content. The contents of chlorophyll a, chlorophyll b and carotenoids were calculated utilizing the following formulae:

Chlorophyll a concentration (mg/L) = $12.21A_{663}$-$2.81A_{646}$
Chlorophyll b concentration (mg/L) = $20.13A_{646}$-$5.03A_{663}$
Carotenoid concentration (mg/L) = **(**$1000A_{470}$-3.27Ca-$104C_b$**)**/229

$$\text{Pigment content (mg/g)} = \frac{\text{Pigment concentration x extracting solution volume x dilution ratio}}{\text{Sample wieght}}$$

Analysis of superoxide dismutase, catalase, and peroxidase activities: Fresh samples were extracted with phosphate buffer (pH 7.8) with a mortar and pestle on ice. After centrifugation, crude enzyme extracts were obtained to determine the SOD and CAT activities. The SOD activity was measured as described by Beauchamp & Fridovich (1971). The CAT activity was determined using the strategies of Kar & Mishra (1976) with the following modifications. The reaction mixture to measure the activity of catalase included 0.5 ml of enzyme and 100 µM H_2O_2. The mixture was placed at 25°C for 5 min and then stopped with 5 ml of 1.8 M H_2SO_4. One millilitre of 20% KI (v/v), 3 drops of $(NH_4)_6Mo_7O_{24}$, 5 drops of starch and the residual H_2O_2 were titrated with 0.05 M $Na_2S_2O_3$ until the blue colour diminished. The control utilized enzyme activity was stopped at time zero. The peroxidase (POD) activity was measured by the method described by Li (2000).

Determination of the relative water content: The relative water content (RWC) was measured in accordance with the method of Morgan (1986).

Analysis of soluble sugar and proline contents: The contents of proline and soluble sugars in the wild barley leaves were measured using the method of Li (2000). The anthrone spectrophotometric technique was used to determine the total soluble sugar content. Samples were homogenized in 70 ml of distilled water using a mortar and pestle. The homogenate was then poured into a 100-ml volumetric flask and heated in a water bath at 80°C for 30 min. The supernatant was subsequently filtered and then diluted 50 times. Afterward, 5 ml of the anthrone reagent was added to 2 ml of the diluent, after which the mixture was kept for 10 minutes. The absorbance at 620 nm was then determined with a spectrophotometer.

To measure the proline content, the samples were homogenized with 5 ml of 3% sulfosalicylic acid and centrifuged for 10 minutes at 3000 r/min. afterward; 2 ml of the supernatant was transferred to a tube. Two millilitres of glacial acetic acid and 2 ml of acidic ninhydrin were then added to the tube, after which the mixture was placed in a boiling water for bath 30 min for incubation. After cooling to room temperature, the reaction solution was extracted with 4 ml of toluene and mixed vigorously for 15 sec. The toluene phase containing the chromophore was used to determine the proline content using a spectrophotometer (SP-723; Shanghai, China) at a 520 nm wavelength.

Statistical Analyses

All tests were repeated three times, and the results were expressed as the mean ± standard deviation (SD). We used SPSS version 19.0 (SPSS, Inc., Chicago, IL) to analyse the data. The effects of salt stress and endophytic fungi on the RWC; the chlorophyll, carotenoid, soluble sugar and proline contents; and the activity of POD, SOD and CAT were determined by two-way ANOVA. Significant differences between the presence and absence of endophytic fungi in wild barley under the same treatment conditions were determined using independent t-tests.

Results

Chlorophyll and carotenoid contents: The amounts of Chla, Chlb and Cars did not differ significantly between the 200 mM NaCl treatment and the control (Fig. 1). However, after 6 days of treatment with 200 mM NaCl, the contents of Chla, Chlb and Cars in the leaves of the E+ wild barley were significantly higher than those in the leaves of the E– plants (Fig. 1). The presence of the endophyte had a significant effect on the Chla, Chlb and Car contents of wild barley leaves (p<0.001) (Table 1).

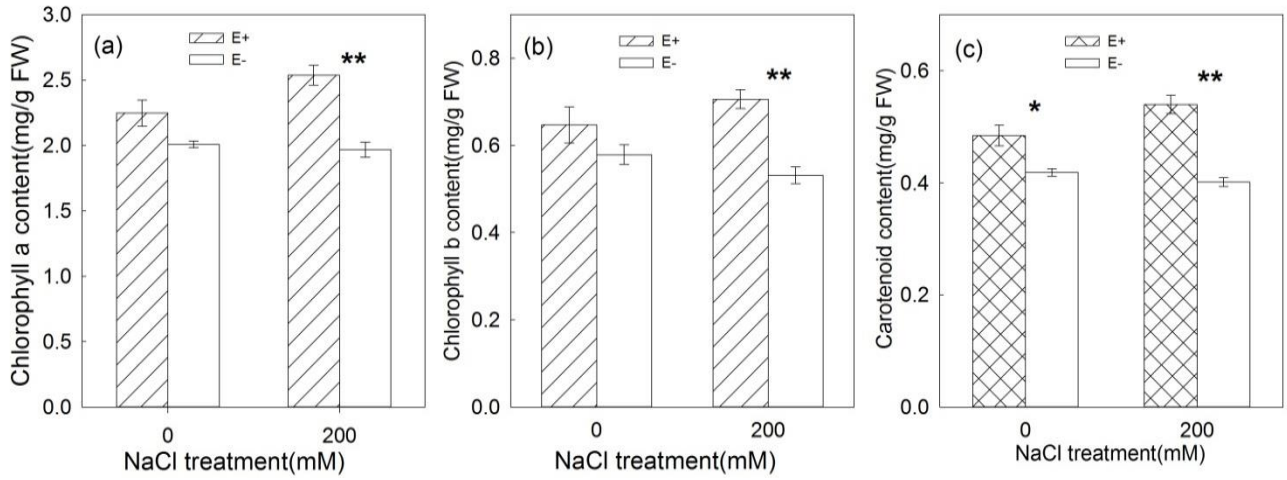

Fig. 1. Contents of chlorophyll a (a), chlorophyll b (b) and carotenoid (c) after 200mM/L sodium chloride treated six days. Asterisk (*) means significant difference (p<0.05) between E+ and E− plants under the same treatment. Double asterisk (**) means significant difference (p<0.01) between E+ and E− plants under the same treatment.

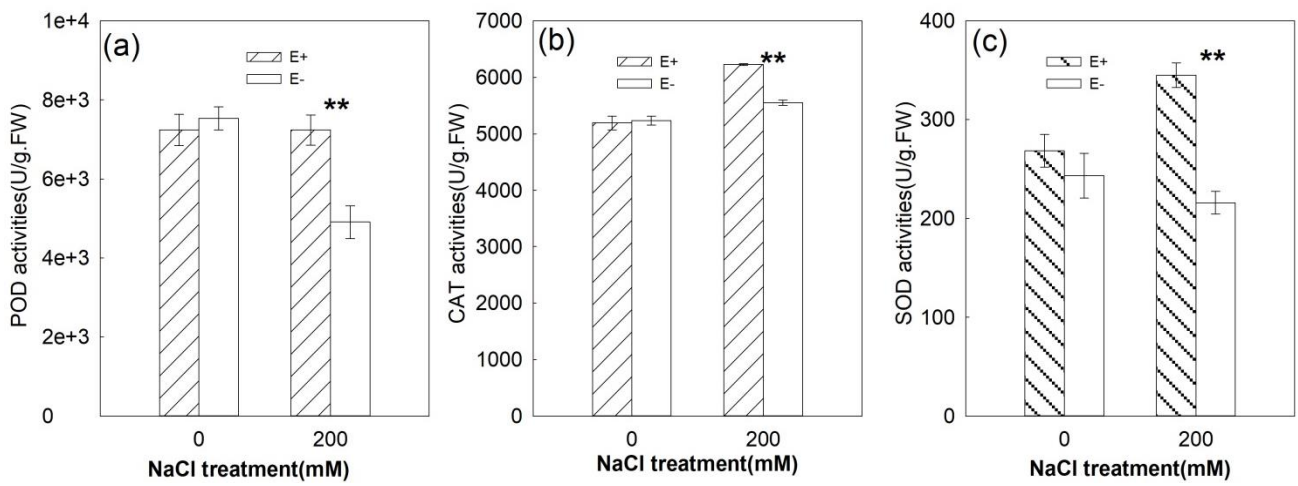

Fig. 2. Activities of POD (a), CAT (b) and SOD(c) after 200mmol/Lsodium chloride treated six days. Double asterisk (**) means significant difference (p<0.01) between E+ and E− plants under the same treatment.

Table 1. Results of two-way ANOVA for the effect of endophyte (E) and salt concentrations(S) on chl content, car content in the leaf of *H. brevisubulatum*.

Treatments	df	Chlorophyll a content		Chlorophyll b content		Carotenoid conten	
		F-value	P	F-value	P	F-value	P
E	1	33.53	<0.001	19.38	<0.001	60.16	<0.001
S	1	3.19	0.093	0.05	0.829	2.12	0.165
E×S	1	5.52	0.032	3.72	0.072	7.67	0.014

Effect on antioxidant enzyme activity: Salt stress significantly affected the activity of antioxidant enzymes in the leaves of both E+ wild barley and E– wild barley (Fig. 2). The POD activity in the E+ plants did not change significantly in the wild barley leaves but was reduced significantly in the E– plants (Fig. 2a). The CAT activity was significantly increased in the E+ plants in the presence of the salt, but there was no definitive change in the E– plants. A similar pattern was observed for the SOD activity. The SOD activity was changed noticeably in the E+ plants after exposure to NaCl. The activities of SOD, CAT and POD in the leaves of E+ wild barley were significantly higher than those in the E– plants treated with 200 mM NaCl (Fig. 2). The measurements of POD, CAT and SOD revealed interactions between the endophyte and salt treatments (Table 2).

Relative water content: The RWC was not significantly different between the endophyte-infected control (E+ ck) and the endophyte-uninfected control (E– ck) plants. The RWC in the E– wild barley leaves was significantly reduced by the 200 mM NaCl stress (Fig. 3). Under salinity stress, the endophyte-infected wild barley plants, exhibited a significantly higher relative water content than

the non-infected plants (Fig. 3). The higher RWC indicated the benefits of the endophytic association, which reduced the adverse effects of salinity stress. Both the endophyte and salt significantly affected the relative water content (Table 3).

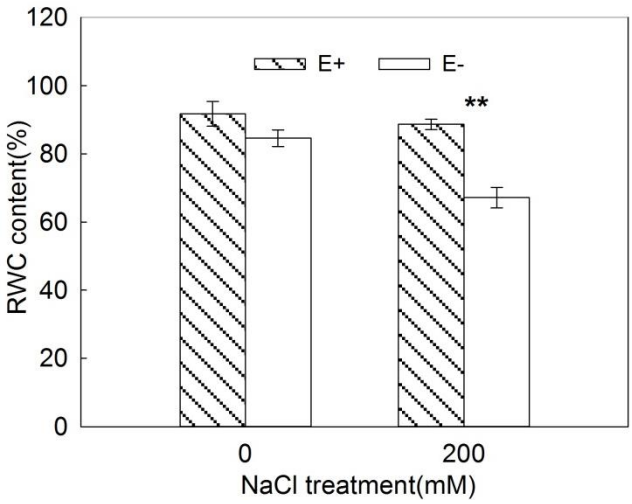

Fig. 3. Contents of relative water content after 200mmol/Lsodium chloride treated six days. Double asterisk (**) means significant difference (p<0.01) between E+ and E− plants under the same treatment.

Osmotic adjustment: The results showed that the proline content did not differ between the E+ ck and E–ck wild barley leaves. The E+ wild barley leaves had a significantly higher proline content than did the E– wild barley leaves after the plants were treated with 200 mM NaCl (Fig. 4a).

In our study, the soluble sugar content was enhanced after treatment with salt (Fig. 4b and Table 3). The control treatment of the E– wild barley leaves had a slightly higher soluble sugar content than did the E+ wild barley leaves. However, this difference was not significant. The soluble sugar content in the E+ wild barley leaves was significantly higher than that in the E– wild barley leaves after treatment with salt (p<0.01) (Fig. 4b). The results showed that there was an interaction between the salt stress and the endophyte (F=25.356, P=0.00029) (Table 3).

Discussion

The aim of this study was to quantify the host performance in the presence of salt stress. Wild barley plants were treated with 200 mM NaCl for six days in a hydroponic system. We determined that the endophyte altered the activities of the antioxidative enzymes, the photosynthetic pigments, the relative water content and the compatible solutes in the host plant. The results further confirmed the protective effects within E+ grasses regarding plant physicochemical properties in response to salt stress (Zhang & Nan, 2007).

Under salt stress, a decrease in chlorophyll content in plant leaves is a general effect (Turan *et al.,* 2009). The contents of photosynthetic parameters and photosynthetic pigments diminish dramatically with increasing salt–alkaline blended stress (Yang *et al.,* 2008, 2009). We observed a similar effect for the E– plants in this study. However, the concentration of the photosynthetic pigments in the presence of 200 mM NaCl was not reduced for the E+ plants. This is consistent with the previous report (Liu & Shi, 2010), in which it was found that photosynthetic pigment was not declined under salt stress. Gou (2007) reported that *Neotyphodium* endophyte-infected *Achnatherum inebrians* had a greater accumulation of chlorophyll when subjected to salt stress. Some researchers also reported an enhancement of chlorophyll a and b in E+ vs. E– *Elymus dahuricus* in the presence of low water stress (Zhang & Nan, 2007). In this study, we found the same result in the presence of salt stress (Fig. 1). Chlorophyll is the basis of photosynthesis, and in reality, some researchers have confirmed that the net photosynthesis in E+ tall fescue is higher than that in E– plants (Newman *et al.,* 2003). Therefore, we can conclude that the wild barley endophyte may improve host plant photosynthesis to resist salt stress.

Carotenoids are tetraterpenoids and can be synthesized by plants and microorganisms but not by animals (Stahl & Sies, 2005). In plants, carotenoids adhere to the photosynthetic apparatus and protect it from photodamage (Rao & Rao, 2007). These compounds also serve as antioxidants in humans (Stahl & Sies, 2005). Carotenoids efficiently scavenge peroxyl radicals and help protect against lipid peroxidation (Burton & Ingold, 1984). In this study, the significantly greater amounts of carotenoids in the E+ plants in the presence of salt stress might protect those plants from sodium chloride stress. In the absence of salt, the content of carotenoids in the E+ wild barley was higher than that in E– wild barley, possibly because the endophytic fungi secreted carotenoids in the E+ plants. However, this hypothesis requires further experiments to confirm.

ROS are the inevitable products of biochemical plant pathways including glycolysis and photosynthesis. As such, plants have produced antioxidant enzyme mechanisms to remove ROS and avoid oxidative stress injury (Apel & Hirt, 2004). Agami (2014) found that the higher activity of SOD, POD and CAT helped barley plants to protect themselves from oxidative damage in salt environments. The combined activity of superoxide dismutase and catalase plays a vital role during plant-endophyte interactions (Baptista *et al.,* 2007). The CAT enzyme is key for the removal of ROS during stress (Ahmad, 2010; Abbasi *et al.,* 2014). It has been demonstrated that salt stress increases the activity of SOD in salt-resistant varieties (Gosset *et al.,* 1994; Neto *et al.,* 2006). In addition, *Piriformospora indica* infection results in a higher concentration of antioxidants in barley roots than in E– barley roots under salt stress (Waller *et al.,* 2005). Our results for superoxide dismutase and catalase activities were similar, although the plants were maintained in a hydroponic system. *Phoma glomerata*-infected cucumber plants produced significantly lower levels of POD under salt stress compared to control plants (Waqas *et al.,* 2012). In our study, the POD activity of the E– plants was lower under conditions of 200 mM salt stress than under the control conditions (Fig. 3a), while the activity of POD in E+ wild barley was almost unchanged in both the salt stress and non-salt stress treatments. Therefore, under the same amount of salt

stress, there was a significant difference in POD activity between the E+ wild barley leaves and the E− wild barley leaves. Greater amounts of POD activity can help plants resist oxidative stress (Scalet *et al.,* 1995). This result suggests that endophytic fungi may degrade ROS, enabling the plants to remove the ROS more effectively or repress ROS production under salt stress conditions. It is clear that the symbiosis helps the plants both adjust to the ROS generation and prevent host cell damage during salt stress. We conclude that these symbiotic-associations can mitigate the effects of salt stress on plants by altering the activities of SOD, CAT and POD.

When plants are subjected to salt stress, they accumulate proline. This phenomenon is an essential defence reaction to maintain osmotic pressure within plant cells. A few studies have demonstrated proline's significant role in modifying the osmotic levels in some salt-sensitive and salt-tolerant crop plant cultivars (Lin *et al.,* 2002; Desingh & Kanagaraj, 2007; Chaum & Kirdmanee, 2009; Joseph *et al.,* 2015). Under drought stress, a large amount of osmotic regulation occurs via proline and soluble sugars at different growth stages (Okunlola *et al.,* 2016). Some researchers have reported that water stress-induced proline accumulation occurs when *E. dahuricus* is infected with an endophyte compared to uninfected plants (Zhang & Nan, 2007). Gou (2007) reported that, under salt stress, E+ *A. inebrians* had a higher proline content than did E− *A. inebrians.* Our results showing that E+ plants accumulated high amounts of proline under salinity stress are consistent with these results. The proline content in the plants containing the endophytes was 2.28-fold higher between the plants subjected to the salt treatment and the controls. The amount of proline was increased 1.33-fold in the E– plants. An increase in free proline and sugars reduces the loss of water (Ashraf & Foolad, 2007). Our results were consistent with the results of this work, as we observed increases in the RWC as well as the proline and soluble sugar contents in E+ wild barley treated with 200 mM sodium chloride for 6 days. The increase in the level of compatible solutes in E+ wild barley plants may help to keep the RWC high enough for plant development. Therefore, we propose that the fungal-inoculated host plants not only avoid salt stress but also help the plant to obtain greater amounts of water from sources that are generally unavailable to E– wild barley plants.

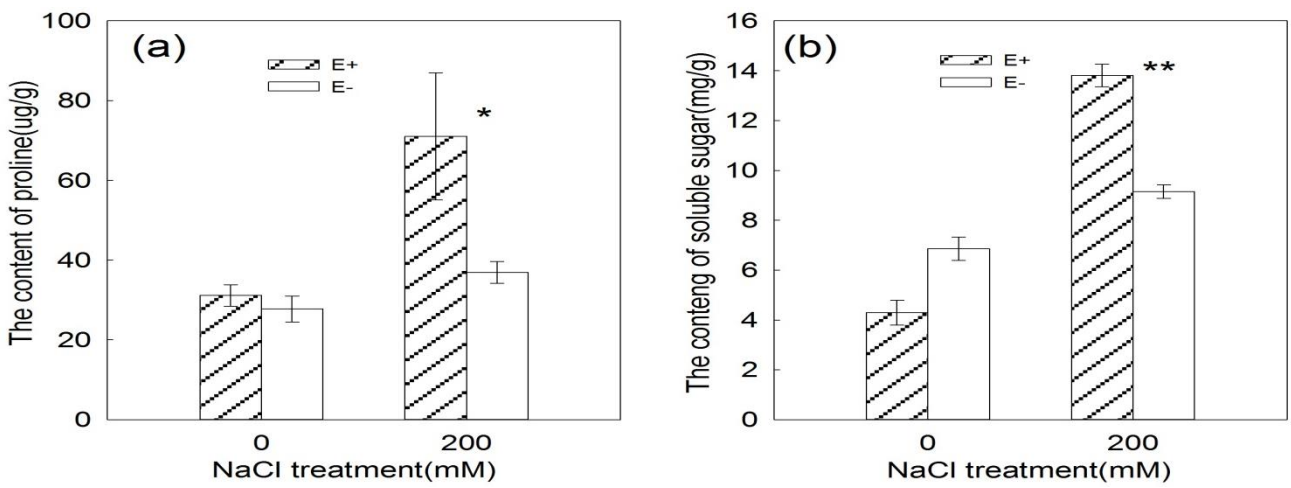

Fig. 4. Contents of proline (a) and soluble sugar (b) after 200mmol/Lsodium chloride treated six days. Asterisk (*) means significant difference (p<0.05) between E+ and E− plants under the same treatment. Double asterisk (**) means significant difference (p<0.01) between E+ and E− plants under the same treatment.

Table 2. Results of two-way ANOVA for the effect of endophyte (E) and salt concentrations (S) on POD, CAT, SOD activity in the leaf of *H. brevisubulatum.*

Treatments	df	POD activity		CAT activity		SOD activity	
		F-value	P	F-value	P	F-value	P
E	1	3.87	0.073	16.94	0.001	22.06	0.001
S	1	8.58	0.013	75.25	<0.001	2.22	0.162
E×S	1	7.81	0.016	21.42	0.001	10.00	0.008

Table 3. Results of two-way ANOVA for the effect of endophyte (E) and salt concentrations(S) on RWC content, soluble sugar content, proline content in the leaf of *H. brevisubulatum.*

Treatments	df	RWC content		Soluble sugar content		Proline content	
		F-value	P	F-value	P	F-value	P
E	1	27.052	<0.001	2.12	0.171	6.73	0.024
S	1	13.859	0.003	67.91	<0.001	18.36	0.001
E×S	1	6.771	.023	25.36	<0.001	1.18	0.300

Conclusion

In conclusion, hydroponically grown E+ and E– wild barley plants exhibit different responses to treatment with 200 mM sodium chloride. Host plants containing the endophytes had altered antioxidative enzyme activities and photosynthetic pigments, and compatible solutes protected against salt stress. We suggest that these phenomena are part of the biochemical adjustment to meet ecological environmental pressures. The molecular mechanism controlling how the endophyte improves the wild barley salt tolerance remains to be further studied.

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