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Antioxidant Enzyme Activity in Salt Tolerant Selected Clones of Stylo 184 (*Stylosanthes guianensis* CIAT 184), an Important Forage Legume

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ABSTRACT

In vitro shoots from five selected clones—one sensitive (T1) and four salt tolerant (T2, T3, T4 and T5)—of *Stylosanthes guianensis* CIAT 184 were multiplied in Murashige and Skoog medium with 0, 0.5 and 1% (weight per volume) NaCl for 1 wk followed by transfer to a recovery medium for a further week. Their relative fresh weight (RFW) and antioxidative enzymes—superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX)—were measured after 7 d on the salt medium and again after another 7 d on a recovery medium (without additional NaCl). Exposure to NaCl reduced the growth of all clones at some stage. For the four clones selected as salt tolerant, the reduction in growth was evident after 7 d (up to 45%) on the NaCl media, however, for the clone selected as salt sensitive this reduction (50%) only occurred after 7 d on the recovery medium. Shoots of clones T2, T3 and T5, however, increased their growth on the recovery medium and this was associated with an increase in POX activity (from 0.5% NaCl medium for T2 and 1% NaCl medium for T3 and T5). The SOD activity of these clones was higher than the salt sensitive clone but decreased at 1% NaCl when shoots had been on the recovery medium for 7 d. In T4, the RFW increased to equal that of the control at 0.5% NaCl after recovery while the SOD activity was reduced and the POX activity was stable after both salt treatment and recovery. The NaCl treatments had no effect on the CAT activity for any of the clones. The lowest SOD activity was found in T1 while the tolerant clone, T5, showed the highest CAT and POX activity, providing the ability to distinguish between clones.

Keywords: oxidative stress, salt stress, superoxide dismutase, catalase, peroxidase, *Stylosanthes guianensis*

INTRODUCTION

Oxidative stress is a major phenomenon associated with osmotic effects and ion toxicity caused by salt stress. It is displayed by the overproduction of reactive oxygen species (ROS) represented predominantly by the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$) (Cavalcanti *et al.*, 2004). ROS is the major cause of oxidative stress and is counteracted by a number of antioxidative mechanisms consisting of

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nonenzymatic (that is, production of tocopherol, carotenoids, ascorbate and glutathione) and enzymatic components (that is, production of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX)) (Jithesh *et al.*, 2006).

The most important mechanism for scavenging ROS is the production of antioxidative enzymes. The primary scavenging enzyme is SOD which converts $O_2^{\cdot-}$ to yield O_2 and H_2O_2. Subsequently, H_2O_2 is scavenged into H_2O and O_2 by CAT and a variety of peroxidase compounds such as APX and glutathione peroxidase and type III peroxidases (Cavalcanti *et al.*, 2004; Jithesh *et al*., 2006). The latter are also referred to as guaiacol peroxidases, an important group of peroxidases oxidizing guaiacol as the most commonly used reducing substrate. These are found in the cytoplasm and the apoplast and are typically involved in H_2O_2 scavenging processes. Various isoenzymes of peroxidase not only assist with ROS activity but are also involved in lignin and ethylene synthesis and can therefore influence plant development (Becana *et al*., 1998; Cavalcanti *et al*., 2004; Tayefi-Nasrabadi *et al*., 2011).

Variation in antioxidant enzyme activity under salt stress has been reported for many plants (Bandeoğlu *et al*., 2004; Arulbalachandran *et al*., 2009; Saha *et al*., 2010) and specific enzyme activity depends upon the species being examined (Cavalcanti *et al.*, 2004; Noreen and Ashraf, 2009). Generally, salt tolerant legumes express increased antioxidant enzyme activity under salt stress. For example, in lentils, enhanced SOD and APX activity occurs (Bandeoğlu *et al*., 2004) while in three *Vigna* species, salt stress increased the POX activity (Arulbalachandran *et al*., 2009). Within-species variation also occurs as illustrated by salt tolerant genotpyes of faba bean producing increased CAT, POD, APX and glutathione reductase (GR) (Azooz, 2009) and lotus (*Lotus filicaulis*) producing higher levels of SOD and GR (Melchiorre *et al*., 2009) when exposed to NaCl. In addition, pretreatment with a sublethal dose (50 mM) of NaCl enhanced the salt tolerant capacity of mungbean. In this species, CAT activity increased in roots but decreased in shoots while the SOD activity increased in both shoots and roots (Saha *et al*., 2010).

Other legumes have been reported to produce more variable enzymatic responses to excess salt exposure. For example, in mature leaves of cowpea (*Vigna unguiculata*), the ability to survive under high salinity was not assisted by the operation of an antioxidant system involving SOD, POX and CAT activities (Cavalcanti *et al*., 2004). In common bean (*Phaseolus vulgaris*), three antioxidant enzymes (APX, CAT and GR) significantly decreased while SOD activity increased along with increasing NaCl concentration (Gama *et al*., 2009). In addition, nine cultivars of pea (*Pisum sativum*), exposed to 0–120 mM NaCl, showed no response with respect to enzymatic (CAT and SOD) and non-enzymetic (H_2O_2), malondialdehyde (MDA) and tocopherols metabolites although oxidative stress was expressed in all clones (Noreen and Ashraf, 2009). Lastly, a significant decrease in SOD, CAT and POX activities of soybean under 100 and 200 mM salt was reported (Amirjani, 2010). Variation in enzyme activity, therefore, has the capacity to identify genetic differences between groups but the response of a particular species needs to be examined before the salt tolerance mechanism or genetic difference can be identified (Bandeoğlu *et al*., 2004; Freitas *et al*., 2011).

Stylo 184 (*Stylosanthes guianensis* CIAT 184) is a short-lived forage legume well adapted to a wide range of soil types from sandy to light clay but is sensitive to saline and sodic soils (pH > 8.5). It is considered a high quality forage legume which has been extensively used throughout Southeast Asia, Northern Australia and other subtropical regions (Skerman *et al*., 1988). Increased salt tolerance would raise the usefulness of this legume as it could be utilized in unproductive saline soil. The current study aimed to measure the antioxidant enzyme activity to evaluate the

salt tolerant capacity of selected Stylo 184 clones and to determine whether these enzyme activities can be used to differentiate between salt tolerant and salt sensitive genotypes.

MATERIALS AND METHODS

Plant material preparation

Stylo 184 seeds were obtained from the Animal Nutrition Division, Department of Livestock, Thailand. Over 37,000 seeds were germinated *in vitro* in different concentrations of NaCl. Four seeds that successfully germinated in 2% NaCl weight per volume (w/v) were considered salt tolerant (clones T2, T3, T4 and T5) and one seed that only germinated after removal from 1% NaCl was considered salt sensitive (clone T1). These seeds were used as a source of material for micropropagation on a Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962) containing 30 $g.L^{-1}$ sucrose at a pH of 5.8. Shoots were multiplied by supplementing the basal medium with 0.01 $mg.L^{-1}$ α-naphthaleneacetic acid (NAA) and 1 $mg.L^{-1}$ benzyladenine (BA; Veraplakorn *et al*., 2012). Media were autoclaved at 121 ^{o}C for 20 min and cultures were maintained under a 16 hr photoperiod (40 $\mu mol.m^{-2}.s^{-1}$) at 24 ± 1 ^{o}C. Shoots were elongated on the MS basal medium under the same environmental conditions for 2 mth (including a subculture after 4 wk) prior to transfer to the NaCl exposure experiments.

Plant culture and salt stress treatment

In vitro shoots of the selected clones were cultured on the above medium supplemented with NaCl (0, 0.5 and 1% w/v) for 7 d and subsequently transferred to the medium without additional NaCl (recovery medium) for another 7 d. The relative fresh weight (RFW = 100 × fresh weight of stressed plant / fresh weight of the control) according to Yacoubi *et al*. (2010) and enzyme activity was measured after 1 wk on NaCl medium and 1 wk on the recovery medium. The experiment was set up using a factorial completely randomized design with four replicates.

Sample extraction and enzyme activity

Enzyme extraction

Shoots (0.5 g) were ground in 0.75 mL of ice cold 0.1 M sodium phosphate buffer pH 7.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonylfluoride and 20 mg of polyvinyl pyrolidone. Insoluble material was removed by centrifuging at 12,000×*g* for 15 min at 4 C (Lokhande *et al*., 2010). All spectrophotometric analyses were conducted at 25 °C on a UV/visible light spectrophotometer (UV-1601; Shimadzu Corp.; Kyoto, Japan).

SOD activity assay

The SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One mL of reaction mixture contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton X-100, 75 μM nitroblue tetrazolium, 2 μM riboflavin and an appropriate aliquot of enzyme extract. The reaction mixtures were illuminated for 10 min under a 36 W daylight fluorescent tube. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium reduction when monitored at 560 nm. The SOD activity was expressed as units per gram fresh weight ($U.g^{-1}$ fwt) as in Noreen and Ashraf (2009).

CAT activity assay

The CAT (EC 1.11.16) activity was determined by monitoring the disappearance of H_2O_2 (Noreen and Ashraf, 2009; Amirjani, 2010). The reaction mixture was prepared by adding 0.16 mL of 33 mM H_2O_2 to 100 mL of 0.1 M potassium phosphate buffer at pH 7.0 and 0.1 mL of enzyme extract. Then the reaction was initiated by adding the enzyme extract. The decrease in H_2O_2 was followed by a decline in the optical density at 240 nm and the activity was calculated using the extinction coefficient of 40 $mM^{-1}.cm^{-1}$ for H_2O_2.

The CAT activity was expressed as $U.g^{-1}$ fwt.

POX activity assay

The POX (EC 1.11.1.7) activity was measured on the basis of determination of guaiacol oxidation at 470 nm (Noreen and Ashraf, 2009; Amirjani, 2010). In the presence of H_2O_2, POX catalyzes the transformation of guaiacol to tetraguaiacol. The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 20 mM guaiacol and 12.3 mM H_2O_2. This reaction was recorded at 470 nm using the extinction coefficient of 26.6 $mM^{-1}.cm^{-1}$. One unit of POX activity was defined as an absorbance change of 0.01 $U.min^{-1}$. The enzyme specific activity was expressed as $U.g^{-1}$ fwt.

Statistical analysis

Three way (clone×NaCl treatment× recovery), two way and one way analysis of variance (within each clone) were performed and equal variances tested using Levene's method (Freund *et al*., 2010). Where significant differences were found due to treatment, Tukey's B multiple range test (Freund *et al*., 2010) was applied. Differences were considered significant at $P \leq 0.05$. All analyses were performed using the PASW Statistics 18 software package (SPSS Inc.; Quarry Bay, Hong Kong).

RESULTS

The overall effects examined using the three way ANOVA indicated that there were significant differences in growth, enzyme activity (between clones), salt treatment and recovery medium (Table 1). The general effects indicated that the RFW increased on the recovery medium while the enzyme activity varied with an increase in POX, decrease in SOD and no change in CAT (Table 1). However, two way interactions between clone, NaCl and time indicated the different clones

Table 1 Means for three way analysis of variance of shoot relative fresh weight (RFW) and antioxidant enzymes activity; superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) treated with NaCl and then recovery by transfer to medium without NaCl.

Clone	RFW	Activity ($U.g^{-1}$ fresh weight)		
	(% of control)	SOD	CAT	POX
T1	89.9±5.3^{a}	19.0±0.9^{c}	2.1±0.2^{c}	6.0±0.6^{b}
T2	85.4±4.9^{ab}	47.4±2.9^{b}	4.1±0.3^{b}	2.6±0.2^{c}
T3	73.5±5.4^{b}	63.3±4.6^{a}	2.1±0.3^{c}	3.8±0.5^{c}
T4	80.5±4.4^{ab}	58.8±5.6^{a}	2.7±0.4^{c}	3.8±0.3^{c}
T5	89.7±5.3^{a}	62.0±3.6^{a}	7.5±0.8^{a}	8.6±1.2^{a}
NaCl (%)				
0	100.0±0.0^{a}	55.4±4.3^{a}	4.0±0.4^{a}	5.8±0.6^{a}
0.5	87.0±4.5^{b}	54.8±4.1^{a}	4.1±0.6^{a}	5.1±0.7^{a}
1	64.4±3.4^{c}	40.0±3.0^{b}	2.9±0.3^{b}	3.9±0.6^{b}
Time				
Treat NaCl	76.5±3.4^{b}	57.4±3.5^{a}	3.9±0.5	4.1±0.3^{b}
Recovery	91.1±2.8^{a}	42.8±2.6^{b}	3.5±0.3	5.9±0.6^{a}
Interactions				
Clone × NaCl	*	*	**	ns
Clone × Time	ns	**	**	**
NaCl × Time	**	ns	ns	ns
Clone × NaCl × Time	ns	ns	ns	ns

Numbers represent mean (± SE). Different superscript letters within columns are significantly different from each other according to Tukey's test (Freund *et al*., 2010) at 5% probability level. For interactions, * = Significant at $P \leq 0.05$, ** = Significant at $P \leq 0.01$, ns = Not significant.

produced a variety of responses with regard to NaCl treatment and the recovery medium (Table 1). The interactions between clones and NaCl showed significant differences in RFW, SOD and CAT but no effect on POX. Clone×time interactions showed no effect of RFW, but were highly significant for all enzymes. Time×NaCl interactions, however, were the reverse, with RFW being highly significant but with no significant effect from the enzymes. The three-way interaction (clones×NaCl×time) was not significant (Table 1).

Generally, as the NaCl increased, the RFW significantly decreased by approximately 36% (100.0±0.0 to 64.4±3.4%) of the control (Table 1). However, when individual clones were examined, the RFW of all clones, except T1, was significantly reduced after 7 d on salt media. The reduction, however, occurred at different concentrations for the four salt tolerant clones. T3 and T4 had a significant reduction at 0.5% NaCl while T2 and T5 had a significant reduction at only 1.0% NaCl (Figure 1a). After 1 wk on the recovery medium, the RFW of T1 was significantly lower at 1% NaCl while there was no significant difference between 0.5% NaCl and the control. T4 produced a partial recovery at 0.5% NaCl. Clones T2, T3 and T5 were able to recover their growth completely as indicated by no significant difference among the shoots obtained from the different salt media (Figure 1b).

Generally the SOD, CAT and POX activities significantly decreased at 1% NaCl (Table 1) and there was a significant difference among clones for each enzyme. T3, T4 and T5 showed high SOD activity (63.3±4.6, 58.8±5.6 and 62.0±3.6 U.g^{-1} fwt respectively), while T1 had the lowest level at approximately one third (19.0±0.9 U.g^{-1} fwt) of these clones. Additionally, T1 produced the lowest SOD activity which did not change over the range of NaCl concentrations or after transferring to the recovery medium. For T2 and T3, the SOD activity was stable on the NaCl media but those transferred from 1% NaCl to the recovery medium had a significant decrease in the SOD activity. In addition, at 0.5% NaCl the SOD activity decreased in T4 but increased in T5 (Figures 2a and 2b).

For CAT, T5 showed the highest activity (7.5±0.8 U.g^{-1} fwt), T1, T3 and T4 had the same

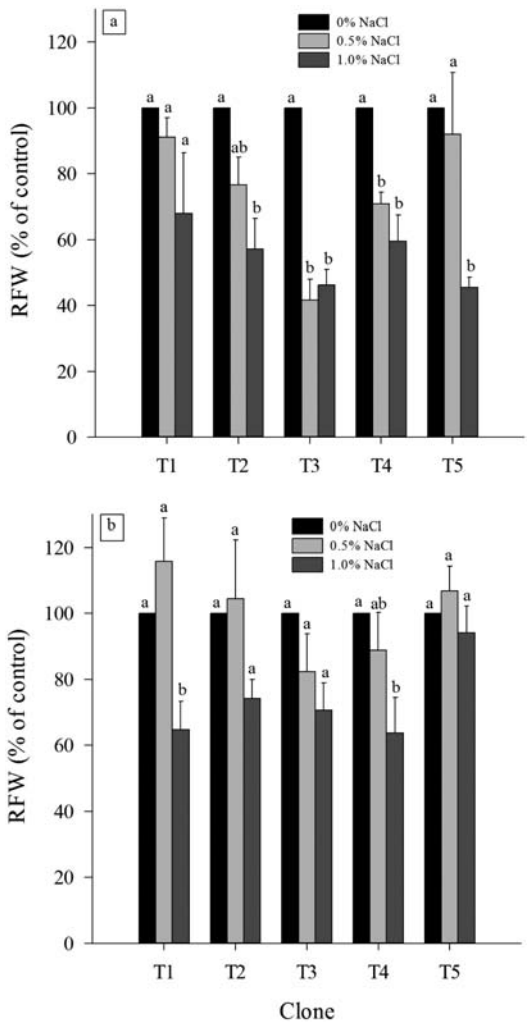

Figure 1 Relative fresh weight of shoots cultured on Murashige and Skoog (1962) basal medium: (a) Treated with 0, 0.5 and 1% NaCl for 1 wk, (b) Recovery on medium without NaCl for 1 wk. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

level and T2 was intermediate (Figure 3). While there appeared to be differences in the CAT levels due to NaCl exposure, this was not evident when individual clones were examined (Table 1, Figure 3a). After 7 d on the recovery medium, the CAT levels were reduced for T5 and T3 while for T2 and T4 they increased and there was no change for T1 on the recovery medium (Figure 3b).

T5 had significantly higher POX activity than all the other clones (8.6±1.2 $U.g^{-1}$ fwt; Table 1) and this was most likely due to its high production after 7 d on the recovery medium (Figure 4). This activity was reduced on the 1%

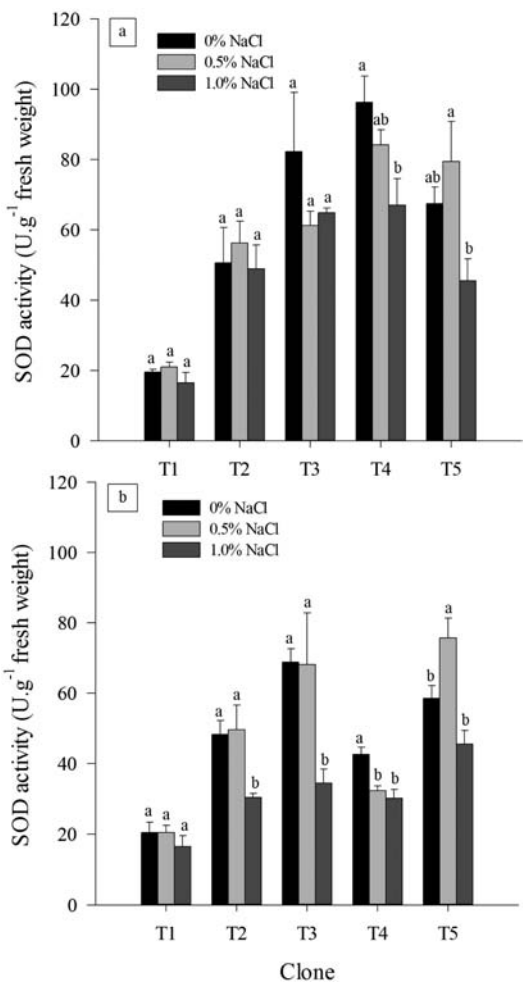

Figure 2 Superoxide dismutase (SOD) activity of shoots cultured on Murashige and Skoog (1962) basal medium: (a) Treated with 0, 0.5 and 1% NaCl for 1 wk, (b) Recovery on medium without NaCl for 1 wk. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

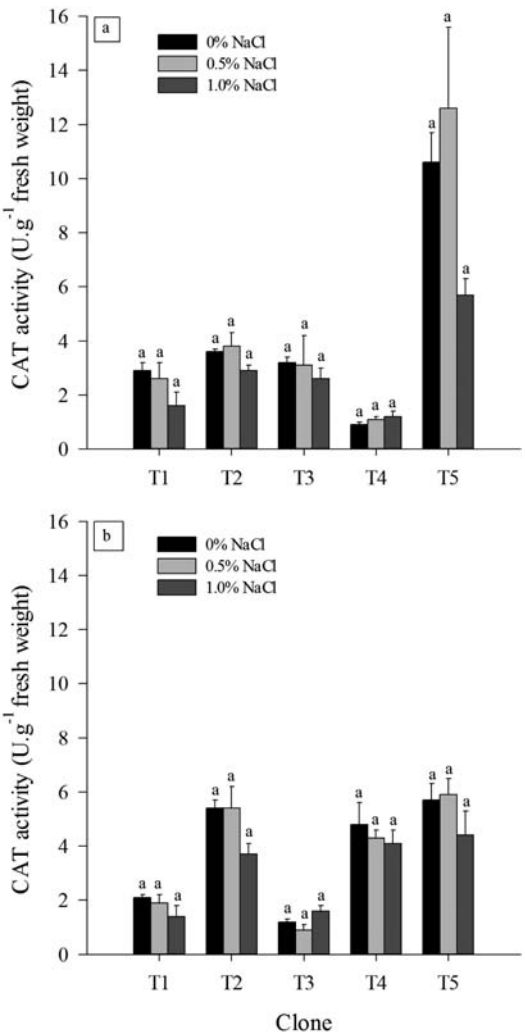

Figure 3 Catalase (CAT) activity of shoots cultured on Murashige and Skoog (1962) basal medium: (a) Treated with 0, 0.5 and 1% NaCl for 1 wk, (b) Recovery on medium without NaCl for 1 wk. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

NaCl medium but this difference was not apparent after recovery (Figure 4b). The POX activity of the other clones varied by having lower levels of the enzyme and having a reduction on 0.5% NaCl (T2 and T3) or having no change (T1 and T4) due to either NaCl or recovery (Figure 4).

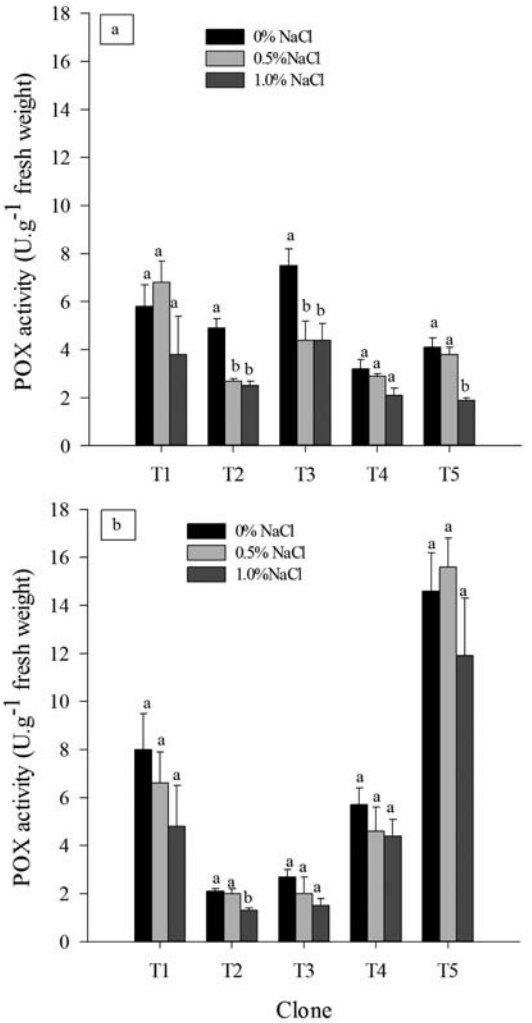

Figure 4 Peroxidase (POX) activity of shoots cultured on Murashige and Skoog (1962) basal medium: (a) Treated with 0, 0.5 and 1% NaCl for 1 wk, (b) Recovery on medium without NaCl for 1 wk. Error bars indicate standard error; n = 4; different letters above bars for each clone indicate significant differences ($P \leq 0.05$).

DISCUSSION

Oxidative stress impairs plant growth and development when antioxidative capacity and ROS are unbalanced (Munns and Tester, 2008; Ellouzia *et al*., 2011). Increasing the antioxidant enzyme activity to reduce the oxidative stress under the influence of salt has been consistently reported (Arulbalachandran *et al*., 2009; Azooz, 2009); however, it appears that for some species, the enzyme increases are insufficient to overcome the oxidative stress (Radyukina *et al*., 2007; Ellouzi *et al*., 2011). In addition, while a correlation between the antioxidant enzyme activity and salt stress has frequently been found, higher increases are not always associated with salt tolerance (Munns and Tester, 2008). For example, salt-sensitive cultivars of potato (Rahnama and Ebrahimzadeh, 2005), wheat (Mandhania *et al*., 2006) and rice (Khan and Panda, 2008)are associated with higher ROS production leading to higher antioxidant enzyme activity.

The SOD activity in T1 was stable when NaCl increased as in T2 and T3; a response similar to that reported for cowpea where the SOD activity was stable throughout the experimental period (Cavalcanti *et al*., 2004). However, the low SOD activity for Stylo 184 may be associated with salt sensitivity, as T1 had lower constitutive SOD levels compared to the other four clones. This may be similar to reports comparing maize and wheat, where the maize was able to resist the potential oxidative damage without requiring additional SOD while wheat, which had lower initial SOD levels, produced SOD when exposed to NaCl (Stepien and Klobus, 2005).

The capacity to maintain SOD activity in Stylo 184 shoots may relate to preferential K^+ selectivity. Increasing in the severity of K^+ deficiency under abiotic stress is associated with the enhanced activity of enzymes involving in the detoxification of H_2O_2 and the utilization of H_2O_2 in oxidative processes (Cakmak, 2005). In T4, however, the SOD activity was high but decreased

significantly at 1% NaCl. In addition, the tolerant clones (T2, T3 and T4) showed decreasing SOD activity after recovery compared to the control. This is similar to the reduction in the SOD activity in pea (*Pisum sativum;* Hernández and Almansa, 2002), green gram (*Vigna radiata;* Panda and Khan, 2009) and soybean (Amirjani, 2010) in stressed and recovered tissues. This may be caused by the scavenging mechanism of the clones (T4 and T5) containing high H_2O_2 which results in a reduction and inactivation of SOD (Bray *et al*., 1974; Khan and Panda, 2008; Panda and Khan, 2009).

The lack of response in CAT activity due to salt treatment in all clones is similar to what has been reported for cotton and sorghum (Freitas *et al*., 2011), cowpea (Cavalcanti *et al*., 2004; Freitas *et al*., 2011) and lentil (Bandeoğlu *et al*., 2004). However, the innate differences in levels of CAT activity between the clones may confer some level of salt tolerance; within Stylo 184, T5 had the highest constitutive levels of CAT. This high CAT activity may be similar to the responses that have been reported for green bean where CAT activity had a greater increase in salt tolerant genotypes (Yasar *et al*., 2008). T5 also had the highest SOD activity among the clones. This may be a reflection of a salt tolerant mechanism that has explained differences between relatively salt sensitive plants such as cowpea and less salt sensitive species such as sorghum and cotton. The low SOD and CAT activities in cowpea can at least partially explain its susceptibility to salt stress (Freitas *et al*., 2011). This relationship of greater salt tolerance being conferred with higher constitutive antioxidant enzyme levels has been reported for a number of species including legumes (Türkan *et al*., 2005; Freitas *et al*., 2011), oilseed rape (Abedi and Pakniyat, 2010), maize (Stepien and Klobus, 2005) and rice (Demiral and Türkan, 2005).

On exposure to NaCl, the POX activity increased in walnut leaves and peaked on the seventh day after exposure to NaCl and this prevented H_2O_2 accumulation (Goharrizi *et al*., 2011). Peroxidases are involved not only in scavenging of H_2O_2 produced in chloroplasts but also in growth and development (Panda and Khan, 2009; Kawaoka *et al*., 2003). Increased POX activity in Stylo 184—even in the control after recovery—may be caused by aging of the plants or be associated with cell wall stiffening (Sánchez *et al*., 1995; Roldán *et al*., 2008). No change in the POX activity in the shoots (either during salt treatment or on the recovery medium) in T1 or T4 was similar to the result seen in the leaves of the glycophyte green gram (Panda and Khan, 2009). On the other hand, the POX activity in T2, T3 and T5 was reduced as NaCl increased—a response similar to that reported in sunflower shoots and soybean leaves (Santos *et al*., 2001; Amirjani, 2010). After recovery, the POX activity increased and reached the control treatment levels for T3 and T5 and at 0.5% NaCl for T2. This was also reflected in the RFW recovery and was similar to what has been reported for green gram where the POX activity significantly decreased in stressed roots but increased in recovered tissues (Panda and Khan, 2009). Increasing activity in recovered tissue can indicate a higher capacity of H_2O_2 decomposition which is generated during the stress. This may be attributed to the increased activity of POX-encoding genes or the increased tissue-specific isozyme activity (Khan and Panda 2008; Panda and Khan, 2009).

Though the CAT and POX activity in Stylo 184 did not increase along with the increase in NaCl, they were constitutively higher in tolerant clones (T5) than the sensitive one. In addition, the SOD activity in the latter was the lowest (Table 1). SOD activity plays a vital role in catalyzing the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2 (Panda and Khan, 2009). Catalase decomposes H_2O_2 to water and O_2, and is mostly confined to peroxisome and glyoxysome. Peroxidase also decomposes H_2O_2 but, unlike catalase, it relies on various organic electron donors to reduce H_2O_2 to water. Plants usually contain many isoenzymes of peroxidases

with nonspecific activity. These isoenzymes are involved in lignin and ethylene synthesis and in plant development and organogenesis (Becana *et al*., 1998). Increasing the amount of H_2O_2 induced antioxidant enzyme activity, GR and CAT (Sairam and Srivastava, 2000). On the other hand, SOD can be reduced and inactivated due to high H_2O_2 levels (Bray *et al*., 1974).

In T5, the high levels of CAT and POX activity appear to have been essential for the high scavenging content of H_2O_2. The SOD activity, however, was high but decreased at 1% NaCl in both the salt treatment and recovery period. This may have been a negative feedback that decreased the SOD activity under exposure to the higher salt concentrations, hence maintaining an efficient scavenging system in tolerant clones. In addition, the expression of CAT activity decreased when the POX activity increased. The antagonistic phenomenon of these enzymes displayed in T1 and T5 is also seen in oilseed rape where high POX activity is associated with CAT inactivation, which might be considered a key point for the decomposition of H_2O_2 (Abedi and Pakniyat, 2010).

Restoration after recovery indicated variation in the salt tolerant capacity between the Stylo 184 selected clones. Short term (8–96 hr) salt stress interrupted the leaf growth of pea with delayed restoration after 8 hr of recovery compared to the controls (Hernández and Almansa, 2002). All salt tolerant clones except T4 showed effective recovery. T4 and T1 showed reduced recovery capacity with significantly decreased RFW after removing the salt treatment. It was noted that clone T1 showed no change in RFW along with all salt treatments suggesting that ion toxicity has no effect on T1 under short term stress. This may be a typical phenotypic response of the glycophyte in Stylo 184 which was able to cope with salt stress by using an exclusion mechanism. According to our previous result, callus of clone T1 regulated salt ions with high K^+ uptake while the tolerant clones accumulated a high content of Na^+ and Cl^- as osmolytes. Differences in the osmotic adjustment mechanism of the tolerant clones may be a result of growth reduction during salt treatment. T1, however, had low growth with poor recovery at 1.0% NaCl and this may be a reflection of the constant low SOD and CAT activities which could have played a role in the scavenging system.

CONCLUSION

Investigation of the antioxidant enzyme activity in shoots of selected clones of Stylo 184 revealed distinct differences between clones. The low activity of SOD and CAT supported low salt tolerant capacity, advocating T1 as the salt sensitive clone which expressed a difference in antioxidant activity compared to the other clones. In addition, within the clones selected as salt tolerant, T5 showed the highest activity of CAT and POX with similar levels of SOD, possibly relating to higher salt tolerance among the salt tolerant clones.

Perhaps the most important differential is the constitutive levels of the enzymes rather than the particular responses or changes due to NaCl exposure. All four tolerant clones had significantly higher levels of CAT and SOD activity. This could be used as a selection criterion in this species without having to expose plants to salt.

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