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Effect of mixed salt stress on malondialdehyde, proteins and antioxidant enzymes of *Leymus chinensis* in three leaf colors

Efecto del estrés producido por la mezcla de sales en la concentración de aldehído malónico, proteínas y enzimas antioxidantes de *Leymus chinensis* de tres colores foliares diferentes

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Abstract. The mixed salt stress is common in nature. Salt stress always affects plant growth. Different plant species have different adaptive capacity to salty soils. *Leymus chinensis* is an herbaceous plant with different leaf colors. However, little research was conducted to explore the different tolerance mechanisms to salt stress among the three different leaf colour genotypes of *Leymus chinensis* (grey green, transitional color, yellow green). Pot experiments for *Leymus chinensis* in three leaf colors were conducted under mixed salt treatments in 2010. Malondialdehyde (MDA) and protein concentrations, and the activity of various antioxidant enzymes [i.e., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR)] were determined and compared among the three leaf color genotypes of *Leymus chinensis*. The concentrations of MDA and protein, and the activity of antioxidant enzymes showed an increasing trend with increasing pHs in almost all three leaf colors, and all of them became highest when salt stress and pH values were also highest. Moreover, antioxidant enzymes were the highest in the grey-green leaf color, and the lowest in the yellow green leaf color after exposure to the same pH treatment. The results suggested that all three leaf colors of *Leymus chinensis* were tolerant to salt stress, and the salt-tolerance declined according to the order of grey green > transitional color > yellow green of *Leymus chinensis*. This study can give us a better understanding of the intra-species adaptation to mixed salt soils.

Keywords: Salt stress; Leaf color; *Leymus chinensis*; Physiological responses; Antioxidant system.

Resumen. El estrés causado por mezcla de sales en el suelo es común en la naturaleza. El estrés salino siempre afecta el crecimiento de las plantas. Plantas de especies diferentes difieren en su capacidad de adaptación al estrés por sales en el suelo. *Leymus chinensis* es una planta herbácea con diferentes colores foliares. Sin embargo, se han conducido pocos estudios tendientes a determinar los diferentes mecanismos de tolerancia al estrés salino entre los tres genotipos de color foliar diferente de *L. chinensis* (grisáceo verdoso, color intermedio, amarillo verdoso). En 2010, se condujeron experimentos en macetas usando genotipos de *L. chinensis* de tres colores diferentes de hoja expuestos o no a tratamientos conteniendo una mezcla de sales. Las concentraciones de aldehído malónico (MDA) y proteínas, y la actividad de varias enzimas antioxidantes [es decir, la superóxido dismutasa (SOD), catalasa (CAT), ascórbico peroxidasa (APX), glutatión reductasa (GR), dehidroascórbico reductasa (DHAR) y monodehidroascórbico reductasa (MDHAR)] se determinaron y compararon entre los tres genotipos de color foliar diferente de *L. chinensis*. Las concentraciones de MDA y proteínas, y la actividad de enzimas antioxidantes mostraron una tendencia a incrementarse a mayores pHs en casi todos los colores foliares, y las tendencias en los tres colores foliares alcanzaron su punto máximo cuando el estrés salino y los valores de pH fueron máximos. Más aún, las concentraciones de las enzimas antioxidantes fueron las más altas en el color grisáceo verdoso, intermedias en el color intermedio, y las más bajas en el color amarillo verdoso después de la exposición al mismo tratamiento de pH. Los resultados sugirieron que los genotipos de los tres colores foliares de *L. chinensis* fueros tolerantes al estrés salino, y la tolerancia a la sal declinó de acuerdo al orden grisáceo verdoso > color intermedio > amarillo verdoso de *L. chinensis*. Este estudio puede proveer un mejor entendimiento de la adaptación intraespecífica de *L. chinensis* a suelos salinos.

Palabras clave: Estrés salino; Color de hoja; *Leymus chinensis*; Respuestas fisiológicas; Sistema antioxidante.

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INTRODUCTION

The salinization and sodication of the soil became a widespread ecological problem (Shi & Sheng, 2005). Salty soils occupy 831 million hectares on earth, and 434 million hectares of them are saline-alkaline soils (Jin et al., 2006). But there are complex compositions in the salty or saline-alkaline soils because neutral (e.g., NaCl and Na_2SO_4) and alkaline (e.g., Na_2CO_3 and $NaHCO_3$) salts often appear simultaneously in saline and sodic soils (Wang et al., 2004; Li et al., 2006; Liu et al., 2011). There are many studies about plant responses to alkaline salt stresses. However, studies on neutral salt stresses are scarce. Thereafter, plant physiology and growth responses to mixed saline-alkaline soil are worth to study.

Survival, biomass production and yield of plants growing on salty soil are seriously affected (Grover et al., 1998). The effects caused by salty soils on plants are various and very complex (Chen & Wang, 2009). According to Munns and Tester (2008), two ways of salinity stress might limit plant growth, development and survival. High levels of salt in soil can keep plant roots (other than halophytes) from absorbing water and nutrients. As a result, growth and associated plant metabolism are affected indirectly. On the other hand, high salt levels might cause large amounts of sodium ion accumulation within plants which can be toxic. Moreover, excess Na^+ induces oxidative damage leading to an imbalance of water and cell constituents in plants. This has a profound negative impact on primary plant physiological processes, and ultimately affects the quality of plants (Forieri et al., 2015). Meanwhile, high pH values in soil are also accompanied with its salinization owed to the elevation of CO_3^{2-}, which can damage plant roots directly by causing the precipitation of Ca^{2+}, Mg^{2+} and H_2PO^{4-} (Yang et al., 2008). This in turn reduces plant nutrient uptake, and breaks the balance of ions (Flowers & Flowers, 2005; Jin et al., 2006). Because of the harmful growing conditions, plants have developed many complex mechanisms at the physiological scale to adapt to the salt stress. This helps plants to avoid the adverse salt effects, thereby contributing to a sustainable plant growth and development (Wang et al., 2008).

When suffering from high level salty stress, the generation and accumulation of reactive oxygen species (ROS) often occurs in plants (Alscher et al., 1997; Dionisio-Sese & Tobita, 1998; Apel & Hirt, 2004; Pancha et al., 2015). These ROS contain superoxide (O_2^-), single oxygen ($^1O_2$), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2), which can seriously disrupt the regular metabolism causing oxidative injure to lipids, some proteins and nucleic acids (Qureshi et al., 2005). Fortunately, large amounts of antioxidant enzymes are present within plants to protect them and resist the damage from the ROS (Dionisio-Sese & Tobita, 1998). The major scavenger is the superoxide dismutase (SOD), which can react with O_2^-, and finally produce H_2O_2 and O_2 (Bowler et al., 1992). The hydrogen peroxide (H_2O_2) produced then reacts with CAT and various peroxidases and finally comes to water (H_2O) (Gupta et al., 1993). Another antioxidant enzyme system is the ascorbic acid (ASA)-glutathione (GSH) (Haliwell-asada circulation system) that also plays an important role against damage caused by radicals in plants. Ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) are main enzymes in this system. In short, the various antioxidant systems are complementary and work coordinated to protect plants growing under harmful conditions (Grams et al., 1999).

Different plant species have different adaptive capacities to salty soils. Some plant species may reduce growth even under low-salinity soils. Other species, however, have a high salt tolerance and can grow and reproduce normally under oceanic salinity (Munns & Tester, 2008; Jampeetong & Brix, 2009). Furthermore, physiological responses and tolerance to salt stress vary not only among species but also among different phenotypes of the same species. For instance, the wild salt-tolerant sugar beet (*Beta maritima*) exhibited a better adaptation to salt stress than the salt sensitive sugar beet (*B. vulgaris*) (Bor et al., 2003). However, the underlying intra-specific differences of tolerance mechanisms have not been clearly understood. Once they are well understood, genetic engineering will be highly beneficial for obtaining salt stress tolerant genotypes of *Beta vulgaris* (Bor et al., 2003; Munns & Tester, 2008).

Leymus chinensis is an herbaceous plant with an economically favorable value. This species is widely distributed in the Songnen plains of the northeastern China (Teng et al., 2006; Zhou & Yang, 2006; Shi & Guo, 2006). *Leymus chinensis* is well adapted to severe conditions of drought and salinity (Wang et al., 2004). Because of the adaptation to different ecological habitats for a long time, *L. chinensis* individuals grow together in three different leaf colors in the same meadow (Zhou et al., 2013). Differences in leaf color on *L. chinensis* have received the attention of many scholars, and these differences currently concentrate on grey green and yellow green leaf colors. Intraspecific differences in genetic diversity, and morphological and physiological traits have been well recorded between the two leaf colors on *L. chinensis* (Wang & Gao, 2001; Liu et al., 2002; Zhou & Yang, 2003; Gong et al., 2007; Chen & Wang, 2009; Zhou et al., 2014). However, very few studies are available on *L. chinensis* in the transitional leaf color. Especially, the physiological mechanisms contributing to determine the three leaf colors on *L. chinensis* are unknown under mixed salinity stress.

We analyzed the physiological strategies based on MDA and protein concentrations and the comprehensive antioxidant system to obtain a further understanding on the underlying differences among the three leaf colors of *L. chinensis* under mixed salt stress. Hypotheses were: (1) tolerance to salt stress differs among *L. chinensis* genotypes of different leaf color, and (2) antioxidant system responses of *L. chinensis* in

the three different leaf colors to mixed salt stress increase as the salt concentration also increases. This study can give us a better understanding on the intra-species adaptation to mixed salt soils. It will help us in improving the use of salty lands for increasing productivity of *L. chinensis* in the salted meadow.

MATERIALS AND METHODS

Plant material and culture conditions. In August 2010, seeds of three leaf colors of *L. chinensis* were collected in the Northeast meadow (44° 30' – 44° 45' N, 123° 31' – 123° 56' E). SPAD values for the three leaf colors were 42.46 ± 2.30 of gray-green, 33.06 ± 1.02 of transitional and 23.93 ± 1.11 of yellow-green. After ripening stage of a winter, seeds were prepared for experiment.

During May to June in 2012, pot experiments were carried out in the Botanic Garden of Liaoning University in China. To sterilization and disinfection, all the seeds were soaked in 0.01% HgCl and gibberellins, respectively, for 10 minutes. Seeds were washed thoroughly by running water and were germinated in plastic pots (28 cm diameter and 20 cm deep) filled with washed sand under natural conditions. There were 36 pots in total with 25 seedlings in each pot. After germination, Hoagland solution (Hoagland & Arnon, 1950) was used for watering the seedlings every two days, and water was replenished every day at a regular time to ensure an adequate growing environment.

Stress treatments. After a growing period of eight weeks, uniform plants from the 36 spots were divided into four groups randomly to subject them to salinity stress treatments of four different gradients; each concentration of salinity can be seen in Table 1. Three replications were performed and a completely randomized block experimental design was used in this study. Two neutral salts (NaCl and Na_2SO_4) and two alkaline salts ($NaHCO_3$ and $NaCO_3$) were mixed in a molar ratio of 1:9:9:1 (NaCl: Na_2SO_4: $NaHCO_3$: $NaCO_3$) to simulate natural salinity conditions (Gao et al., 2011). We selected four salt concentrations: 0, 50, 200 and 350 mmol/L, and the pH we measured were 7.00, 8.55, 8.79 and 8.94, respectively. These concentrations were referred to all salts including NaCl, Na_2SO_4, $NaHCO_3$ and Na_2CO_3.

Table 1. Concentration of mixed salts.
Tabla 1. Concentración de mezcla de sales.

Group	NaCl: Na_2SO_4: $NaHCO_3$: Na_2CO_3 = 1:9:9:1 (mmol/L)	
	pH	concentration (mmol/L)
1	7.00	0
2	8.55	50
3	8.79	200
4	8.94	350

MDA concentration determination. Malondialdehyde (MDA) reflects the status of membrane damage due to lipid peroxidation under salinity stress. MDA concentration was measured according to the method of disulfide barbituric acid (Gao, 2006). Three to five leaves were cleaned carefully and cut into small segments of 0.5 cm. Leaves (0.3 g) were ground into homogenate with little silica sand and 5 mL TCA of 5%. The homogenate was centrifuged at 3000g for 15 minutes, and 5 mL 5% TBA were added into 2 mL supernatant. The mixture was boiled in boiling water for 15 minutes and cooled down in a cold bath. The solution was centrifuged at 3000g for 15 minutes. The supernatant was separated for measurements of absorbance under 450 nm, 532 nm and 600 nm, having a 0.5% TBA as a control. The unit of MDA was nmol MDA/g FW (Ilhami et al., 2012).

Determination of protein. The determination of protein was conducted according to the method reported by Bradford (1976) and taking the BSA (Bovine Serum Albumin) as standard.

Enzyme extractions and assays. One gram fresh plant material was ground into fine powder with liquid nitrogen and a small amount of quartz sand in a mortar. Little enzyme extract homogenate was added on the ice to extract soluble protein. An extraction of 8 mL containing 50 mM phosphate buffer (pH 7.0), 1 mM EDTA and 2% (w/v) PE polyethylene pyrrolidone (PVPP) was centrifuged at 13000g for 40 min at 4 °C. In order to extract APX and DHAR, two kinds of reagent were also needed to ensure the activity of the enzyme (1mM ascorbic acid and 2 mM mercaptoethanol, respectively). The supernatant of mixture was prepared for enzyme activity measurements (Lu et al., 2007).

Superoxide dismutase (SOD) activity was measured according to the method of Beauchamp and Fridovich (1971), based on the reduction of SOD and nitroblue tetrazolium (NBT) under light. Reaction system contained 50 mM $Na_2HPO_4^-$- NaH_2PO_4 buffer (pH 7.0); 13 mM methionine (Met); 75 μL NBT; 10 μM EDTA; 2 μM riboflavin and enzyme extract. The reaction was carried out at 25 °C under a fluorescent light intensity of 120 μmol/m²/s for 20 min. The absorbance was recorded at 560 nm (Beauchamp & Fridovich, 1971). Suppression of NBT photoreduction by 50% was defined as an enzyme activity unit.

Activity of the catalase (CAT) was determined referring to the method of Knoraer et al. (1996). The reaction system contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract. Changes in H_2O_2 absorbance was noted at 240 nm for 3 minutes under 25 °C.

The activity of POD was measured according to the method described by Chance and Maehly (1955). The rise of guaiacol absorbance was measured at 470 nm for 2 minutes at room temperature. The reaction system contained 50 mM phosphate buffer (pH 7.0), 0.25% guaiacol, 5 mM H_2O_2 and enzyme extract.

The determination of APX activity was done according to Nakano and Asada (1981). The decline in ASA absorbance was measured at a wavelength of 290 nm under 25 ˚C for 3 minutes. The reaction system contained 50 mM phosphate buffer (pH 7.0), 0.5 mM ASA, 0.1 mM H_2O_2, 0.5 mM EDTA and enzyme extract.

Activity of the GR was determined according to Halliwell and Foyer (1978). The oxidation of NADPH declining rate of absorbance values were measured at 340 nm under 25 ˚C for 3 minutes (Baloğlu et al., 2012). The reaction system contained 50 mM Tris - HCl (pH 7.5), 0.5 mM GSSG, 5 mM $MgCl_2$, 0.2 mM NADPH and enzyme extract.

The activity of the MDHAR was determined referring to Hossain et al. (1984). The decrease in NADH absorbance was measured at 340 nm within 5 minutes. The reaction system contained 50 mM Tris - HCl (pH 7.5), 0.2 mM NADH, 2.5 mM ascorbic acid, 0.15 units and exzyme extract.

The activity of DHAR was measured by the method of Hossain and Asada (1984). Changes of ascorbic acid absorbance were recorded at 265 nm under 25 ˚C for 3 minutes. The reaction system contained 50 mM phosphate buffer (pH 7.0), 0.5 mM single dehydrogenation bad blood acid, 5 mM GSH, enzyme extract.

The units of CAT, POD, APX, GR, DHAR and MDHAR were all expressed by 1 μmol product of 1 mg protein per minute.

Statistical analyses. All statistical analyses were made using SPSS21.0 software. A two-way ANOVA (leaf color × salt stress) was performed at a P<0.05 significance level.

RESULTS

MDA concentration. Lipid peroxidation in three different leaf color *L. chinensis*, expressed by MDA concentration, is shown in Fig. 1 I. Two-way ANOVA showed that MDA concentration in *L. chinensis* was significantly affected by the salinity stress (P<0.01) (Table 2). Compared to the control groups, the concentration of MDA in each leaf color had an increase of 29.2%-104.1%, 13.4%-98.6% and 26.3%-114.1%, respectively. For grey green leaf color, salty stress induced a progressive increase in MDA concentration and was remarkably highest among the three leaf color at pH 8.79. Although the increase was minimal at the transitional leaf color of *Leymus chinesis* at pH 8.79, a sharp increase of MDA was followed by the exposure to an enhancement of salt stress. In yellow green leaf color, MDA concentration under pH 8.79 was slightly low compared to groups of pH 8.55. When pH value increased to 8.94, MDA concentration in transitional leaf color was higher than grey green leaf color of *L. chinensis.* However, in yellow green leaf color, it was greater than in transitional leaf color of *L. chinensis* when pHs were 8.55 and 7.00.

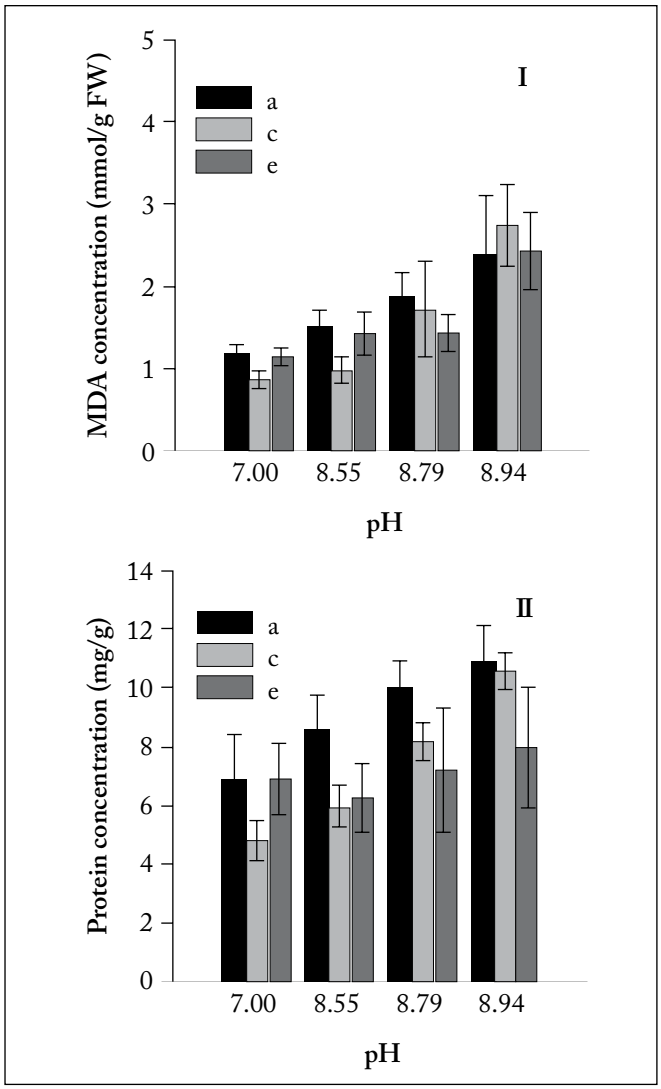

Fig. 1. Effects of salt stresses on MDA and protein concentrations on *Leymus chinesis* of three leaf colors (a: grey green leaf color, c: transitional leaf color, e: yellow green leaf color). Vertical bars represent ± 1 S.E. of the mean.

Fig. 1. Efectos del estrés salino en las concentraciones de MDA y proteínas en *Leymus chinensis* de tres colores foliares (a: color de hoja gris verdoso, c: color de hoja intermedio, e: color de hoja amarillo verdoso). Barras verticales representan ± 1 E.E. del promedio.

Effects of salt stress on soluble protein concentrations. Protein concentration increased observably as salinity also increased in all three leaf colors of *L. chinensis* compared to control groups (Fig. 1 II). Only in grey green leaf color it first decreased by 9.8% under salt stress of pH value 8.55, and thereafter it appeared to increase slowly. Two-way ANOVA revealed that salt stress affected protein concentration in *L. chinensis* remarkably (P<0.05). However, the effect of leaf color on protein concentration was not so significant. In the case of pH 8.55, protein concentration on yellow green leaf color became intermediate between the grey green and transitional

Table 2. Two-way ANOVA of the salinity stress and leaf color effects on the antioxidant activity.
Tabla 2. ANOVA de dos vías (estrés salino × color foliar) para los efectos sobre la actividad antioxidante.

Variation	df	MDA			Protein			SOD		
		MS	F	P	MS	F	P	MS	F	P
LC	2	0.083	0.490	0.635	14.557	4.088	0.076	385.130	14.958	0.005
pH	3	3.658	21.698	0.001	23.612	6.631	0.025	54.362	2.111	0.200
LC×pH	6	0.169	0.416	0.861	33.561	0.756	0.611	25.747	0.271	0.945
Variation	**df**	**CAT**			**POD**			GR		
		MS	F	P	MS	F	P	MS	F	P
LC	2	0.106	30.384	0.006	11.805	14.283	0.005	22.081	8.937	0.016
pH	3	2.172	272.86	0.001	3.397	4.094	0.067	16.338	6.613	0.025
LC×pH	6	0.008	0.108	0.004	0.830	0.640	0.698	2.471	1.966	0.111
Variation	**df**	**APX**			**MDHAR**			**DHAR**		
		MS	F	P	MS	F	P	MS	F	P
LC	2	10.215	10.239	0.012	68.675	24.982	0.001	89.943	28.541	0.001
pH	3	36.688	36.774	0.001	202.134	73.531	0.001	400.581	127.12	0.001
LC×pH	6	0.998	0.098	0.162	2.749	0.046	1.000	3.151	0.146	0.988

Note: LC: leaf color; df: degree of freedom; MS: mean squares.

leaf colors. Nevertheless, when the concentration of the salt treatment increased to pH 8.79 and 8.94, the protein concentration became the maximum in grey green and the minimum in yellow green among the three leaf colors.

Effects of salt stress on the antioxidant defense systems. On the whole, SOD activity did not show a remarkable difference to salinity stress. However, it was significantly affected by leaf color ($P<0.05$). In the case of grey green leaf color of *L. chinensis*, the activity of SOD did not have a pronounced change with the enhancement of salt stress. However, SOD activity increased in the transitional leaf color as pH values also increased; highest values were reached when pH was 8.79, and then decreased when pH came to 8.94 (Fig. 2 I). Unlike those two leaf colors, the increase of SOD activity in yellow green leaf color was just seen under pH 8.55, and it declined above this treatment concentration. The activity of SOD was different in the three different leaf colors of *L. chinensis*. Yellow green leaf color had the lowest SOD activity in all treatments. The activity of SOD in the transitional leaf color of *L. chinensis* was higher than that in the yellow green leaf color, except when pH values were 8.55 and 8.79.

Treatments with salt stress caused that the CAT activity significantly increased over the control in all three leaf colors (Fig. 2 II). The high level of salinity stress resulted in a high activity of CAT. The catalase activity differed among leaf colors. Similar to the untreated groups, the CAT activity was highest in the grey leaf color and lowest in the yellow green leaf color of *L. chinensis* when it was under pH 8.94. Both the salinity concentration and leaf color affected significantly the CAT activity ($P<0.01$ and $P<0.05$, respectively). It meant that the salty stress and leaf color had a common effect on CAT activity.

The activity of POD had varying degrees of rise in the three leaf colors when treated with increasing salt stress (Fig. 2 III). The activity of POD in the grey green *L. chinensis* was maximum at pH 8.94. For the transitional leaf color, POD activity had a tendency of increasing with the enhancement of salt stress, and reached a maximum at pH 8.99. Differently, POD activity in the yellow green achieved a maximum at pH 8.55, and then slightly decreased with increasing pH values. The activity of POD in the grey green leaf color was greater than that in the other cultivars at any pH. Two-way ANOVA showed than that in the order POD activity was significantly affected by the leaf color ($P<0.01$), while not by the pH.

The activity of the glutathione reductase (GR) increased in all three leaf colors with increasing salt stress compared to the control (Fig. 3 I). However, the increase in grey green *L. chinensis* was most pronounced among the three leaf colors. In non-stressed and pH 8.55 treated groups, GR activity was intermediate in the transitional leaf color between the grey green and yellow green leaf colors. Leaf color had a significant action on GR activity ($P<0.05$). Moreover, GR activity was also significantly affected by the salinity level ($P<0.05$).

The activity of the APX was significantly increased in three leaf colors of *L. chinensis* with the enhancement of the salinity level compared to the control. The transitional leaf color exhibited an intermediate APX activity between the grey green and yellow green leaf colors under any treatment (Fig. 3 II). Two-way ANOVA showed that APX activity was significantly affected by leaf color ($P<0.05$) and salt stress ($P<0.01$). Simi-

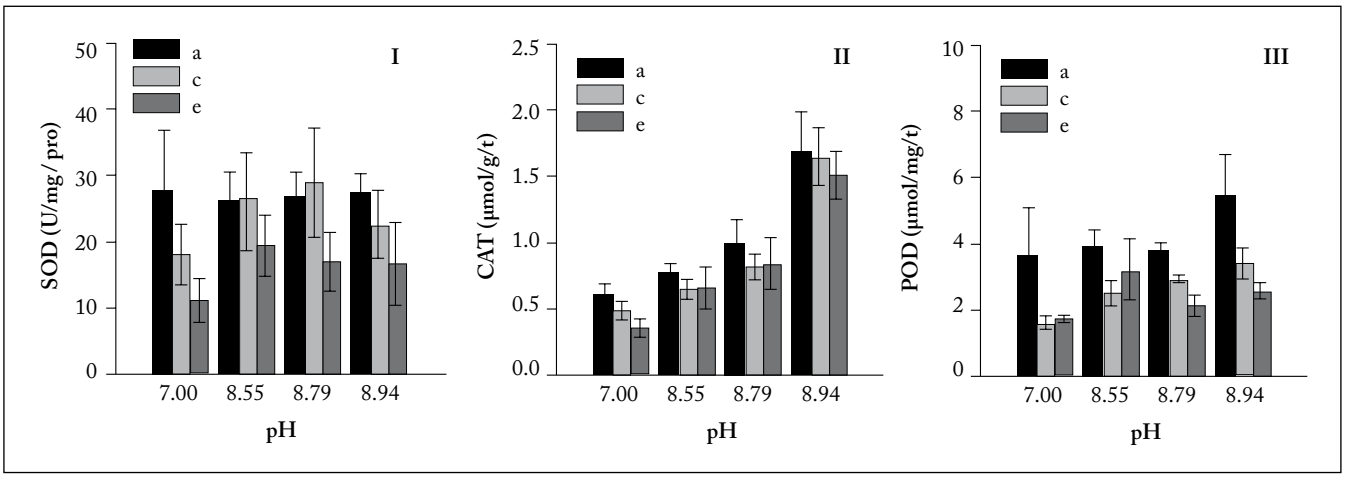

Fig. 2. Effects of salt stresses on SOD, CAT and POD on *Leymus chinesis* of three leaf colors. (a: grey green leaf color, c: transitional leaf color, e: yellow green leaf color). Vertical bars represent ± 1 S.E. of the mean.
Fig. 2. Efectos del estrés salino sobre SOD, CAT y POD en *Leymus chinensis* de tres colores foliares (a: color de hoja gris verdoso, c: color de hoja intermedio, e: color de hoja amarillo verdoso). Barras verticales representan ± 1 E.E. del promedio.

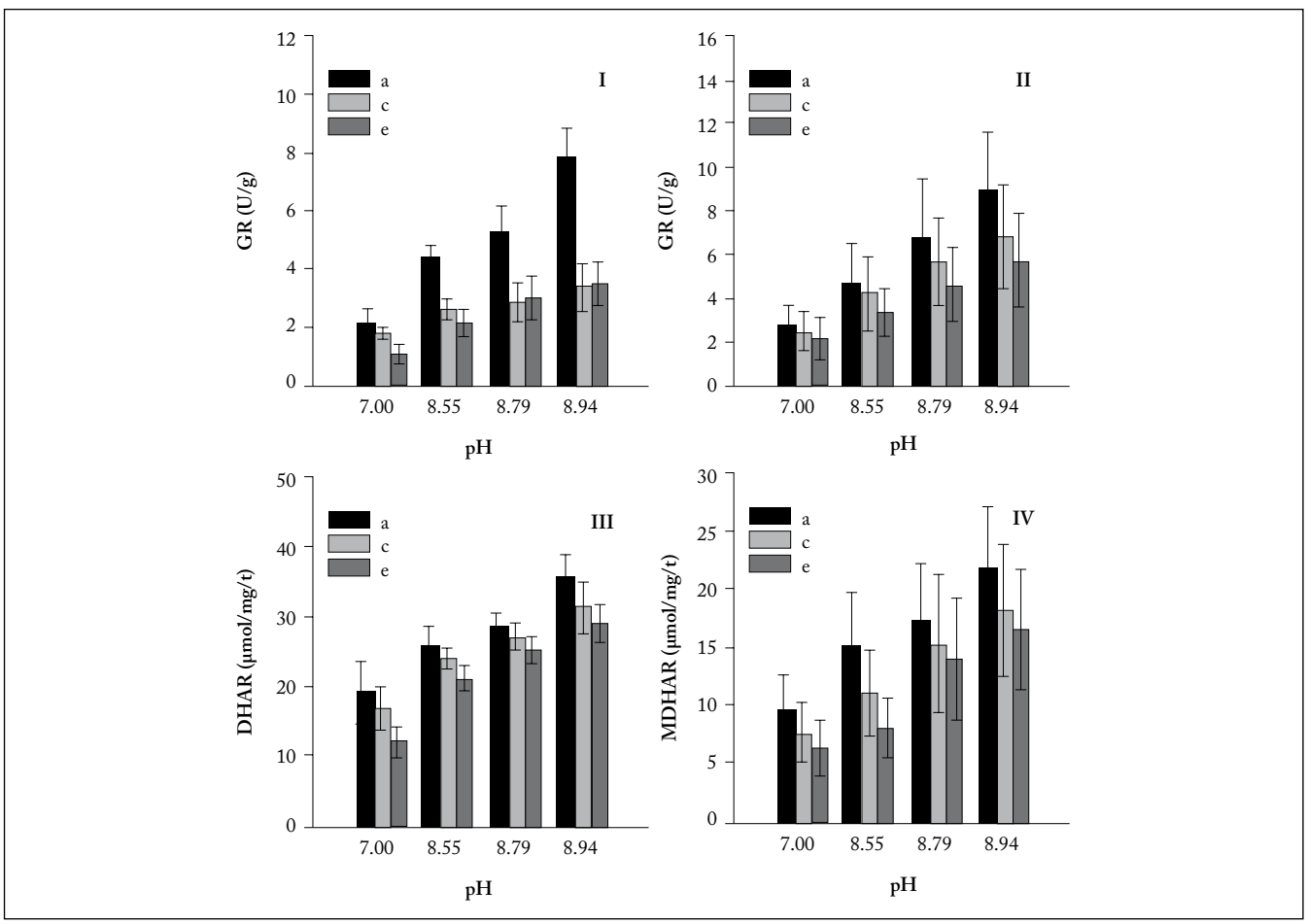

Fig. 3. Effects of salt stresses on GR, APX, DHAR and MDHAR on *Leymus chinesis* of three leaf colors. (a: grey green leaf color, c: transitional leaf color, e: yellow green leaf color). Vertical bars represent ± 1 S.E. of the mean.
Fig. 3. Efectos del estrés salino sobre GR, APX, DHAR y MDHAR en *Leymus chinesis* de tres colores de hoja (a: color de hoja gris verdoso, c: color de hoja intermedio, e: color de hoja amarillo verdoso). Barras verticales representan ± 1 E.E. del promedio.

larly, when increasing the salinity level, MDHAR and DHAR activities (Fig. 3 III, IV) showed a same trend than the activity shown by APX. Both the leaf color and salt stress significantly affected the activity of the three enzymes ($P<0.01$).

DISCUSSION

Hypothesis (1) was supported by our results regarding MDA concentrations. MDA, a final product responding to lipid peroxidation, is often considered an important indicator of plant resistance to stress (Rao & Sresty, 2000). When plants were subjected to salt stress, AOS attacked membranes resulting in the peroxidation reaction which followed the generation of MDA (Bor et al., 2003). The lower level of MDA concentration in the plant demonstrates that it may have been better defended against oxidative damage under salinity stress. In our study, we can observe that concentration of MDA in grey green leaf color was the lowest at 350 mm/L salt concentration (pH 8.94). This may account for the elevated activity of antioxidant enzymes reacting with O_2^- and H_2O_2, and then alleviating cell membrane damage, indicating the strongest resistance to salt stress of grey green *L. chinensis* when the three leaf colors are compared. At pH 7.00 and 8.55, MDA concentration was higher in yellow green than in transitional leaf color of *L. chinensis*. It means that perioxidation was much more serious in yellow green leaf color, indicating a greater salt-resistance in transitional than in yellow green leaf colors.

Stress proteins play an important role of accelerating the cellular metabolism and maintaining homeostasis in the plant when it is under risk (Slos & Stoks, 2008). In this study, the associated increasing protein concentration response to the increasing salt stress in all three leaf colors indicated that they have a better salt-tolerance. Similar results have also been reported by various researchers on cotton (Garratt et al., 2002) and other cultivars (Bor et al., 2003; Hu et al., 2012). Salt-induced protein concentration was highest in grey green leaf color of *Leymus chinensis* when treated with pH 8.55 and pH 8.94, demonstrating a better resistance to salt stress. The salt-tolerance of the three leaf colors of *L. chinensis* was significantly different. This is in good agreement with hypothesis (1).

Salt-tolerance capability is often related to an efficient antioxidative system (Panda & Upadhyay, 2004). Our data showed that the activity of the antioxidant enzymes increased as the salt treatment also increased in the three leaf colors, which is consistent with hypothesis (2). Our results are in good agreement with those in citrus (Gueta-Dahan et al., 1997) and maize (Neto et al., 2006). Once the AOS was generated in plant tissues, SOD collaborated with other enzymes such as POD, CAT and APX to achieve the goal of AOS scavenging. Willekens et al. (1997) reported that SOD plays a key role in the decomposition of O_2^-. The superoxide dismutase (SOD) also seems to be the first antioxidant enzyme against the AOS when facing oxidative stress. Our data showed that SOD activity increased both in transitional and yellow green leaf colors when *L. chinensis* was exposed to salts of pH 8.55 and pH 8.79. The activity of SOD was higher in transitional than yellow green leaf colors, indicating a stronger capacity for protecting from oxidative damage in transitional leaf color genotypes. However, SOD did not show an obvious change in grey leaf color which showed the highest activity among the three leaf colors when exposed to high pH values. This suggests that grey leaf color was better adapted to salt stress than the other two cultivars.

Increases in the activity of SOD were accompanied by increases in the activity of CAT and POD throughout the salt stress period, although the activity of POD changed slightly. Previous studies showed that CAT is one of the most effective antioxidant enzymes in the process of scavenging H_2O_2 associated with SOD (Scandalios, 1993; Bor et al., 2003). Meanwhile, POD also plays an important role in preventing cellular damage by AOS in chloroplasts (Asada & Takahashi, 1987). In our study, the highest CAT activity was observed in grey green leaf color of *L. chinensis* in all treatments, indicating that the grey green leaf color genotype was the most tolerant to salinity stress. The higher CAT activity in the transitional than in the yellow green leaf color of *L. chinensis* in the control and the highest pH suggests that the transitional leaf color is more resistant to high salinity stress than the yellow green leaf color. The POD activity in the grey green was highest among the three leaf colors in all treatments, which also demonstrated a strongest resistance to salt stress. However, the activity of POD gradually decreased in the yellow green leaf color after exposure to pH 8.79 and 8.94. This suggests its insufficient ability for removal of H_2O_2, and then its weak salt-tolerance. Additionally, the lower activity of CAT and POD at pH 8.55 in the transitional than in the yellow green color may be due to the fact that the production of AOS was detoxificated by other coordinated antioxidant enzymes.

In the mechanism of antioxidative adaptation to salinity stress, the ascorbate-glutathione cycle (Haliwell-asada) is an important part for scavenging AOS, involving ascorbate, glutathione, NADPH catalyzed by GR, APX, MDHAR and DHAR. Several studies of these antioxidant enzymes were reported in salt-tolerant cultivars (Gossett et al., 1994; Dionisio-Sese & Tobita, 1998; Azevedo Neto et al., 2004). In these studies, increases of antioxidant enzyme activities in plants exposed to salt stress are consistent with our results. Among the antioxidant enzymes, APX has been reported to be the most effective catalyst, and better at decomposing H_2O_2 than CAT and POD (Benavides et al., 2000). It has been shown that the activity of the APX has an almost identical increasing trend than the GR, MDHAR and DHAR when salinity stress increases in *L. chinensis* of the three leaf colors. This suggests that these four enzymes are very efficient when working together in the ascorbate-glutathione cycle to decompose H_2O_2. In our work, these antioxidant enzyme activities were all highest in grey green leaf color of *L. chinensis* and lowest in the yellow green color. In addition to agreeing with our

first hypothesis, this indicated that the resistance to salinity stress followed the order of grey green > transitional > yellow green leaf color in *L. chinensis*.

Overall, the increase of the antioxidant enzymes of *L. chinensis* in this study demonstrated that the antioxidant defense mechanism plays a crucial role in the process of adaptation to salinity stress. To remove the harmful AOS, it was very helpful to induce the activity of SOD, CAT, POD, GR, APX, MDHAR and DHAR. All of these antioxidant enzymes make up a close-knit circle as a protecting mechanism since their enhancement and mutual cooperation appears to be more efficient. In conclusion, our results were in good agreement with our hypotheses This is, tolerance of *L. chinensis* to increasing salt concentration was clearly leaf color dependent, and the antioxidant response system of *L. chinensis* to salt stress in the three different leaf colors was greatly increased as the salt concentration also increased. Among the three leaf colors, the grey green leaf color of *L. chinensis* was the most salt-tolerant when compared to the other two leaf colors; the yellow green leaf color of *L. chinensis* showed the weakest tolerance to salt stress. For a further interpretation of the physiological responses of *L. chinensis* to mixed salt stress, much more efforts are necessary to understand the effects on ionic and osmotic adjustment, and on the mechanism of protection at the incellular and subcellular scales.

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