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SALINITY-INDUCED CALLUS BROWNING AND RE-DIFFERENTIATION, ROOT FORMATION BY PLANTLETS AND ANATOMICAL STRUCTURES OF PLANTLET LEAVES IN TWO *MALUS* SPECIES

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Abstract

Apple (*Malus domestica* L.) is widely grown in northern China. However, soil salinization has become one of the most severe factors limiting apple productivity in some regions including the Loess Plateau. In our study, the regeneration system of both rootstock Rehd (*Malus robusta* Rehd) and scion Fuji (*Malus domestica* Borkh. cv. Fuji) was established *In vitro*. The two *Malus* species were cultured on the MS medium containing 0 or 150 mM NaCl to examine salt-induced effects on callus browning and re-differentiation, root formation of plantlets and anatomical structures of plantlet leaves at 15 days old callus and plantlet stages. Salt stress caused a marked increase in callus browning rate, while a decrease in re-differentiation rate, rooting rate, root number and length in both species. Additionally, anatomical structures of plantlet leave showed salt-induced damage such as reduced palisade tissue and intracellular chloroplast, incomplete development of xylem and severe damage of the phloem tissue. Salt stress also caused a few adaptive structural features in leaves including increased thickness of upper and lower epidermis, elevated proportion of spongy tissue and formation of lignified vessels. The responses of the two *Malus* species did not differ significantly at the differentiation stage. However, they were more sensitive to salinity at the callus stage than those at the plantlet stage in each species. Therefore, callus stage has been found to be more suitable for evaluating responses of the two apple species to salt stress. The Fuji and Rehd could be treated as a good scion/rootstock combination of apple to adapt to soil salinity based on their similar degree of salt stress-tolerance.

Key words: Salt tolerance; Callus browning; Re-differentiation; Root formation; Anatomical structures; *Malus* species.

Introduction

Over 800 million land hectares are inflicted by salt, which constitutes about 6% of the total world land (Munns & Tester, 2008). Salinity has been considered as one of the main limiting factors in crop production including apple (Ashraf & Akram, 2008; Ashraf *et al.,* 2012; Azhar *et al.,* 2012; Wang *et al.,* 2013). *Malus robusta* Rehd (Rehd) and *Malus domestica* Borkh. cv. Fuji (Fuji) are the most commonly used rootstock and scion combination for enhancing apple production. Rehd was a moderately salt tolerant species while evaluating the growth performance of 19 species or genotypes of apple rootstocks under saline stress (Du *et al.,* 2002). Therefore, to understand the biological mechanisms responsible for salt tolerance of both Rehd and Fuji, it is necessary to explore up what extent and how these plants respond to saline stress at different ontogenic phases (Ashraf *et al.,* 2012; Wang *et al.,* 2013).

One of the strategies against saline conditions is to delay growth rate (Al-Khayri, 2002; Zhang *et al.,* 2004; Patade *et al.,* 2008; Wang *et al.,* 2013). The growth retardation helps plants to conserve the metabolic energy against stress. Plant tissue culture is an expedient means to examine the relationships between whole plant and cellular responses to saline stress (Zhang *et al.,* 2004). The comparison of undifferentiated calli arising from Malus plantlets and the subsequent organs developed on the plant may enable differentiation between salt-induced cellular responses and tissue-specific reactions (Ashraf *et al.,* 2012; Wang *et al.,* 2013).

Several researchers have shown a positive relationship of growth with salt tolerance in a number of *Malus* species/cultivars (Fu *et al.,* 2013; Du *et al.,* 2002; Wang *et al.,* 2013; Vilaplana & Valentines, 2006). The callus growth and physiological responses under salinity were evaluated to select salt-tolerant *Malus* species in other studies (Visarada *et al.,* 2002; Wang *et al.,* 2013).

Most studies conducted so far have used plant seedlings of rootstock or scion in pot trials to examine the responses to salinity (Ashraf *et al.,* 2008, 2012; Wang *et al.,* 2013). In view of our present knowledge, there is little information available in literature about the effects of salt stress on biological and cellular responses of callus and plantlet in Rehd and Fuji species, i.e., a typical combination of scion/rootstock (Wang *et al.,* 2013). Considering above findings, we hypothesized that callus browning and re-differentiation, root formation by plantlets and anatomical structures of plantlet leaves under salt stress could be sound criteria for evaluating salt tolerance of *Malus* species and better combination of rootstock and scion species adapted to salty soils. Thus, the present study was focused to examine the salt-induced callus browning and re-differentiation, root formation of plantlets and alterations in anatomical structures of plantlet leaves in two *Malus* species.

Materials and Methods

Induction of callus and regeneration: An *In vitro* study was carried out using a callus and plantlets of Rehd and Fuji. Fresh leaf samples were subjected to sterilization in 75% ethanol for 30 s and in 0.1% $HgCl_2$ for 8 min. After washing the samples with sterile water, they were cut into small pieces of 0.5 cm × 1 cm size. Each explant was cultured onto MS medium (pH 5.8) that contained 0.2 mg L^{-1} 6-benzyladenine (6-BA), 30 g L^{-1} sucrose, 2 mg L^{-1} indole acetic acid (IAA), 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 6 g L^{-1} agar to induce callus (Li, 2001). The callus was isolated from the explants after 25 days and for further proliferation it was placed on a fresh media in a controlled growth chamber at a light intensity of 2000 lux for 14 h and temperature 25 ± 2°C. For inducing adventitious buds, the calli were shifted to the MS medium containing, 4 mg L^{-1} 6-BA, 0.2 mg L^{-1} IAA, and 30 g L^{-1} sucrose (Yu *et al.,* 2005). Before autoclaving, the pH of all growth media was adjusted to 5.8. The adventitious buds appeared after 30 days. The explants were then shifted to a fresh media and kept there for 25 days under the conditions mentioned below. One-leaf plant material having length greater than 0.5 cm was considered as a plantlet.

Salt stress treatment: All treatments were induced by supplying the MS medium containing 0 and 150 mM sodium chloride (NaCl). The callus and plantlets were shifted to the media as described earlier. For performing different assays, the calli and plantlet leaf samples were collected after 15 days of growth and washed well with distilled water (Zhang *et al.,* 2004). All leaf samples from each plantlet were of uniform size. A randomly selected sample from each of eight replicates was chosen and the 4th leaf from the base detached.

Callus browning and re-differentiation assay: Callus browning rate and callus re-differentiation rate were estimated according to the followings relationship (Ganesan & Jayabalan, 2005; Niroula *et al.,* 2007).

$$\text{Callus browning rate (\%)} = \frac{\text{Number of browning calli}}{\text{Total number of calli}} \times 100$$

$$\text{Callus re-differentiation determination (\%)} = \frac{\text{Number of adventitious bud-forming calli}}{\text{Total number of calli}} \times 100$$

Root formation of plantlets: Uniform plantlets (20-day old) with 1 cm height (5-6 leaves per plantlet) of two species were cultivated after cutting aging calli formed on the base from root induction MS medium (pH 5.8) containing 0 or 150 mM NaCl that was supplemented with 1 mg L^{-1} indole-3-butytric acid (IBA), 30 g L^{-1} sucrose and 6 g L^{-1} agar to induce root, then after 10 days on the 1/2 MS adventitious root elongation medium (pH 5.8) containing 0 or 150 mM NaCl containing 6 g L^{-1} agar and 15 g L^{-1} sucrose. Number and length of root were determined after 20 days. Average root number and rooting rate were calculated following Khatun *et al.* (2010).

Leaf anatomical structures: Leaf anatomical structures were observed using paraffin sections and photo-microscopy following the protocols described by Uaboi-Egbenni *et al.* (2009). After 15 days of salt treatment, rootless plantlets were excised from the top of the first two leaves and cut along both sides of the chief vein about 0.4 mm. The fixative solution was prepared using formalin 5%, acetic acid 5% and ethanol 90%. For dehydration all samples were passed for one hour through varying concentrations of alcohol, i.e., 50, 70, 80 and 95%. Thereafter the samples so treated were placed in ethanol for 16 h, and then transferred to alcohol: chloroform mixtures (3:1; 1:1; 1:3, v/v) and finally to pure chloroform. By adding parafilm slices to the chloroform at 45 min interval, the mixture was heated at 37°C for 48 h and then raised the temperature to 60°C in a water bath. The leaf samples were added to the molten paraffin and then they were transferred to paper boats. When the paraffin containing samples was solidified sample sectioning was carried out using a rotary microtome (Model RM2015), and then stained the sections with 1% methylene blue and mounted with euparol. Dewaxing and staining were done as follows: xylene two times, each time for 5 min; 1:1 xylene: ethanol, 10 min; 100% ethanol, 3 min; 100% ethanol, 3 min; 95% ethanol, 3 min; 85% ethanol, 3 min; 70% ethanol, 3 min; 1% safranin, 3 min; 70% ethanol, 1 min; 85% ethanol, 1 min; 95% ethanol, 1 min; 0.5% fast green, 6 s; 100% ethanol twice for 1 min; 1:1 xylene: ethanol, 1 min; xylene twice for 1 min. Finally, the sections were mounted with neutral gum and covered with coverslip. The sections were viewed and photographed using a microscope imaging system (OLYMPUS-BX5) at field of view 25 mm and a 40X objective lense. The size bar indicates 100 μm.

Results

Callus browning: A drastic increase in browning rate was observed in calli of both species after 15 days of salt stress (Table 1). The browning symptoms such as soft, watery, non-structured and black brown color were observed (Fig. 1). The browning rates of both species were above 80% (significant) in salt stressed tissues but less than 3% in control tissues (Table 1).

Callus re-differentiation: The differentiation rate of calli exhibited distinct performance when two *Malus* species were cultured on media treated differently (Table 1). Rehd and Fuji were able to produce numerous adventitious buds with a similar re-differentiation rate. The re-differentiation rates were observed after fifteen days of salt treatment. The re-differentiation rates of both species decreased due to salt stress. The ability of re-differentiation in Fuji was similar to Rehd irrespective of treatments (Table 1).

Root formation: In contrast to controls, saline conditions had significantly effects on root growth of two species. The rooting rates, root number and length of Fuji and Rehd decreased significantly under salt stress. Nevertheless, no significant difference on above root parameters was observed between two species under all treatments.

Table 1. Effects of salt stress on callus browning, callus re-differentiation and root formation of plantlets in *Malusrobusta* Rehd (Rehd) and *Malus domestica* Borkh. cv. Fuji (Fuji).

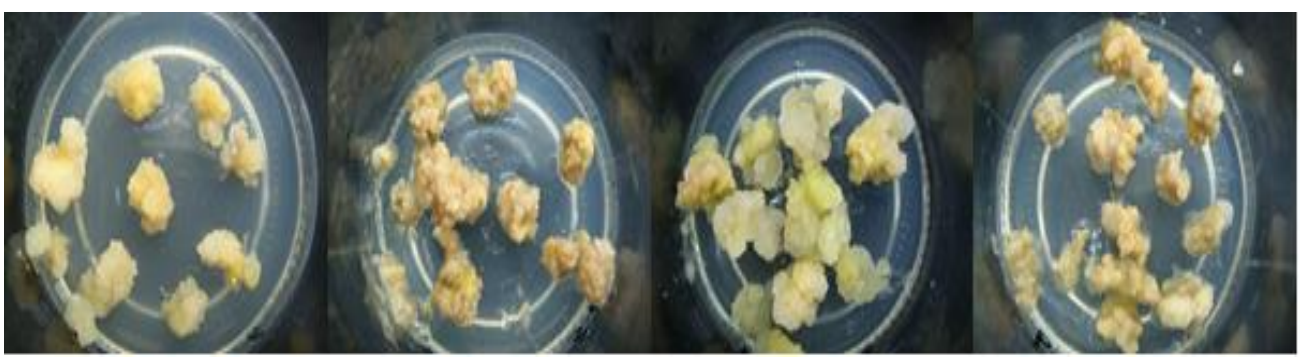
Parameter	Rehd		Fuji	
	Control	Salt stress	Control	Salt stress
Browning rate (%)	2.90 ± 0.40 b	87.7 ± 3.80 a	2.40 ± 0.30 b	90.80 ± 2.80 a
Re-differentiation rate (%)	55.40 ± 4.20 a	1.50 ± 0.50 b	60.00 ± 4.80 a	0.80 ± 0.05 b
Rooting rate (%)	100.0 ± 0.00 a	48.70 ± 4.20 b	100.00 ± 0.00 a	53.30 ± 5.60 b
Root number in average	9.07 ± 0.64 a	0.73 ± 0.23 b	9.53 ± 0.49 a	0.67 ± 0.25 b
Root length (mm)	47.58 ± 4.91 a	17.67 ± 3.18 b	50.15 ± 1.76 a	14.33 ± 2.03 b

Browning rate and re-differentiation rate were determined after 15 days of salt treatment. Root number and length were measured after 20 days of salt treatment. Values are means ± S.E. (n=8) means, which were compared using LSD test (p< 0.05) between any pair of data. Different superscript letters indicate significant differences between cultivars at p<0.05.

Table 2. Effects of salt stress on anatomical features of plantlet leaves in *Malus robusta* Rehd (Rehd) and *Malus domestica* Borkh. cv. Fuji (Fuji).

Parameter	Rehd		Fuji	
	Control	Salt stress	Control	Salt stress
Leaf thickness (μm)	102.33 ± 2.33 a	108.07 ± 3.06 a	102.67 ± 2.40 a	106.67 ± 6.77 a
Upper epi-cuticle thickness (μm)	12.33 ± 0.33 b	15.33 ± 1.76 ab	12.67 ± 0.67 b	17.33 ± 0.67 a
Palisade tissue thickness (μm)	24.00 ± 1.15 a	18.30 ± 1.15 ab	23.33 ± 1.33 a	14.67 ± 2.91 b
Proportion in leaf thickness (%)	23.52 ± 1.58 a	16.75 ± 1.55 b	22.71 ± 0.98 a	13.53 ± 1.87 b
Spongy tissue thickness (μm)	54.67 ± 3.71 a	60.67 ± 0.67 a	54.67 ± 1.76 a	60.67 ± 4.06 a
Proportion in leaf thickness (%)	53.31 ± 0.67 a	56.24 ± 2.31 a	53.26 ± 1.15 a	56.94 ± 2.31 a
Lower epicuticle thickness (μm)	11.33 ± 0.88 a	14.01 ± 1.81 a	12.34 ± 1.07 a	14.60 ± 2.28 a

The anatomical features were observed after 15 days of salt treatment. Values are means ± S.E. (n=8). Means were compared using LSD test (p<0.05) between any pair of data. Different superscript letters indicate significant differences between cultivars at p<0.05



CR-C **CR-S** **CF-C** **CF-S**

Fig. 1. Effects of salt stress on callus browning of *Malus robusta* Rehd (Rehd) and *Malus domestica* Borkh. cv. Fuji (Fuji). CR-C and CR-S indicate callus of Rehd cultured on the MS medium which contained 0 mM or 150 mM NaCl for 15d, respectively; CF-C and CF-S indicate callus of Fuji cultured on the MS medium which contained 0 mM or 150 mM NaCl for 15 d, respectively.

Anatomical structures: The anatomical structures of plantlet leaves were found changed after salt treatment in both species. Salinity led to reduced size of the leaf mesophyll and the whole leaf, butincrease upper thickness and lower epidermis (Table 2). The epidermis thickness in Fuji was similar to that in Rehd. However, the spongy parenchyma became thicker and shorter in salt-stressed plantlets than that in controlled, while leaf thickness increased considerably. The palisade tissue thicknesses of both species declined under salt stress.

Cross-sections of plantlet leaves of Fuji and Rehd showed a typical structure similar to that of *Malus* leaf (Fig. 2). The size of the chief veins was similar in control and salt-treated plantlets. In controls, palisade parenchyma was found to have two layers of dense cells, containing abundant number of chloroplasts. In most part of the mesophyll there were loose spongy parenchymtous cells with extensive intercellular spaces. The lower epidermis had smaller cells than the upper one (Fig. 2A, B). No significant difference was observed in terms of palisade tissue and spongy tissue between Fuji and Rehd plantlets under salt stress (Fig. 2C, D). Compared to controls, the chief vein became thinner and conducting tissue degenerated with less distribution of tiny vessels under salt stress. (Fig. 2E-H). Consequently, the cell structure was unorganized and irregular in both species.

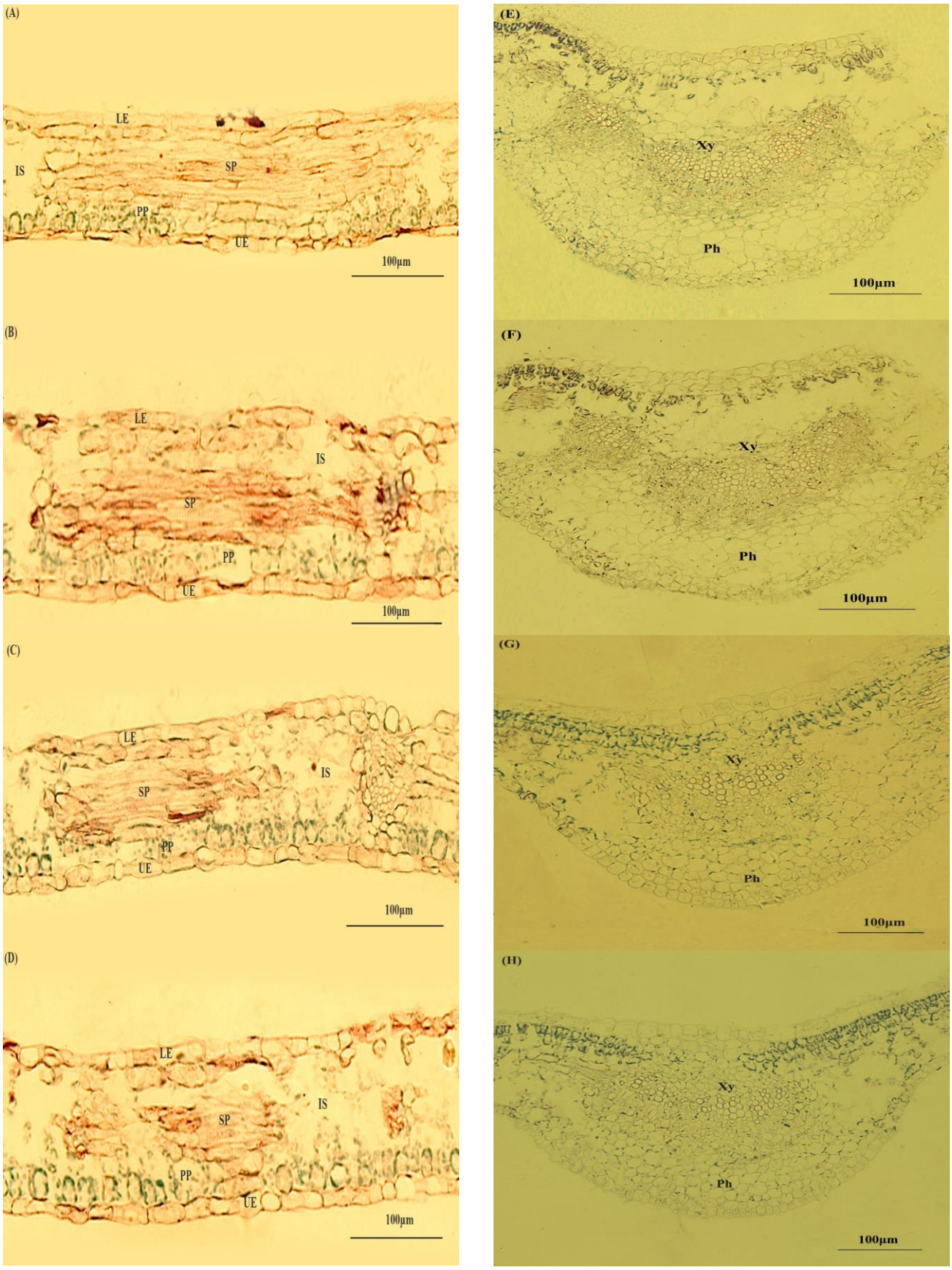


Fig. 2. Effects of salt stress on anatomical features of plantlet leaves in *Malus robusta* Rehd (Rehd) and *Malus domestica* Borkh. cv. Fuji (Fuji) (400X). A & B represent leaf of Rehd and Fuji, respectively, under control; C & D represent leaf of Rehd and Fuji, respectively, under salt stress; E & F represent chief veins of Rehd and Fuji, respectively, under control; G & H represent chief veins of Rehd and Fuji, respectively, under salt stress. UE (upper epidermis), PP (palisade parenchyma), SP (spongy parenchyma), LE (lower epidermis), IS (intercellular spaces), Xy (xylem), Ph (phloem).

Discussion

Salinity is known to suppress growth and yield of several crops including apple which could be due to perturbance in different metabolic processes (Ashraf *et al.*, 2012). Appraisal of salt-induce adverse effects on growth in some plants has been carried out at different differentiation levels (Ericson & Sharon, 1984; Tang & Newton, 2005). Cherian and Reddy (2003) reported that salt stress caused a marked inhibition in callus cultures of *Suaedanudiflora*. Although Watanabe *et al.* (2000) observed a significant inhibition in *Populuseuphratica* plantlets growth under saline stress (50 mM NaCl), they were unable to reveal the reason for differential responses of callus and plantlet to saline stress.

In our former and present studies, the calli of Rehd and Fuji grew well under 150 mM NaCl for 15 days. In both *Malus* species, a similar rise in MDA (malondialdehyde) content and RGR (relative growth rate) was observed at both different stages under saline regime (Wang *et al.*, 2013). The calli were more sensitive to salinity than plantlets in both species. The findings reported earlier suggest that the growth of callus was more sensitive to salt stress than that of plantlets of both species. Thus, Fuji and Rehd can be considered as a good scion/rootstock combination for apple to adapt to soil salinity.

Browning was reported to be a major problem during *In vitro* culture of bamboo (*Phyllostachys edulis* L.) which enhanced phenolic accumulation. The multiple shoots became blackish brown and ultimately dried up (Ganesan & Jayabalan, 2005). To evaluate the browning disadvantages, different species are incorporated into the media or soaked in the explants in different chemicals mentioned earlier elsewhere (Saxena & Dhawan, 1999). In the present study, callus cultured on a MS medium without NaCl was light green (Fig. 1, Table 1), and it effectively differentiated adventitious buds. However, when treated with 150 mM NaCl, the color became brown and it was unable to differentiate. Callus browning could lead to necrosis, tissue damage and generation of stress responsive metabolites/compounds such as phenolics. Callus browning suppresses callus growth and inhibits adventitious shoot formation (Tang & Newton, 2005). Change in callus color due to saline stress was noted in *Sorghum* bicolor (a relatively salt sensitive species of sorghum), whereas no change in callus color appeared in S. *halepense* (a relatively salt-tolerant species) (Yang *et al.*, 1990). In addition, Ogawa *et al.* (1999) claimed that the callus character depends on the genotype, regardless of the tissue or organ used as explants. We observed that the calli of Rehd were darker brown than those of Fuji, which might have resulted from their differential genetic expression at cellular level.

Two important biological processes i.e. bud differentiation and root formation exist in higher plant development (Munns & Tester, 2008). In our study, the capacity of adventitious bud formation from callus dedifferentiation and adventitious root formation from plantlets was greatly decreased under salt stress, resulting in generating less adventitious root formation compared with controls (Table 1). These results suggest that salt stress significantly inhibited root formation of plantlets of Rehd and Fuji. Similar reductions have been reported elsewhere. For example, 1% NaCl treatment inhibited callus differentiation of *Dendrobium* (Li, 2011) and 75 mM NaCl treatment decreased considerably the rooting rate of *Elaeagnus* (Xu, 2011).

Salt stress is believed to cause dramatic structural and anatomical changes (Wang *et al.*, 2011). The observation of leaf adaptive structures is considered meaningful for understanding the mechanism of salt-tolerant cultivars exposed to high salinity (Huang & Van Steveninck, 1990). In the current study, leaf structures of both species were found to be adversely affected, e.g., reduced size of palisade tissue and intracellular chloroplast, incomplete development of vessel structure in the conducting tissues and severe damage to phloem tissue of the plantlet leaves. In contrast, a couple of adaptive structural features appeared in the plantlet leaves including increased thickness of the upper and lower epidermis, elevated proportion of spongy tissue and the formation of lignified vessels (Fig. 2, Table 2). These anatomical characteristics may play a vital role in storing ions in large-sized vacuoles inside the plant body. Furthermore, the structures of Fuji showed similar deteriorative traits as found in Rehd.

Overall, in both species the callus stage was found to be more sensitive to saline stress than plantlet, because higher accumulation of MDA and lower RGR were found in the callus, quite similar to what has been observed earlier by Wang *et al.* (2013), as well as the greater browning rate and re-differentiation rates of adventitious buds observed in the callus in our present study. Rehd and Fuji had similar responses to salt stress as is evident from a similar increase in RGR and MDA content at both differentiation stages, similar callus browning and re-differentiation, root formation of plantlets and anatomical structures of plantlet leaves. These results indicate that Rehd and Fuji both could tolerate salt-stress through modifying leaf morphological traits, reflecting a similar adaptive potential to salinity. On the basis of structures of callus and plantlets, Rehd was as good as Fuji in terms of salt resistance. Thus, Rehd can be effectively used to form a scion/rootstock combination with Fuji.

In a nutshell, both *Malus* species were affected similarly due to saline stress. Furthermore, the callus stage was more sensitive to salinity than the plantlet stage as was evident from the browning rate and re-differentiation rates of adventitious buds in the callus. The *In vitro* study could be more beneficial to appraise salt-induced biological, physiological and cellular responses of two *Malus* species. Rehd and Fuji had similar responses to salinity in terms of callus browning and re-differentiation, root formation of plantlets and anatomical structures of plantlet leaves. This suggested that Rehd is suitable as a scion/rootstock combination with Rehd to adapt to saline soils.

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