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INFLUENCE OF SALT STRESS ON GROWTH AND ANTIOXIDANT RESPONSES OF TWO *MALUS* SPECIES AT CALLUS AND PLANTLET STAGES

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Abstract

Soil salinization has become a major threat to apple (*Malus domestica* L.) production in some regions of China. In this study, we investigated the effects of salt stress i.e. 150 mM NaCl on growth and antioxidant responses of 15-d old callus and plantlets of two *Malus* species i.e., rootstock *Malus robusta* Rehd (Rehd) and scion *Malus domestica* Borkh. cv. Fuji (Fuji) grown on Murashige and Skoog (MS) medium. Salt stress induced an increase in activities of superoxide dismutase (SOD), peroxidase (POD) and glutathione reductase (GR) in two *Malus* species, while a decrease in catalase (CAT) activity in Fuji and unchanged pattern in that in Rehd species respectively at callus and plantlets stages. Additionally, salt stress led to an increase in hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\bullet -}$) contents, along with an enhanced accumulation of malondialdehyde (MDA) resulting in a decrease in relative growth rate (RGR) of both species. The activities of SOD, POD, CAT and GR of both species at plantlets stage were greater while H_2O_2, $O_2^{\bullet -}$ and MDA contents lower than those at callus stage. The two *Malus* species showed similar accumulation of MDA and RGR at both differentiation stages, although they showed different patterns of H_2O_2 and $O_2^{\bullet -}$ accumulation and their corresponding scavenging capacity. The results of this study suggested that callus of both species was more sensitive to salt stress than plantlets. The responses of two *Malus* species to salt stress were similar at a given differentiation stage. Therefore, callus stage is more appropriate for evaluation of plant responses to salt stress. The scion (Fuji) can be matched with rootstock (Rehd) to adapt to soil salinity stress.

Introduction

The devastation of arable land area due to soil salinization has been increasing in many regions of the world. Soil salinity stress limits plant growth and yield of most crops including apple (*Malus domestica* L.) (Grattan & Grieve, 1998; Zhu, 2001; Ashraf & Akram, 2009; Ashraf *et al*., 2012; Azhar *et al*., 2012). The *Malus robusta* Rehd and *Malus domestica* Borkh. cv. Fuji are most important commercial rootstock and scion respectively in China. However, the apple trees with the above scion/rootstock combination are sensitive to salinity stress due to their obvious growth retardation and fruit yield decrease in some parts of northern China (Ma *et al*., 1992; Du *et al*., 2002).

One of the biochemical changes occurring in plants subjected to environmental stresses including salt stress is the production of reactive oxygen species (ROS), i.e. superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), resulting in oxidative stress (Hendty, 1994; Zhu, 2001; Ashraf & Harris, 2004; Panda & Upadhyay, 2004; Jehan *et al*., 2012). ROS interact with a wide range of molecules to cause multiple damage including lipid peroxidation, membrane destruction, protein denaturation and DNA mutation, which are determined by malondialdehyde (MDA) accumulation (Mittler, 2002). To alleviate the deleterious effects of ROS, plants have evolved specific antioxidative mechanisms to scavenge ROS. Thus, antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7) and glutathione reductase (GR, EC 1.8.5.1), the key members of antioxidant protective system, seem to be main important determinants of plant tolerance to salt stress (Vranova *et al*., 2002; Dalmia & Sawhney, 2004; Ashraf *et al*., 2008, 2009). SOD is a major scavenger of $O_2^{\bullet -}$ and its enzymatic action results in the formation of H_2O_2. POD decomposes H_2O_2, by oxidation of co-substrates, such as phenolic compounds and/or antioxidants, whereas CAT breaks down H_2O_2 into water and molecular oxygen (Mittler, 2002). Increasing body of evidence suggests that together with osmotic adjustment and ion compartmentalization, an efficient antioxidant system is also vital in mitigating the adverse effects of salinity stress (Ashraf, 2009).

Many studies support that antioxidant response was well correlated with NaCl tolerance in several *Malus* species such as rootstock *Malus prunifolia* (Fu *et al*., 2012), MM 106 (Molassiotis *et al*., 2006), EM 9 (Molassiotis *et al*., 2006) and *Malus hupehensis* Rehd (Du *et al*., 2002) and scion 'Golden Smoothee' apples (Vilaplana & Valentines, 2006). However, most of the above studies were carried out using whole plant seedlings of rootstock or scion under pot experiments (Ashraf *et al*., 2008, 2012). As compared with cell and tissue culture, the whole plant culture study always exhibits lower and insensitive responses and longer generation period, especially in tree species that have long reproductive cycles. The former can be treated as a better tool to investigate the salt tolerance mechanisms (Zhang *et al*., 2004). Thus, the objective of this study was to investigate the growth and antioxidant system responses to salt stress at callus and plantlet stages of two *Malus* species, i.e. rootstock (*Malus robusta* Rehd) and scion (*Malus domestica* Borkh. cv. Fuji).

Materials and Methods

Plant materials, callus induction, regeneration and culture conditions: The experiment was conducted using callus and plantlets of two *Malus* species i.e. rootstock (*Malus robusta* Rehd) and scion (*Malus domestica* Borkh.

cv. Fuji) from an *in vitro* culture. Leaves were sterilized for 30s in 75% ethanol and 8 min in 0.1% $HgCl_2$, then were subsequently washed with sterile water five times and finally cut into pieces (0.5cm×1cm). Each explant was cultured in Murashige & Skoog (MS) medium.(Murashige & Skoog, 1962) supplemented with $30gl^{-1}$ sucrose, $0.2mgl^{-1}$ benzyladenine (BA), $2mgl^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and $2mgl^{-1}$ indole acetic acid (IAA) to induce callus as described by Li and Ma (1999). After four weeks, callus was separated from the explant and subcultivated to fresh media after 25d for further proliferation in a growth chamber at 25 ± 2°C and 14 h photoperiod (2000 lx). The callus was moved onto the MS medium containing $30gl^{-1}$ sucrose, $4mgl^{-1}$ BA and $0.2mgl^{-1}$ IAA to induce adventitious buds as described by Yu *et al*., (2005). The pH of all growth media was adjusted to 5.8 before autoclaving at 121°C for 20 min, and the media was dispensed in 250 ml flasks. Adventitious buds emerged 30 d later after which time the explants were incubated in fresh media for 25 d in the aforementioned conditions for salt treatments.

Salt stress treatments: The experiment was conducted with two treatments:. (1) salt stress, MS medium supplemented with 150 mM sodium chloride (NaCl) (2) Control, no NaCl in medium. The callus and plantlets were transferred into the above media in a growth chamber at 25 ± 2°C and 14 h photoperiod (2000 lx). Calluses and leaves of plantlets were collected after 15d and washed in distilled water, then blotted with a filter paper for assay.

Relative growth rate (RGR): Samples were weighed at the time of their transfer (Wi) and after 15d of culturing (Wf). The mean RGR was calculated according to the ratio, (Wf-Wi)/Wi. The RGR in the control was considered as 100% and the effect of salt stress treatment on the growth rate of callus was obtained following Zhang *et al*., (2004).

Preparation of extracts and antioxidant enzymes activities assay: The callus and leaves of plantlets were homogenized in a cold mortar and pestle in a three-fold volume of 50 mM phosphate buffer (pH 6.5) containing 1.0 % (m/v) soluble polyvinylpyrrolldone (PVP). To prepare crude extracts for the SOD assay, the plant material was homogenized in an extraction buffer consisting of 50mM Tris (pH 7.8), 1.0% (m/v) PVP and 1 mM EDTA. The homogenates were centrifuged (10000*g* for 20 min at 4°C) and the supernatants used for enzyme assays. Protein content was determined by the method of Bradford (1976), using bovine serum albumin as the protein standard.

SOD activity was estimated by recording decrease in absorbance at 560nm of superoxide-nitroblue tetrazolium complex by the enzyme. One unit of SOD was considered to be the amount of enzyme required to inhibit tetrazolium (NBT) reduction by 50% (Beauchamp & Fridovich, 1971). Peroxidase (POD) activity was assayed as described by Ngo & Lenhoff (1980). POD activity specifically with guaiacol at 470 nm and one unit of enzyme activity was taken as the rate of guaiacol which was oxidized in three min. Catalase (CAT) activity was assayed by measuring the residual H_2O_2 by the tris-HCl reagent according to Wang (1995). Absorbance was recorded immediately at 240 nm every four min and one unit of enzyme determined the amount necessary to decompose 1μmol of H_2O_2 per min at 25°C. Glutathione reductase (GR) activity was measured as described by Grace & Logan (1996). The reaction was started by the addition of 0.05 mM NADPH. The rate of nonspecific NADPH oxidase activity was subtracted. A molar extinction coefficient of 6200 cm^{-1} was used to calculate GR activity.

Determination of H_2O_2, $O_2^{\bullet -}$ and MDA contents: The H_2O_2 content was estimated according to Pazdzioch-Czochra and Widenska (2002). Fresh leaf tissue (1.0 g each sample) was ground in cold acetone (10 ml) and centrifuged at 3000 *g* for 10 min. One ml of the supernatant was mixed with 0.1ml titanium reagent and 0.2ml of 17 M ammonia solution and then it was centrifuged at 3000*g* for 10 min. The precipitate was washed 5 times with acetone by resuspension, drained, and dissolved in 3 ml of 1 M H_2SO_4. The absorbance of the solution was measured at 410 nm against blanks, which had been prepared similarly but without plant tissue. The $O_2^{\bullet -}$ content was analyzed using the method of Elstner and Heupel (1976). One ml of the enzyme extract as described above for SOD was mixed with 1 ml of 1 mM hydroxylammonium chlroride, and then incubated for 30 min at 30°C. One ml of the incubated solution was then added to 1 ml of 17 mM 3-aminobenzenesulfonic acid and 1 ml of 7 mM 1-naphthylamine, and then further incubated for 20 min at 30°C. The absorbance of the solution was monitored at 530 nm. Malonaldehyde (MDA) was extracted with 10% trichloroacetic acid and absorbance measured at 450, 532 and 600 nm with 0.6% thiobarbituric acid as described by Heath and Packer (1968). All spectrophotometric analyses were conducted with a Shimadzu UV-1700 spectrofluorimeter (Shimadzu, Kyoto, Japan)

Statistical analysis: Each treatment included 4 replications (flasks). Analysis of variance was calculated using the SPSS-11 statistical software and the significance among mean values was determined at 95 % confidence limit.

Results

Analysis of variance for response parameters: Analysis of variance showed that the effects of salt stress (Ss), differentiation stage (D), and species (Sp) were significant on most parameters measured, except the effects of Sp on RGR and contents of $O_2^{\bullet -}$ and MDA. Two-way interactions were also significant for: (1) Ss×D for RGR, H_2O_2 and MDA contents and GR activity; (2) Ss×Sp for MDA content, activities of SOD, POD and GR; (3) D×Sp for RGR, H_2O_2 content and activities of CAT, POD and GR. The magnitude of *F* values for most of the parameters were in the order: D > Ss > Sp, except the order of Ss > D > Sp for RGR. This study showed that choice of species and differentiation level are important to overcome the adverse effects of salt stress in terms of plant growth and antioxidant responses (Table 1).

Table 1. *F* values for the effects of salt stress (Ss), differentiation stage (D) and species (Sp) and their interactions on different growth and antioxidant parameters of the callus and plantlets of two *Malus* species.

Source of variation	Salt stress (Ss)	Differentiation level (D)	Species (Sp)	Ss×D	Ss×Sp	D×Sp	Ss×D×Sp
RGR	816.133***	633.236***	0.002	9.236**	0.868	5.716*	1.788
H_2O_2 content	202.112***	654.651***	110.665***	60.523***	3.192	24.305***	0.005
$O_2^{\bullet-}$ content	33.482***	41.121***	0.200	1.337	2.608	3.337	1.857
MDA content	130.336***	163.501***	0.698	30.878***	5.110*	0.124	0.194
SOD activity	259.178***	515.76***	9.184**	0.710	36.422***	1.747	1.929
CAT activity	3.829	152.088***	51.984***	0.574	1.505	8.821**	0.470
POD activity	21.417***	228.246***	23.429***	1.814	6.501*	6.449*	0.085
GR activity	169.779***	686.445***	16.250**	83.695***	14.474**	43.365***	2.659

*, **, *** significance at 5%, 1% and 0.1 % level of significance, respectively. CAT, catalase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase; RGR, relative growth rate; SOD, superoxide dismutase; $O_2^{\bullet-}$, superoxide radicals

RGR: The RGR of callus and plantlets in both Rehd and Fuji species was decreased under salt stress above the control. The above negative effects were greater on callus as compared to those on plantlets in both species. The two *Malus* species showed similar values of RGR at both differentiation stages (Fig. 1).

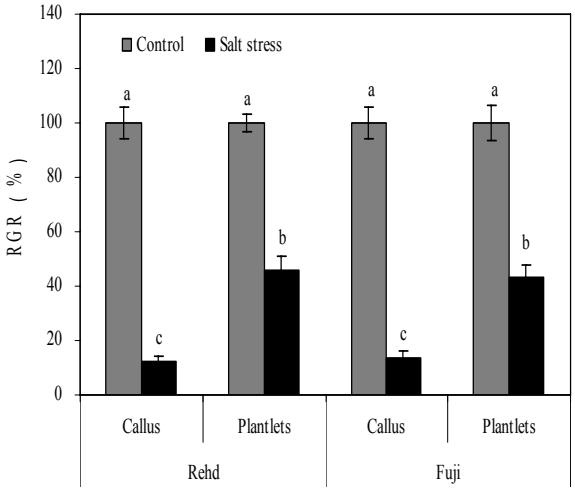

Fig. 1. Relative growth rate (RGR) of callus and plantlets of two *Malus* species grown for 15 days in Murashige and Skoog (SM) medium with 0 (control) and 150 mM NaCl. Values are means ± S.E. (n=4). At the top of all bars, different letters indicate significant differences among the mean values (p<0.05).

Antioxidant enzymes activities: By comparison with control conditions, salt stress increased SOD activities in callus and plantlets of Rehd by 156% and 76%, and in those of Fuji by 81% and 25%, respectively. The SOD activities in plantlets were greater than those in callus of both species. The SOD activities in callus or plantlets were greater for Rehd than those for Fuji (Fig. 2A). Salt stress decreased activities of CAT in callus and plantlets of Fuji by 20% and 14% respectively above the control. The CAT activities in callus and plantlets of Rehd were not significantly influenced by salt stress. The CAT activities of both species were greater in plantlets as compared to those in callus. The CAT activities in callus were greater for Fuji as compared to those for Rehd (Fig. 2B).

The POD activities were increased by 15% in callus and 53% in plantlets of Fuji respectively unde salt stress above the control. While the effects of salt stress on POD activities of Rehd were significant but only in plantlets. Fuji maintained a higher level of POD activities than Rehd with the same differentiation stage. The POD activities in callus were greater than those in plantlets of both species (Fig. 3A). Salt stress increased GR activities of Rehd and Fuji in callus by 55% and 175%, and those in plantlet by 59% and 224%, respectively, as compared with control conditions. The GR activities in plantlets were greater than those in callus of both species. GR activities of Rehd were greater than those of Fuji at plantlet stage regradless of salt stress treatments, while the reverse was true at callus stage (Fig. 3B).

Contents of H_2O_2 and $O_2^{\bullet-}$: Salt stress increased H_2O_2 contents of Rehd and Fuji at callus stage by 95% and 63% respectively, and $O_2^{\bullet-}$ contents of those at the same stage by 40% and 63% respectively above the control. The corresponding increase rates of Rehd and Fuji at plantlets stage were 50% and 53% for H_2O_2 and 21% and 15% for $O_2^{\bullet-}$ respectively. The H_2O_2 and $O_2^{\bullet-}$ contents in callus were greater than those in plantlets of both species. H_2O_2 contents of Fuji were greater than those of Rehd, while the converse was true for $O_2^{\bullet-}$ contents but only at callus stage (Fig. 4A & B).

Lipid peroxidation: Salt stress increased MDA contents in callus of Rehd and Fuj by 55 and 49 %, and in plantlet of those by 28% and 38%, respectively. The greater MDA content was recorded in callus than in plantlets of both species. At a given differentiation stage, the MDA accmulation was similar in two *Malus* species (Fig. 5).

Discussion

Salinity stress inhibits vegetative and reproductive growth of many crops by inducing severe physiological dysfunctions and causing direct and indirect harmful effects (Ashraf *et al.*, 2008, 2012; Ashraf & Ali, 2008; Iqra *et al.*, 2012). The evaluation of salt stress on growth responses were conducted in some plants at different differentiation levels such as callus of *Helianthus annuus* and *Nicotiana tabacum* (Ericson & Alfinito, 1984;

Silvana *et al.*, 2003) and plantlets of *Pinus virginiana* (Wei & Newton, 2005). Zhang *et al.* (2004) reported that the maximum RGR of callus induced from *Populus euphratica* was observed at low concentration of NaCl (50 mM), whereas significant growth inhibition at higher concentrations of NaCl (150 and 250 mM). In the present study, the callus survived in a medium containing 150 mM NaCl over 15 d. Cherian & Reddy (2003) stated that growth of the callus cultures of *Suaeda nudiflora* Moq was significantly inhibited at 50, 100, 150 and 200mM NaCl. Watanabe *et al*., (2000) showed that the growth of *Populus euphratica* plantlets slightly decreased under salt stress (50 mM NaCl). However, the reason for different responses to salt stress between callus and plantlet remains unclear. Our results showed that salt stress i.e. 150 mM NaCl significantly inhibited the growth of callus and plantlets of Rehd and Fuji. The RGR at plantlet stage was greater than that at callus stage for both *Malus* species under salt stress. The RGR of both species was similar at a given differentiation stage (Fig. 1). It is concluded that the callus growth was more sensitive to salt stress than plantlet growth of two *Malus* species, and the scion (Fuji) could be matched with rootstock (Rehd) to adapt to soil salinity.

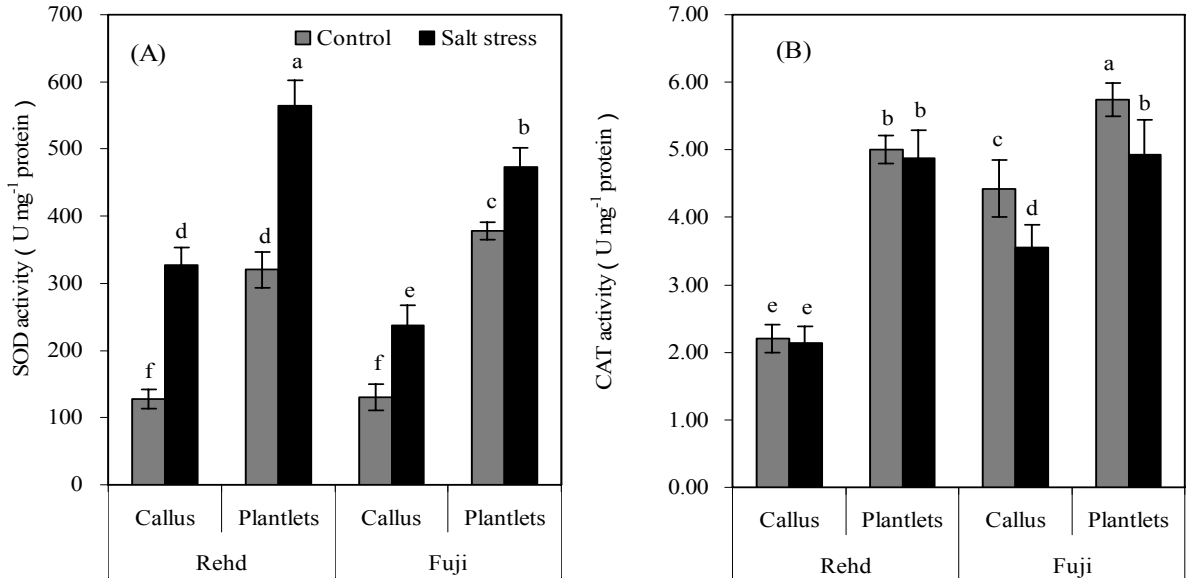

Fig. 2 Superoxide dismutase (SOD) (A) and catalase (CAT) activity (B) in callus and plantlets of two *Malus* species grown for 15 days in Murashige and Skoog (SM) with 0 (control) and150 mM NaCl. Values are means ± S.E. (n=4). At the top of all bars, different letters indicate significant differences among the mean values (p< 0.05).

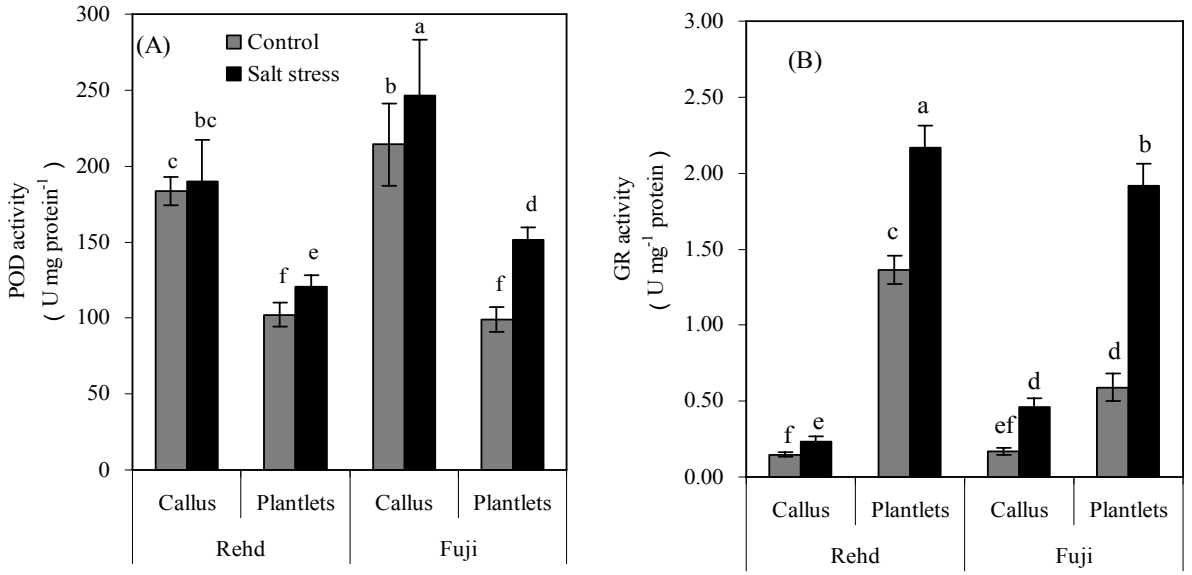

Fig. 3 Peroxidase (POD) activity (A) and glutathione reductase (GR) activity (B) in callus and plantlets of two *Malus* species grown for 15 day in Murashige and Skoog (SM) medium with 0 (control) and 150 mM NaCl. Values are means ± S.E. (n=4). At the top of all bars, different letters indicate significant differences among the mean values (p< 0.05).

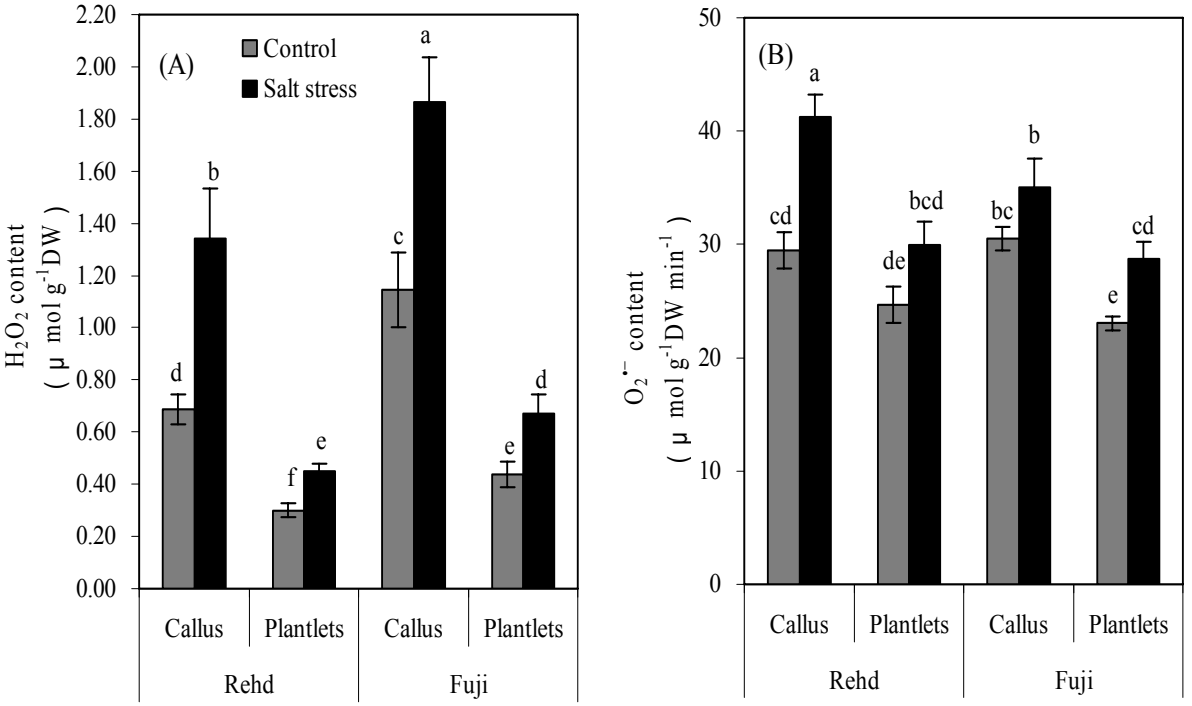

Fig. 4. Hydrogen peroxide (H_2O_2) (A) and superoxide radicals ($O_2^{\bullet-}$) (B) contents in callus and plantlets of two *Malus* species grown for 15 day in Murashige and Skoog (SM) medium with 0 (control) and 150 mM NaCl. Values are means ± S.E. (n=4). At the top of all bars, different letters indicate significant differences among the mean values (p<0.05).

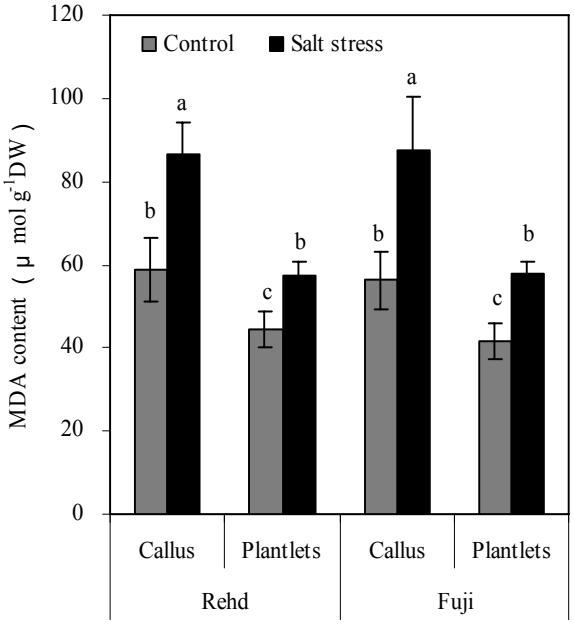

Fig. 5. Malondialdehyde (MDA) content in callus and plantlets of two *Malus* species grown for 15 days in Murashige and Skoog (SM) medium with 0 (control) and 150 mM NaCl. Values are mean ± S.E. (n=4). At the top of all bars, different letters indicate significant differences among the mean values (p< 0.05).

Plants exposed to salinity stress generally show enhanced generation of ROS including $O_2^{\bullet-}$ and H_2O_2 in plant cells, which cause considerable damage to cell membranes due to lipid peroxidation (Blokhina *et al.*, 2003). The level of MDA is considered as a good indicator of degree of lipid peroxidation in stressed plants (Dhindsa *et al.*, 1981; Masia, 2003; Apel & Hirt 2004). Molassiotis *et al*., (2006) reported production of H_2O_2 and $O_2^{\bullet-}$ and MDA accumulation were increased in apple rootstock MM 106 under salt stress. Our results showed that salt stress induced similar increases in contents of H_2O_2 and $O_2^{\bullet-}$ and MDA of scion Fuji and rootstock Rehd at a given differentiation stage (Figs. 4-5).

The salt-induced harmful effects can be alleviated by activating antioxidant enzymes such as SOD, POD, CAT and GR, which can effectively scavenge ROS (Dalmia & Sawhney, 2004; Ashraf *et al.*, 2008). Of these antioxidant enzymes, SOD functions as the first line of defense against oxidation at the membrane boundaries. POD together with CAT are believed to be two efficient H_2O_2 scavenging systems which facilitate rapid removal of H_2O_2 in cells of plants including *Malus* (Mittler, 2002; Blokhina *et al.*, 2003). Antioxidant defense system in plants, is characterized by ROS (H_2O_2 and $O_2^{\bullet-}$) accumulation and enhanced activities of antioxidant enzymes, which determine lipid peroxidation i.e. extent of accumulation of MDA (Gossett *et al.*, 1994; Ashraf, 2002; Dalmia & Sawhney, 2004; Shan *et al.*, 2006; Ashraf & Ali, 2008; Ashraf *et al.*, 2012).

The results of our study demonstrated that salt stress induced increases in activities of SOD, POD and GR, and a decrease in that of CAT. The increased H_2O_2 and $O_2^{\bullet-}$ contents resulted in enhanced MDA accumulation in callus and plantlets of both *Malus* species (Figs. 2-5). The H_2O_2 and $O_2^{\bullet-}$ production in callus of both species was greater, while activities of SOD, CAT and GR (except

that of POD) were lower as compared with those in plantlets, resulting in greater MDA accumulation under salt stress. POD activity was greater in Fuji as compared to that in Rehd under salt stress. The reverse was true for SOD and GR activities. The CAT activity was greater in Fuji than that in Rehd but only at callus stage. The H_2O_2 contents of Fuji were greater than those of Rehd while the reverse was true for $O_2^{\bullet -}$ contents at callus stage under salt stress. In salt-stressed plants, the MDA accumulation and plants growth performance are determined by the activities and change patterns of antioxiant enzymes to remove ROS (Dalmia & Sawhney, 2004; Ashraf *et al.*, 2008). Although activities and change patterns of these antioxidant enzymes and contents of H_2O_2 and $O_2^{\bullet -}$ were dependent on enzymes type, *Malus* species and differentiation level. MDA accumulation and RGR of callus and plantlets had no significant difference between two *Malus* species under salt stress (Figs. 1-5). Our results showed that accumulation of MDA was greater while RGR was lower at callus stage as compared to plantlets stage of both species under salt stress. The two *Malus* species showed similar MDA accmulation and RGR at a given differentiation stage (Figs. 1 & 5). These findings suggest that the differentiation stage of plant tissue might affect the salinity tolerance due to variation in gene expression across different differentiation stages (Jamil *et al.*, 2011). The plant tissue at callus stage was more sensitive to salt stress than that at plantlets stage. Therefore, callus might be regarded an appropriate differentiation stage for evaluating plant responses to salinity stress. The scion (Fuji) and rootstock (Rehd) hold a similar growth and lipid peroxidation responses at a given differentiation stage.

Conclusion

In conclusion, the negative effects of salt stress were similar in two *Malus* species. The callus stage of both species was more sensitive to salt stress as compared to the plantlet stage as evident from greater production of H_2O_2 and $O_2^{\bullet -}$ and lower functioning of the antioxidant enzyme system, which resulted in greater MDA accumulation and lower RGR in the former as compared to those in the latter. The callus culture study can be more useful to evaluate growth and antioxidative responses to salt stress for *Malus* species due to its relatively fast and sensitive responses, short generation time, and controlled environment. The scion (Fuji) can be matched with rootstock (Rehd) to adapt to soil salinity.

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