

ENHANCEMENT OF ANTIOXIDANT ENZYMES ACTIVITIES AND GROWTH PERFORMANCE OF *ZOYSIA MACROSTACHYA* FRANCH. & SAV. SUBJECTED TO SALT HETEROGENEITY

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Abstract

Clone integration plays an important role in ecological adapting to salinity heterogeneous habit of clonal plant. However, the impact of clonal integration on the physiological and developmental responses of *Zoysia macrostachya* Franch. & Sav. under salinity heterogeneous habit remains poorly understood. This research took clonal fragments from stoloniferous *Z. macrostachya* that had six consecutive internodes as experiment materials, the daughter ramets were grown in different salinity concentrations solution, while the parent ones were watered normally, stolons were severed or kept intact. The results showed that clonal integration significantly increased the activities of Ascorbate Peroxidase (APX), Glutathione Peroxidase (GPX) and Superoxide Dismutase (SOD), and reduced $\bullet\text{O}_2^-$ production rate, H_2O_2 and MDA contents and electrolyte leakage of daughter ramets subjected to salinity heterogeneous habit, however, there was no effect on Peroxidase (POD) activity of daughter ramets. Clonal integration benefited clonal fragment and daughter ramets growth, but there was no significant effect on growth of parent ramets. Which indicated that physiological integration occurred between parent and daughter ramets, enabling the latter to benefit from the parent ramets under salinity heterogeneous habit, which was a important mechanism for *Z. macrostachya* adaption to salt stress.

Introduction

The clonal plant is a kind of plant which grows in natural environment and can produce numerous genetically identical ramets through asexual reproduction which are similar in appearance and physiology (Zhang *et al.* 2019). Clonal integration is most prominent characteristic of clonal plant (Benedek and Englert 2019). Physiological integration of clonal plant under heterogeneous habits had been well documented (Yu *et al.* 2002, Zhang *et al.* 2002). In most studies, it was found that parent ramets had physiological integration on daughter ramets, but daughter ramets had no physiological integration on mother plants (D'Hertefeldt and Falkengren-Grerup 2002, Chen *et al.* 2006,). However, some studies showed that physiological integration can lead to the relationship change between sources and sinks, and there is a feedback mechanism. Chen *et al.* (2001) found no physiological integration between parent ramets and daughter ramets. These studies suggested that there might be species specificity in clonal integration, and the direction of integration might change under different heterogeneous habits.

Zoysia macrostachya is a kind of perennial warm-season turfgrass that originated from China, and it can be used as the soil-conserving, dike-protecting and sand-fixing turf. It is a kind of euhalophyte, which employs strong tolerance to salinity, and is promising to be taken in landscaping of the saline-alkali lands. To date, there are still few studies on physiological integration of *Z. macrostachya* under salt heterogeneous habit. In this paper, the changes of growth and physiology indexes of *Z. macrostachya* under salinity heterogeneity habit were investigated,

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with aims at enriching the theory about physiological integration of clonal plant and revealing the salt tolerance mechanism from the perspective of groups, which was also of great practical significance for the establishment, maintenance and management of *Z. macrostachya* in saline alkali soil.

Materials and Methods

This study collected 24 clonal fragments with size uniformity from *Z. macrostachya* that had six consecutive internodes in May 2021. Parent and daughter ramets, respectively, were grown in the river sand-filled plastic pots (diameter 10 cm, height 15 cm). Plants were grown under natural light with photosynthetically active radiation ranging from 650 to 1,840 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ and the 14.5 h/9.5 hrs light/dark photoperiod, with the daily minimum and maximum air temperatures of 13.5°C and 37.4°C, respectively, the average air temperature of approximately 25.7°C and the relative humidity of 78 \pm 10%. The plants were watered at intervals of three days, and irrigated weekly with 200 mL 1/2 Hoagland's nutrient solution during culture.

After 30 d growth, the parent ramets were watered normally, while the daughter ramets were placed in turnover boxes with the 0 (control), 100, 200 and 300 mM salinity solution, respectively. Stolons connected to parent ramets were either cut off or linked. This test was carried out for a period of 30 days. Thereafter, the expanded and completely mature leaves from daughter ramets were selected to measure oxidative stress (OS) severity ($\bullet\text{O}_2^-$ production rate, electrolyte leakage, H_2O_2 and MDA levels) and activity of antioxidant enzymes (APX, GPX, POD and SOD).

$\bullet\text{O}_2^-$ production rate was measured according to Liu *et al.* (2014). Leaf H_2O_2 level was measured according to Loreto and Velikova's (2001) method, Leaf malondialdehyde (MDA) content was measured according to Heath and Packer (1968) and leaf electrolyte leakage (EL) was measured according to Dionisio-Sese and Tobita (1998).

Enzymes were extracted at 4°C adopting the method proposed by Reza and Hassan (2014). The activity of APX was measured according to the reduction of OD value at 290 nm (extinction coefficient, 2.8 $\text{mM}^{-1}\text{cm}^{-1}$) due to the oxidization of ascorbate (Nakano and Asada 1981). The activity of GPX was measured based on the changes in OD value at 470 nm after guaiacol was oxidized by H_2O_2 (Chance and Maehly 1955), which was presented in the manner of U mg^{-1} protein min^{-1} . The activity of POD was determined according to oxidation of guaiacol by H_2O_2 (Chance and Maehly 1955). The activity of SOD was measured based on the enzyme amount required to suppress 50% nitroblue tetrazolium (NBT) at 560 nm. Typically, one SOD activity unit referred to the protein amount needed to suppress 50% original decrease in NBT in the presence of illumination, which was presented in the manner of U mg^{-1} protein (Hernandez and Almansa 2002).

Parent as well as daughter ramets, were collected and classified as shoot and root mass, and dried under 80°C until the weights remained unchanged. Then, the dry weight ratio of root to shoot and the total biomass were determined for all parent and daughter ramets.

The experiment was performed under the complete randomization condition with four salinity levels, when stolons kept intact or severed, and each treatment was repeated three times. Differences in data obtained between 2021 and 2022 were not significant. Therefore, integrated data obtained in these two years was used to calculate the average for later analysis. The two-way ANOVA was used for data analysis using SAS (Version 9.4, SAS Institute, Cary, NC, USA). At the same time, the means were compared by Duncan's multiple range test.

Results and Discussion

Salt stress, stolon connection and their interaction significantly affected the $\bullet\text{O}_2^-$ production rate and H_2O_2 content in the daughter ramets, and they gradually increased with the increase in salinity concentrations (Table 1). Under each salinity level, $\bullet\text{O}_2^-$ production rate and H_2O_2 content of the daughter ramets with stolons severed were significantly higher than those of stolons connected (Fig. 1 A, B).

Table 1. F values of two-way ANOVA to test the effects of salt stress, stolon connection, and their interaction on oxidative stress of the daughter ramets of *Z. macrostachya*.

Effect	df	$\bullet\text{O}_2^-$ production rate	H_2O_2 content	MDA content	EL
Salt stress	3, 23	112.93**	254.68**	265.26**	563.04**
Stolon connection	1, 23	83.61**	102.14**	99.37**	110.79**
Salt stress \times Stolon connection	3, 23	13.49**	16.02**	29**	20.42**

** $P < 0.01$.

MDA content and EL of the daughter ramets were also significantly affected by salt stress, stolon connection and their interaction (Table 1). Whether the stolon kept severed or connected, MDA content and EL in the daughter ramets increased with the increase of salinity concentrations. Except MDA content of daughter ramets had no significant difference between the stolon severed and connected at 100mM salinity level. MDA content and EL in daughter ramets with stolon connected were significantly less than those with stolon severed at others salinity concentrations (Fig. 1 C, D).

The activities of APX, GPX and SOD in the daughter ramets were significantly affected by salt stress, stolon connection and their interaction, but the POD activity was only affected by salt stress, but not by stolon connection or their interaction (Table 2).

The higher the salinity concentration, the greater the enzyme activities. Concerning four antioxidant enzyme activities, there was no significant difference between the stolon severed and connected when daughter ramets were subjected to 100 mM NaCl level, but APX, GPX and SOD activities with the stolon connected were significantly lower than those of daughter ramets with the stolon severed when subjected to 200 and 300mM NaCl level (Fig. 2 A, B, C). The intact or severed stolon had no significant effect on POD activity of daughter ramets subjected to 200 and 300 mM NaCl level (Fig. 2 D).

Salt stress, stolon connection and their interaction had significant effect on biomass accumulation of the daughter ramets, but not on parent ramets. As to clonal fragments, their biomass were significantly affected by salt stress and stolon connection instead of their interaction (Table 3).

Whether the stolon was connected or not, the biomass accumulation of the daughter ramets and clonal fragments firstly increased and then decreased with the increase of the NaCl concentration (Fig. 3). When the stolon kept connection, biomass accumulation in the daughter ramets subjected to 100 mM NaCl level was not significantly different compared with the stolon severed. However, when daughter ramets were subjected to 200 and 300mM NaCl level, biomass accumulation in daughter ramets with the stolons connected were significantly higher than that of stolons severed. Besides, biomass accumulation of clonal fragments with the stolons connected were significantly higher than those of the severed clonal fragments when subjected to salinity heterogeneous habit (Fig. 3).

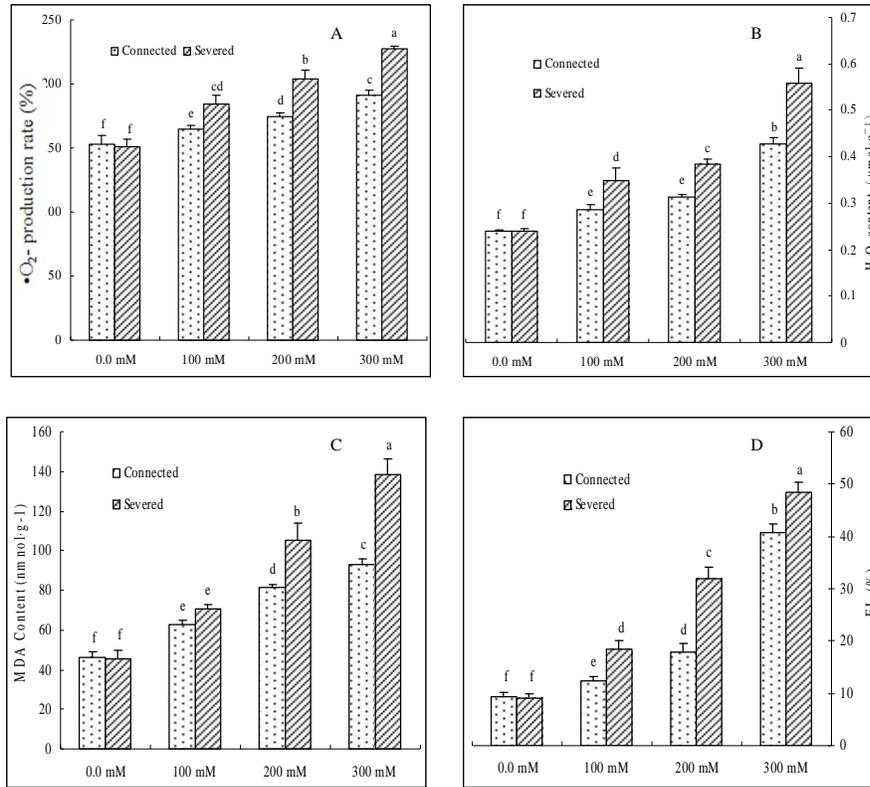


Fig. 1. Oxidative stress of the daughter ramets: A. $\bullet\text{O}_2^-$ production rate, B. H_2O_2 content, C. MDA content and D. Electrolyte leakage. Means followed by the same superscript lowercase letter are not significantly different.

Ratio of root to shoot dry weight in the parent ramets was significantly affected by salt stress, stolon connection and their interaction (Table 3). However, the opposite pattern was observed in the daughter ramets. The proportional biomass allocation to roots by the daughter ramets decreased gradually with the increase in NaCl level. Under 100 and 200 mM NaCl level, ratio of root to shoot dry weight in daughter ramets with the stolons connected had no significant difference compared with the stolons severed, but the ratio in daughter ramets was higher than that of the stolons severed when daughter ramets were subjected to 300 mM NaCl level (Fig. 4).

Table 2. F values of two-way ANOVA to test the effects of salt stress, stolon connection and their interaction on antioxidant enzymes activities of the daughter ramets of *Z. macrostachya*.

Effect	df	APX	GPX	SOD	POD
Salt stress	3, 23	478.15**	101.57**	185.99**	630.97**
Stolon connection	1, 23	168.04**	12.46**	92.5**	0.74
Salt stress \times Stolon connection	3, 23	67.84**	5.07*	35.98**	0.52

** $P < 0.01$, * $P < 0.05$.

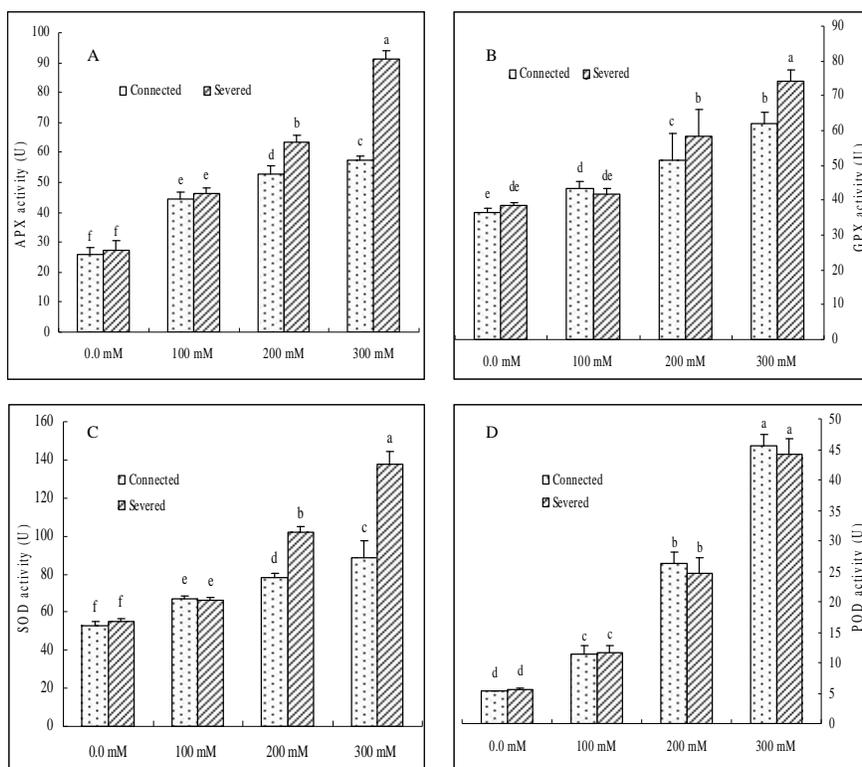


Fig. 2. Antioxidant enzymes activities of the daughter ramets. A. APX activity, B. GPX activity, C. SOD activity, D. POD activity.

Salt stress is one of the main factors affecting the growth and physiological metabolism of plant (Borsani *et al.* 2003, Céccoli *et al.* 2015). It can induce the production of reactive oxygen species (ROS) in plant cells, thus leading to membrane peroxidation (Hazman *et al.* 2015). $\bullet\text{O}_2^-$ production rate and H_2O_2 content were induced by salt stress and caused $\bullet\text{O}_2^-$ and H_2O_2 accumulation. For the stoloniferous herb *Z. macrostachya*, the stolons connected significantly decreased $\bullet\text{O}_2^-$ production rate and H_2O_2 content in the daughter ramets subjected to each salinity level. Over production of $\bullet\text{O}_2^-$ and H_2O_2 damaged cell membranes, and the increase in the permeability of cell membranes led to electrolyte diffuse and EL increase. MDA is the product of membrane lipid peroxidation (Cao *et al.* 2005). Therefore, MDA content and EL reflected the cell membrane damage degree. Daughter ramets with connected stolons exhibited lower MDA content and EL compared to those with severed stolons under salinity heterogeneous conditions, which obviously indicated that clonal integration reduced oxidative damage in the daughter ramets.

The activity of antioxidant enzymes is commonly utilized as a key biomarker for assessing plants' resistance to stress-induced damage. In general, a positive correlation was observed between the enzyme's activity and the extent of cellular damage (Cao *et al.* 2005). In the present study, it was showed that activity of four antioxidant enzymes increased significantly under salinity heterogeneous habit. When the parent ramets and the daughter ramets were connected by stolons, APX, GPX and SOD activities were fewer than these of the stolon severed daughter

ramets, but as for POD activities, there was no significant difference, which indicated parent ramets of *Z. macrostachya* exhibited significant physiological integration on APX, GPX and SOD activities, but not on POD activity under salinity heterogeneous habit.

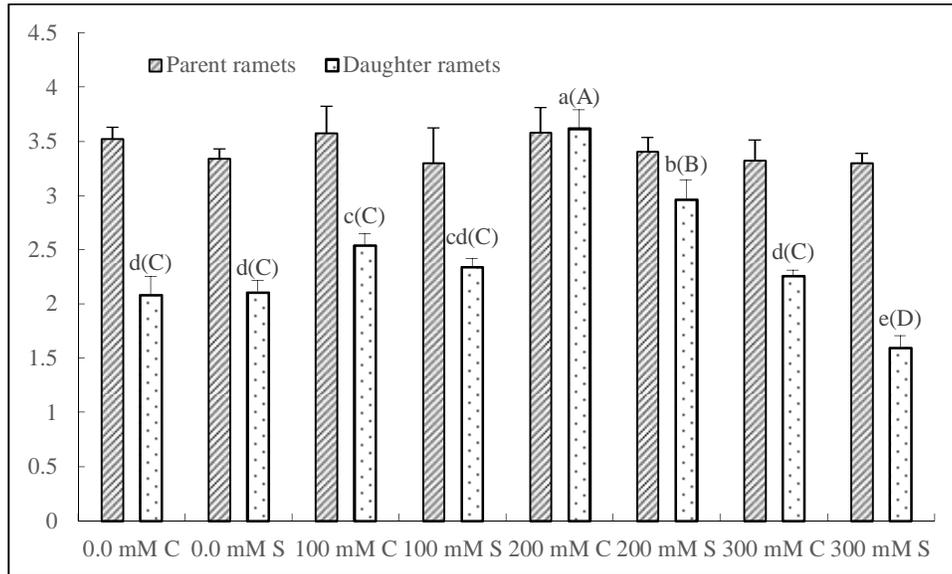


Fig. 3. The biomass accumulation of the parent ramets, daughter ramets and clonal fragments. The biomass accumulation of clonal fragments results from the combined biomass of both parent and daughter ramets. Vertical bars marked with the same superscript lowercase letters indicate that there is no significant difference between the parent and daughter ramets at $P < 0.05$ level, however, with same superscript capital letters mean clonal fragments is not significantly different at $P < 0.01$ level. The C on the horizontal axis represents Connected, while S represents Severed.

Study showed that clonal integration can mitigate the negative effects of stress on their own growth (Yu *et al.* 2004). Furthermore, in some research, it was found that low salt stress promotes plant growth (Zhang *et al.* 2020). In this experiment, whether the daughter ramets connected to the parent ramets or not, 100 and 200 mM salinity level significantly enhanced the growth of the daughter ramets of *Z. macrostachya*, but under 300 mM NaCl stress, the opposite result appeared. The growth of daughter ramets of *Z. macrostachya* with the stolon connected was significantly higher than that of daughter ramets with the stolon severed. At the same time, the growth of clonal fragments increased when stolons kept intact, which indicated that clonal integration was beneficial to the growth of daughter ramets and clonal fragments. The present results are consistent with the findings of Wei *et al.* (2019).

Clonal integration had no significant effect on the growth of parent ramets, which was in line with other experimental results (Janecek *et al.* 2008), might be because of the parent ramets transferred their resources to the daughter ramets without affecting their own growth (Kleunen and Stuefer 1999), or clonal integration changed the relationship between sources and sinks among ramets, thus stimulating the compensatory growth of parent ramets (Janecek *et al.* 2008).

Table 3. F values of two-way ANOVA to test the effects of salt stress, stolon connection, and their interaction on biomass accumulation and ratio of root/shoot of in *Z. macrostachya*.

Effect	df	Biomass accumulation			Ratio of root/shoot	
		Parent ramets	Daughter ramets	Clonal fragments	Parent ramets	Daughter ramets
Salt stress	3, 23	0.89	116.36**	47.9**	0.69	73.81**
Stolon connection	1, 23	4.22	43.77**	30.77**	0.68	12.34**
Salt stress×Stolon connection	3, 23	0.41	9.23**	2.26	0.71	4.72*

** $P < 0.01$, * $P < 0.05$.

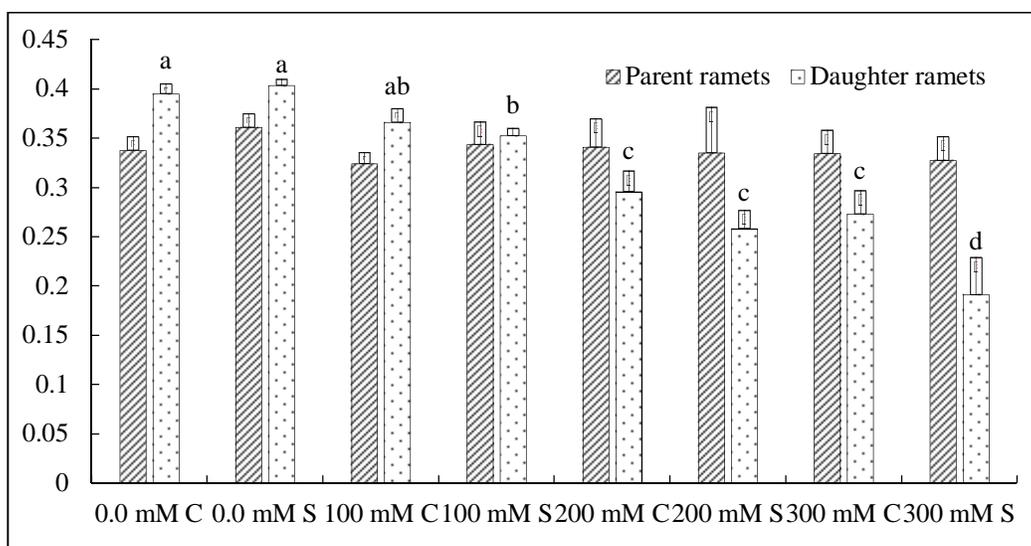


Fig. 4. Ratio of root to shoot dry weight of daughter ramets, Vertical bars sharing the same lowercase letter are not significantly different. C = Connected, S= Severed.

Under salinity heterogeneous habit, clonal integration alleviated adverse effects of salt stress on growth of daughter ramets *Z. macrostachya*, significantly improved APX, GPX and SOD activities and decreased peroxide damage of daughter ramets, but it did not cause significant biomass loss of parent ramets, and enhance the growth of the clonal fragments. Therefore, there was clonal integration from parent ramets to daughter ramets in *Z. macrostachya*, which enhanced the growth performance and antioxidant enzymes activity of daughter ramets subjected to salinity heterogeneous habit, and improved the species population adaptability to salt stress.

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