Physiological and Morphological Responses of the 'Dargazi' Pear *(Pyrus communis*) to *in vitro* Salinity

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Summary

Salinity is one of the most important factors limiting growth and production of plants worldwide. Improving tolerance to salinity in plants is the most effective way to increase performance. Influence of salinity on growth, physiological and biochemical responses of *Pyrus communis* 'Dargazi' explants was investigated in *in vitro* conditions. Explants were cultured on Murashige and Skoog (MS) medium containing 1 mg L^{-1} BAP (6-banzyl amino purine) and 0.1 mg L^{-1} NAA (Naphthaline acetic acid) supplemented with different sodium chloride (NaCl) concentrations 0 (control), 40, 80, 120 and 160 mM. With increased salinity, the fresh weight, dry weight, the number of leaves, shoot length, chlorophyll index, multiplication index, leaf relative water content and total protein decreased. In contrast, proline, soluble sugar and activity of catalase increased.

Key words

salinity, chlorophyll index, catalase, protein, proline, *in vitro*

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Introduction

The pear tree (*Pyrus communis*) is a dicotyledon plant belonging to the *Rosaceae* family. Genus *Pyrus* consists of approximately 30 species. The pear tree is more sensitive to frost than apples, growing well in the sandy loam soils, while the heavy and wet soils are not suitable for it. In dry and calcareous soils, pear is grafted on the wild pear rootstocks (Jalili Marandi, 2008).

Salinity restricts the vegetative and reproductive growth of the plant by creating severe physiological disorders and producing common direct and indirect damaging effects, even at the low level of NaCl (Shannon et al., 1994). It is difficult breeding the plants in terms of tolerance to salinity in conventional methods, due to the multiplicity of the genes of this trait. Plants respond to different environmental stresses, and the ability of plants to adapt depends on the type, intensity and duration of stress and also on the species of plants and stage of stress (Munns and Tester 2008). Tissue damage is caused not only by the effects of osmotic salt but also is encouraged by specific toxic effects of chlorine and sodium accumulation (Hasegawa et al., 2000). Salt stress affects all the major processes such as growth, protein synthesis, and energy and lipid metabolism. There is considerable research information about the response to salt stress (Parida and Das, 2005).

At the beginning and during salt stress all the main physiological activities such as photosynthesis, protein synthesis, and energy are influenced within a plant. However, whole plant mechanism can participate in the inhibiting of stress through the plant's life cycle, endurance can also happen at the cellular level (Yokoi et al., 2002). Among the most common reactions to stress in higher plants is the production of some organic soluble materials for compatibility (Serraj and Sinclar, 2002). These materials are mostly carbohydrates such as sugars, amino acids, and proteins that play a role as osmolytes. One of them is proline that is normally produced in large quantities in response to environmental stresses (Kishor et al., 2005). A large number of carried researches suggest a positive correlation between proline production and environmental stress (Hayat et al., 2012). Osmotic pressure regulation is a way of resistance to osmotic stress. Cellular turgor stability, especially under stress conditions, is posible by the accumulation of metabolites such as betaine, glycine, proline, mannitol and sugars solution is possible (Heidari Shaif Abad, 2002; Houimli et al., 2010). Proline is a rich source of carbon, nitrogen and neutralizing free radicals. Proline acts as a protector for the structure of cell membranes and proteins (Jalili Marandi, 2009). Another function of proline is the maintenance of cell buffering capacity under salt stress (Heidari Shaif Abad, 2002). It inhibits plasmolysis and creats cell turgor. In the plant cells under some environmental stresses such as drought and salinity, larger molecules such as starch convert into sucrose, and then the later ones breake into smaller molecules such as glucose and fructose. This results in the negative potential of water in cells and osmotic modification. Reducing of sugar utilization is another factor in increasing the concentration of cell sugar content (Jalili Marandi, 2009). As well as, the decline of cell growth diminishes the transformation of soluble carbohydrates into structural polysaccharides and hemicelluloses. In the results, the accumulation of soluble sugars in plants happens (Jalili Marandi, 2009).

In vitro experiments are a desirable system for studying the genetic potential of woody plants that can be investigated under controlled conditions with definite space and time. Such cultures can supply useful information on the physiological and biochemical responses of woody species when exposed to salt stresses, especially if this is a response to the salinity that may be encountered in normal plant conditions. The routine reaction of most plants to the stress of salinity, both in *in vitro* condition and in the natural environment, is a decline in growth (Vijayan et al., 2003; Molassiotis et al., 2006).

The induced enzyme activity can reflect the defense response to cellular damage caused by high concentrations of NaCl in the culture medium, such as seen in the apple rootstock *in vitro* (Molassiotis et al., 2006).

Dimassi-Theriou (1998) claimed that low levels of sodium chloride concentration display a significant effect on shoot reproduction *in vitro*. NaCl at low concentrations *in vitro* performs a positive impact on plant growth because of the improved osmolarity (Flowers and Läuchli, 1983). Therefore, this study was carried out to evaluate the tolerance of pear (*Pyrus communis* 'Dargazi') to different levels of salinity. This cultivar is a popular rootstock due to its high graft compatibility, dwarfing and precocity potential. Despite its full testing in many different parameters, the knowledge of the responses of this rootstock to salinity is still unknown.

Material and methods

Healthy and uniform explants (approximately 2-3 cm in length) were transferred on Murashige and Skoog's medium (Murashige and Skoog, 1962). Medium contained macro and microelements, vitamins, 3% (w/v) sucrose, 1 mg L^{-1} BAP (Benzyl amino purine), 0.1 mg L^{-1} NAA (naphthaline acetic acid) and 6 g L^{-1} agar. The media were adjusted to pH 5.7 ± 0.2 with 1 N NaOH or HCl before autoclaving at 120°C and 1 kg cm^{-2} (15 psi) for 20 minutes. Explants were directly subcultured to the different levels of sodium chloride (NaCl 0, 40, 80, 120 and 160 mM). All cultures were kept in the growth room with temperature of 25±2°C, relative humidity 70%, under a 16/8 h (day/night) photoperiod with a light intensity of 50 µM m^{-2} s^{-1} provided by the cool white fluorescent lamps for six weeks. At the end of six weeks, fresh weight, dry weight, multiplication index (the multiplication index was calculated from the number of newly formed shoots (> 0.5 cm) per initial shoot tip recorded in each treatment), chlorophyll index, leaf relative water content, total protein, proline, soluble sugar and total catalase activity were measured. Leaf chlorophyll content was recorded in leaves by using a SPAD meter (Konica Minolta 502, Japan). Leaf relative water content was measured according to Barr and Weatherley's method (1962).

Proline content

Proline content was estimated according to Bates et al. (1973). Proline concentration was specified using calibration curve and expressed as µmol g^{-1} FW. Fresh plant material (0.1 g) was homogenized in 10 ml of 3% sulfosalicylic acid and the mixture was filtered. The filtrated material (2 ml) was treated with 2 ml ninhydrin reagent (1.25 mg ninhydrin in 30 ml of glacial acetic acid and 20 ml 6 M H_3PO_4) and incubated at 100°C for 1 h. The reaction was ended with placing in an ice bath. The reaction mixture was vigorously homogenized with 4 ml toluene. The absorbance of colored solutions was read at 520 nm after warming at 25°C. L-proline was applied as a standard.

Soluble sugar content

For extracting and analyzing of the leaf soluble sugar content, the method of Irigoyen et al. (1992) with some modification was followed. Fresh leaves (0.3 g) were blended in 5 ml of 98% ethanol;

0.1 ml of supernatant was combined with 3 ml of anthrone reagent. The absorbance of the colored solutions was recorded at 625 nm.

Total protein

Total protein content was determined according to Bradford (1976), using bovine serum albumin as a standard.

Catalase (CAT)

Catalase activity was measured according to the method Chance and Maehly (1995) based on H_2O_2 decomposition. Absorbance was read at a wavelength of 240 nm. Enzyme activity was measured in terms of absorbance per minute/ protein content.

Statistical analysis

The experimental design was a completely randomized design with four replicates (a vessel in each replicate). Each tissue culture vessel containing three uniform explants was used. Statistical analysis was performed using SAS 9.1 program. Means were compared by Duncan's multiple range test at $P < 0.05$.

Results and discussion

Results of this experiment revealed that the fresh weight, dry weight, leaf number, chlorophyll index, multiplication index and shoot length were significantly affected by levels of salinity. With increasing salinity, the fresh weight, dry weight, leaf number, chlorophyll index, multiplication index and shoot length were decreased (Table 1 and Fig. 1).

These results were in agreement with the study of Sotiropoulos (2007) that reported that by increasing the concentration of NaCl and $CaCl_2$ in the culture medium the number of branches, branch length, seedling fresh weight and leaf chlorophyll content in the apple rootstock M4 were significantly reduced compared to the control. Moreover, the results were also consistent with the results of Metwali et al. (2014) that reported that the salinity stress reduced significantly the length of shoots and the number of formed shoots (shoot multiplication index) *in vitro* culture of three fig cultivars of Saudi Arabia.

Table 1. Analysis of variance related to the effects of salinity stress on fresh and dry weight, leaf number, chlorophyll index, shoot length and physiological traits (proline, protein, catalase activity, soluble sugar and relative water content) of 'Dargazi' pear

S.O.V.[a]	df	Fresh weight	Dry weight	Leaf number	Chlorophyll index	Shoot length
salt	4	0.0441**	0.0047**	3.115**	1.825**	0.1594**
error	25	0.0095	0.000144	0.444	0.951	0.0352
CV(%)		8.914	9.3	12.62	5.36	12.28
		Proline	Protein	Catalase	Soluble sugar	RWC[b]
salt	4	0.1733**	0.000245**	26.364**	0.0289*	0.2313*
error	10	0.0031	0.0000055	1.0983	0.00046	0.0584
CV(%)		6.4	9.6	28.18	7.9	3.06

[a] Sources of variation, [b] Relative water content, *and ** - significant at 5 and 1%, respectively

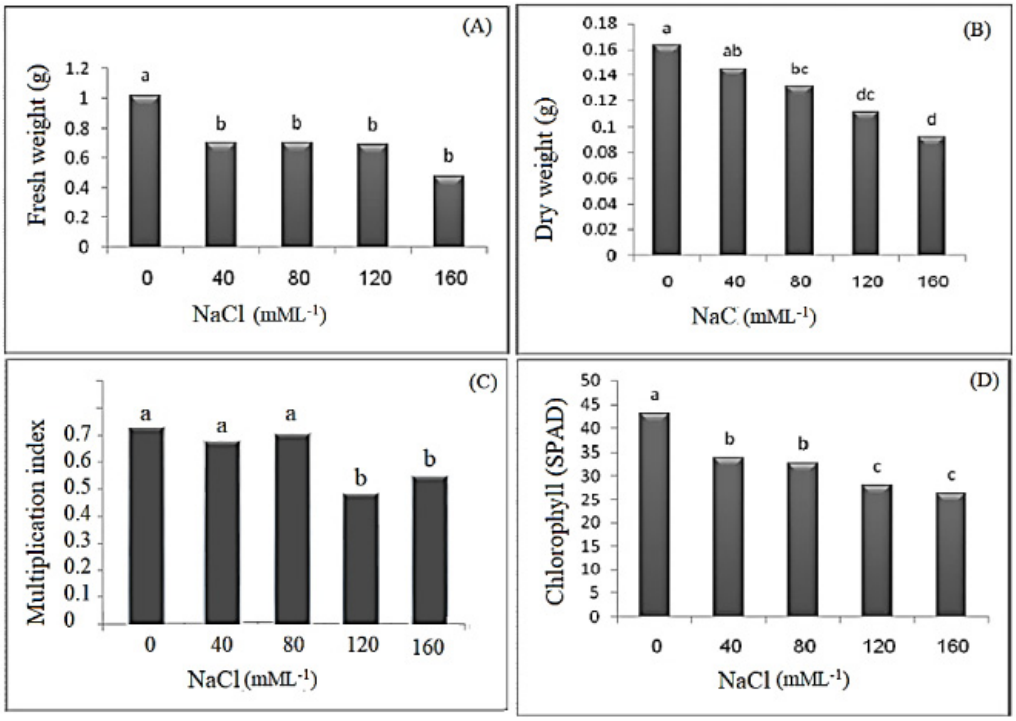

Figure 1. Effect of salinity on the fresh weight (A), dry weight (B), multiplication index (C) and chlorophyll index(D) in 'Dargazi' pear explants. Note: means followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$

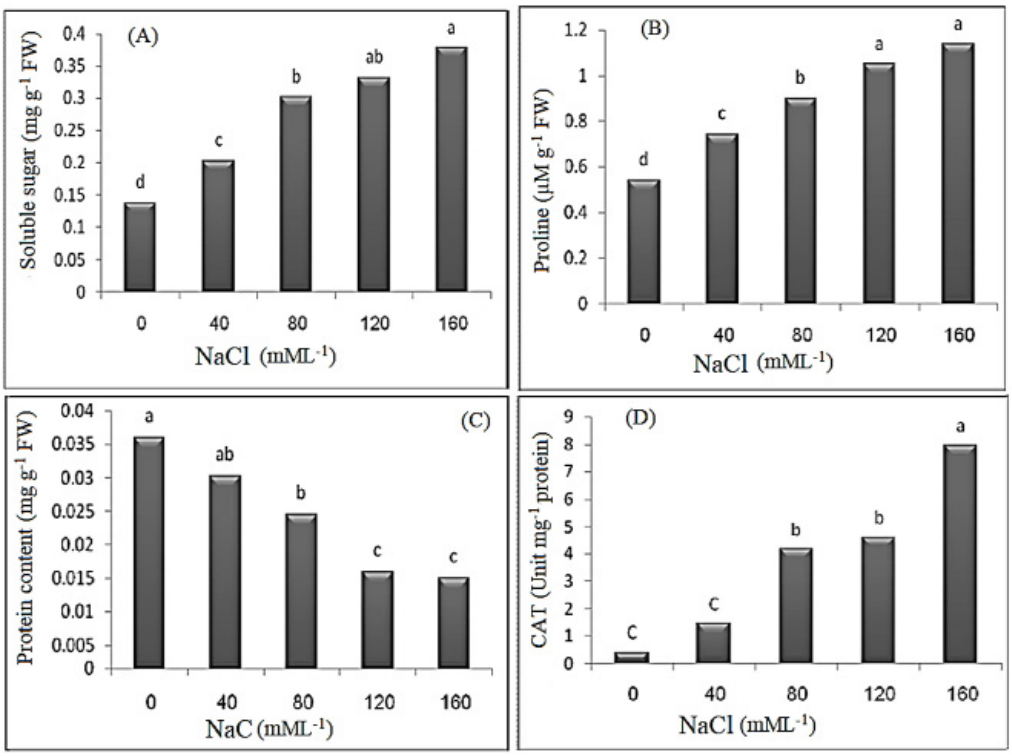

Figure 2.
Effect of salinity on soluble sugar (A), proline (B), total protein (C) and catalase activity (D) in 'Dargazi' pear explants; Note: means followed by the same letter are not significantly different according to Duncan's multiple range test at $P < 0.05$

It can be considered that reduction of leaf area is one of the first reactions of plants to the salinity stress. The accumulation of dry matter and leaf area are decreased with salinity, and the decrease in leaf area may be major reasons for the decrease in growth due to salinity. Salinity stress by decreasing cell proliferation and dry matter accumulation shortens the internodes and plant height and reduces the growth of aerial parts, resulting in reduced leaves dry weight (Zadorian et al., 2011).

The physiological parameters of pear explants such as total protein, proline, catalase activity, soluble sugar, and leaf relative water content (RWC) were significantly affected by salinity under *in vitro* conditions (Table 1).

The result of the means comparison showed that with increasing salinity levels, RWC of the leaves decreased (Table 1). Therefore, the lowest and maximum leaf water content (22.26 and 56.47%) was related to treatments of 160 and 0 mM NaCl, respectively. Under the stress conditions, plants face water deficiency; this occurs by reducing the external water potential due to high salt concentrations in the solution of the soil and its accumulation in the extracellular region of the plants (Romero-Aranda et al., 2001).

The result showed that different salinity levels had the highly significant effect on the chlorophyll index (Table 1 and Fig. 1). With increasing the salinity level, chlorophyll content (SPAD units) of the explants was declined. The least amounts (28.15 and 26.15 SPAD) were attributed to salinity levels of 120 and 160 mM NaCl (Fig. 1D). It could be associated to the breakdown of chlorophyll due to Cl^- accumulation in leaf tissue (Zekri, 1991; Melgar et al., 2008).

The effect of salinity treatments on the total protein content of the explants was very significant (Table 1). The lowest protein content was observed in the treatment of 120 and 160 mM (Fig. 2C).

Many proteins become hydrolyzed during stress and, accordingly, reduction in the concentration of soluble protein can be due to stress. But tensions reveal specific genes that help plant compatibility in adverse conditions. Therefore, it is possible to increase the concentration of soluble protein (Azooz et al., 2009). Moreover, reduction of N-synthesis happens in tissue. The protein loss caused by salinity can be due to degradation of protein structure, damage of biosynthesis pathway and reduction of the synthesis of protein. The effect of reactive oxygen species (ROS) on DNA and RNA can be one of reasons of protein reduction in salinity conditions (Grattan and Grieve, 1999). Salinity reduces the Mg uptake. It is one of the components in the ribosome, thus its deficiency affects protein synthesis (Bar et al., 1997).

As shown in Fig. 2B, salinity affects leaf proline so that proline in leaves is increased with increased salinity. Most of the proline content was obtained in 120 and 160 mM NaCl treatments, with 1.0504 and 1.1404 µM g^{-1} FW, respectively. This is consistent with the results of Doulati Baneh et al. (2013), Sayed et al. (2007) and Roussos and Pontikis (2003) that reported that salinity stresses increased proline in the grapevine, pineapple and jojoba. Proline is an amino acid that normally accumulates in large quantities in response to environmental stresses (Kishor et al., 2005). A large number of carried researches suggests a positive correlation between proline production and environmental stress (Hayat et al. 2012). Proline is a rich source of carbon, nitrogen and neutralizing free radicals. Proline acts as a protector of the structure of cell membranes and proteins (Jalili Marandi, 2009).

As shown in Fig. 2A, the number of soluble sugars increased with increased salinity. The highest sugar was obtained in 160 mM NaCl treatment. The same results were obtained by Masataka et al. (2000) on the effect of salinity stress on two Asian pear cultivars

in vivo. According to many researches, environmental stresses, especially drought and salt stress, increase soluble sugars such as sucrose, glucose, and fructose in plant leaves (Kafi and Masomi, 2003; Heidari sharif Abad, 2002; Sotiropoulos, 2007). In addition, the effect of salinity stress on soluble sugars accumulation in grapes was reported by Munns (2003). He proposed that the carbohydrate accumulation can act as a factor in maintaining turgor pressure and reducing osmotic pressure. One of the reasons for rising soluble sugar content due to salinity stress may be due to the decrease in growth (Munns, 2003). This can also be related to the lower mobility of sugar from the leaves and its lower consumption in the whole plant due to lower growth and breaking of the starch molecules (Sanchez et al., 2005).

The results of analysis of variance showed significant differences in the level of activity of catalase enzyme at $P < 0.01$ (Table 1). With increase of salinity, the activity of catalase enzyme increased and the lowest activity in control treatment was 0.3943 (Unit mg^{-1} protien), while the highest activity was 7.956 in the treatment of 160 mM (Fig. 2). This is in agreement with the results of Dolati Baneh et al. (2013) in study of the salinity stress on four grape varieties, and Erturk et al. (2007) on cherry and Masataka et al. (2000) on two pear varieties that reported that increased activity of catalase enzyme is greater in the treatments of salinity than in the control. It is suggested that the increase in salinity levels will increase the activity of the catalase enzyme, which is likely to limit the cell damage and increase the antioxidant capacity of the plant for resistance to the stress. This increase is an adaptive attribute to overcome the damage of tissue metabolism by reducing the level of toxicity produced by H_2O_2 during cellular metabolism and to protect against oxidative stress.

Conclusion

According to the results of the study on growth and physiological and biochemical reactions of 'Dargazi' pear explants, it can be suggested that with increased the salinity levels, the growth is reduced and this is a general response in the most plants except some halophyte plants. In addition, the salt tolerance had a negative correlation with the amount of sodium and chloride in plant tissue while osmolytes such as proline, soluble sugars, and increased antioxidant enzyme catalase showed positive correlation with increased salt tolerance. In all, there was not found any perished plant in the end of treatments, thus, it can be claimed that 'Dargazi' pear can tolerate up to 160 ml per liter of sodium chloride in *in vitro* condition.

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