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Effects of salt and drought stress conditions on callus growth, proline content and antioxidant enzyme avtivity of *Punica granatum* 'Nana'

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ABSTRACT

Callus of *Punica granatum* 'Nana' were cultured on MS medium supplemented with various concentrations of BA and NAA to evaluate callus growth. The highest callus fresh weight (0.61 g), dry weight (0.068 g) and diameter (16.16 mm) were observed in 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA. In a separate experiment, growth and antioxidant activity (Super oxidismutase (SOD), Catalase (CAT) and Peroxidase (POD)) of callus were studied in plants grown under saline (0 -5 g L^{-1} NaCl) and drought (0 to 8% PEG) conditions. Results showed that under both conditions, growth of callus decreased with increasing in stress treatments. Under saline condition, increasing in salinity increased antioxidant enzyme activity. The highest CAT, POD and SOD activities observed in 4, 5, 2 g L^{-1} NaCl, respectively. While under drought conditions, the highest SOD and CAT activities observed in 6% PEG and highest POD activity occurred in 8% PEG. The survived tolerant calli obtained had potential to produce salt and drought tolerant cultivars.

KEYWORDS

Dwarf pomegranate;
Proline;
Antioxidant enzyme;
Salt;
Drought.

INTRODUCTION

Drought and soil salinity are two major environmental a biotic stresses especially in arid and semi arid regions of the world. These stresses can lead to change in water availability in plants and reduce growth and CO_2 fixation. *In vitro* culture of plant cells and tissues on the medium containing selective agent such as NaCl for salt tolerance and polyethylene glycol (PEG) for drought tolerance is an effective method to improve cultivars. When the cells are exposed to a broad range of selective agents for a long time, only cells capable sustaining such adverse conditions could be selected. Plant cells have developed complex antioxidant systems including superoxidismutase (SOD), catalase (CAT), peroxidase (POD) to protect themselves from salt stresses[12,20,26]. Simiraly, characterization of selected drought tolerance plants is also based on accumulation of compatible solute mainly proline as well as estimation antioxidative enzyme[16,19,21,24]. Identification and selection of drought and salt tolerance cells are also useful to produce salt and drought tolerance cultivars. Pomegranate is an important tree cultivated as fruit tree, medicinal plant and also it's used as ornamental plant.

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Identification and selection salt and drought tolerant pomegranate trees could be used to increase cultivation area of this plant in arid conditions.

EXPERIMENT

A dwarf cultivar of *Punica granatum* 'Nana' ('Minature') with 30 to 50 cm height was used in this investigation. Plants were grown in greenhouse of the Department of Horticultural Science, Shiraz University, Iran. Calli were derived from leaf explants culured on MS medium supplemented with various concentrations of BA (0, 0.5 and 1 mg L^{-1}) and NAA (0, 0.5 and 1 mg L^{-1}) for selecting the best calli growth medium. All treatments were maintained for 4 weeks under dark condition and callus fresh and dry weights and diameters were measured. To induce *in vitro* salt and drought stresses, green and friable pieces of calli about 5 mm in length were cultured on MS basal medium supplemented with 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA (control treatment) plus various concentrations of NaCl (1, 2, 3, 4, 5, 6 and 7 g L^{-1}) and PEG (2, 4, 6 and 8 %). All treatments maintained under a 16-hour photoperiod with 1500 lux light intensity emitted from cool-white fluorescent lamps. After 30 days, dry and fresh weights of calli were measured. Then calli were transferred to new fresh medium and after 2 subsequent subcultures, treatments were evaluated in relation to proline contents and antioxidant enzymes activity (SOD, CAT and POD). To determine antioxidant enzymes activity in each treatment, 1 g fresh callus sample was homogenized in 2 ml ice cold 0.1 molar phosphate buffers (pH 7.5) containing 0.5 mM EDTA. The supernatant was used for the enzymatic assay. The enzymes activity of SOD was measured by a method suggested by Beauchamp and Fridovich[5] and CAT and POD were measured using Chance and Mahley[6] method. SOD activity was estimated by recording the decrease in absorption of superoxide-nitroblue tetrazolium complex by the enzyme at 560 nm. CAT and POD were estimated as consequences of H_2O_2 consumption at 240 nm and purpurogallin formation at 420 nm, respectively. Enzyme activity was expressed in U (U = 1mM of H_2O_2 reduction min^{-1} mg^{-1} protein). Extraction and estimation of free proline were conducted according to the procedures described by Bates *et al.*[4] at 520 nm. All experiments were conducted in a complete randomized design with 3 replications, and each replication consists of 4 explants. Means separation was carried out by LSD test with MSTAT-C experiment.

RESULTES

Callus growth

Maximum callus growth was obtained in MS medium supplemented with 1 mg L^{-1} BA and 1 mg L^{-1} NAA. This treatment had the highest callus fresh weight (0.61 g), dry weight (0.068 g) and diameter (16.1 mm) and was significantly different *(P<5%)* with other treatments except a treatment containing 1 mg L^{-1} BA and 0.5 mg L^{-1} NAA (TABLE 1).

TABLE 1 : Effects of BA and NAA concentrations on callus growth of dwarf pomegranate

BA (mg L^{-1})	NAA (mg L^{-1})	Fresh weight (g)	Dry weight (g)	Diameter (mm)
0	0	0.225 d*	0.025 e	10.75 b
0	0.5	0.313 cd	0.035 de	11.67 b
0	1	0.30 cd	0.035 de	11.66 b
0.5	0	0.366 bc	0.048 cd	12.08 b
0.5	0.5	0.345 bcd	0.038 de	12.80 b
0.5	1	0.423 bc	0.046 cd	13.50 ab
1	0	0.461 b	0.053 bc	12.58 b
1	0.5	0.605 a	0.065 ab	15.75 a
1	1	0.611 a	0.068 a	16.16 a

***Values with the same letters in each column had no significant differences (P<5%) using LSD test**

Salt stress condition

Salinity decreased the fresh weight of callus. The maximum fresh weight was observed in control medium (296.7 mg). Increasing in salinity decreased the fresh weight of callus, so that in 5 g L^{-1} NaCl minimum fresh weight (233.3 mg) was observed which had significant differences (*P*<5%) with other treatments except 4 g L^{-1} NaCl. In higher concentration of NaCl (up to 6 g L^{-1}) browning and death of tissue were occurred (TABLE 2). Similar to these results, dry weight of callus decreased with increasing the salinity. Maximum dry weight observed in control medium (43.67 mg). Adding NaCl to medium decreased dry weight and minimum dry weight observed in 5 gL^{-1} NaCl (30.6 mg). There were significant differences (*P*<5%) between 4

and 5 g L^{-1} NaCl compated to control (TABLE 2).

TABLE 2 : Effects of NaCl concentrations on fresh and dry weights and proline content of callus of dwarf pomegranate.

NaCl (g L^{-1})	Fresh weight (mg)	Dry weight (mg)	Proline (µM g^{-1} f.w.)
0	296.7 a	43.67 a	3607 d
1	290 ab	38.43 ab	4166 c
2	260 cd	36 ab	5368 b
3	273.3 bc	34 ab	5232 b
4	246.7 d	31 b	5732 ab
5	233.3 d	30.6 b	6180 a
6	-	-	-

*** Values with the same letters in each column had no significant differences (P<5%) using LSD test**

Proline content of callus gradually increased with increasing in salinity. Maximum proline content was 6180 (µM g^{-1} f w) in 5 g L^{-1} NaCl which had significant differences (P<5%) with other levels of salinity. (TABLE 2). The proline content of callus in 2 to 4 g L^{-1} NaCl was stable and then significantly increased in 5 g L^{-1} NaCl and was about 2 folds of control.

Activity of all antioxidant enzymes increased under saline conditions. Peroxidase activity gradually increased and received to maximum (73.24 µmol min^{-1} mg^{-1} fw) in 4 g L^{-1} NaCl. There was a significant (P<5%) differences between 4 g L^{-1} NaCl with 1, 2 and 5 g L^{-1} NaCl (Figure 1)

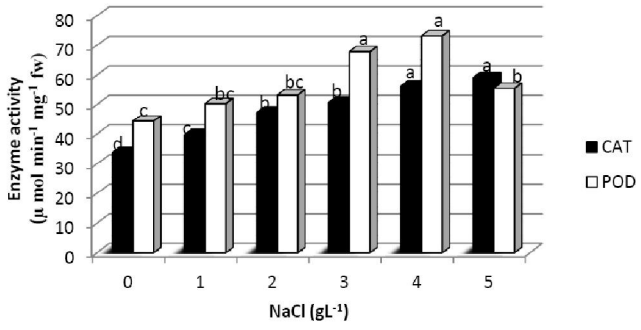

Figure 1 : Changes in CAT and POD activities in callus of dwarf pomegranate under saline conditions. Values with the same letters in each enzyme had no significant differences (P<5%) using LSD test

Catalase activity was measured by measuring H_2O_2 content. Minimum level of CAT activity observed in control (32.88 µmol min^{-1} mg^{-1} fw). Increasing in salinity gradually increased CAT activity, so that in 5 g L^{-1} NaCl rechead to 59.12(µmol min^{-1} mg^{-1} fw). There were significant (P<5%) differences between 4 and 5 g L^{-1} NaCl with other treatments.

Maximum SOD activity during salinity observed in treatments containing 2 g L^{-1} NaCl (444.7 µmol min^{-1} mg^{-1} fw), which had significant differences *(P<5%)* in comparison to control. However, there were no significant differences between treatments containing NaCl. Increase or decrease in salinity decreased SOD activity accordingly (Figure 2).

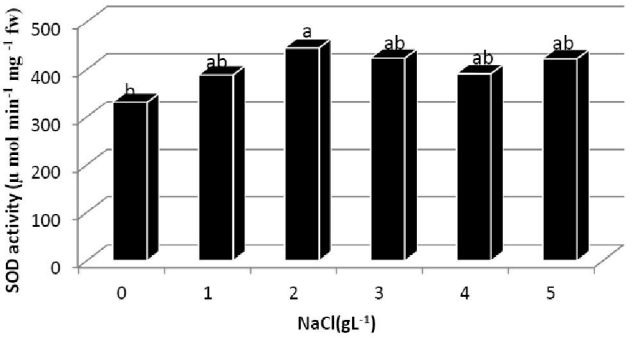

Figure 2 : Changes in SOD activity under saline conditions in callus of dwarf pomegranate. Means with the same letters had no significant differences by LSD test (P<5%)

Drought stress condition

Calli were cultured in MS medium containing various concentrations of PEG to induce drought stress. Data presented in TABLE 3 showed the effect of various concentrations of PEG in fresh weight of callus. Fresh weight of callus increased with increasing PEG concentrations, the highest fresh weight obtained in 4% PEG (330 mg). Increasing in PEG concentrations up to 4% decreased fresh weight of callus and reached to 146.7 mg in 8% PEG. There was a significant (P<5%) difference between 4% PEG with other levels of PEG in relation to fresh weight of callus. Effects of PEG on dry weights of callus are shown in TABLE 3. Similar to fresh weight, the maximum dry weight observed in 4% PEG (58.67 mg). Increasing or decreasing PEG contents of medium, significantly (P<5%) decreased dry weight of callus. Minimum dry weight (35.67 mg) was observed in 8% PEG which had significant differences (P<5%) with other treatments. The proline content of callus under drought stress condition slowly increased with increasing in PEG concentrations of medium, the highest proline content was 4471(µM g^{-1} f. w) in 8% PEG which had significant (P<5%) differences with control and 2% PEG (TABLE 3).

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TABLE 3 : Effects of PEG concentrations on fresh and dry weights and proline contents of callus of dwarf pomegranate.

PEG (%)	Fresh weight (mg)	Dry weight (mg)	Proline (µM g^{-1} f w)
0	296.7 ab	43.67 b	3637 b
2	300 ab	46.67 b	3309 b
4	330 a	58.67 a	3848 ab
6	206.7 b	46.67b	3908 ab
8	146.7 b	35.67c	4471 a

*** Values with the same letters in each column had no significant differences (P<5%) using LSD test**

The activity of CAT and POD under drought condition are shown in Figure 3. Peroxidase activity of callus significantly (*P*<5%) increased with increasing in drought stress conditions. The maximum POD activity

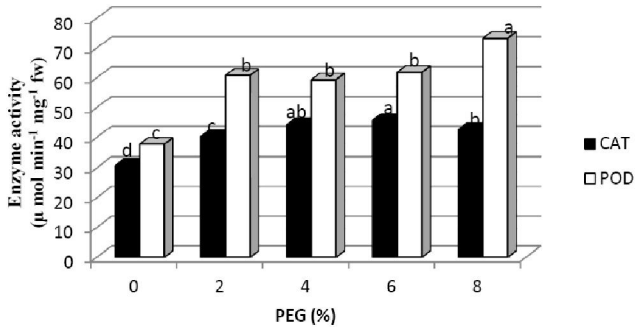

Figure 3: Changes in CAT and POD activities under drought conditions in callus of dwarf pomegranate. Values with the same letters in each column had no significant differences (P<5%) using LSD test

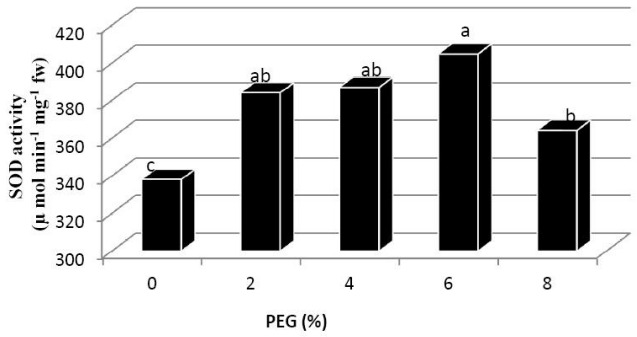

Figure 4 : Changes in SOD activities under drought conditions in callus of dwarf pomegranate.Values with the same letters in each column had no significant differences (P<5%) using LSD test

was 73.31 µmol min^{-1} mg^{-1} fw observed in 8% PEG which had significant (*P*<5%) differences with other treatments. Catalase activity measured as H_2O_2 content. Increasing in PEG concentrations of medium, to induce drought stress resulted in gradually increasing in CAT activity. In 6% PEG the highest activity of enzyme was observed (45.68 µmol min^{-1} mg^{-1} fw) which had significant (*P<5%)* differences with control, 2 and 8% of PEG. There were significant (*P<5%)* differences between treatments in relation to SOD activity. The maximum SOD activity observed in 6% PEG (404.7 µmol min^{-1} mg^{-1} fw) which had significant (*P<5%)* differences with control and 8% PEG. Increasing in PEG concentration gradually increased SOD activity. However, in 8% PEG, SOD activity was decreased (364 µmol min^{-1} mg^{-1} fw). (Figure 4)

DISCUSSION

Plant tissue culture has been proposed as a useful, quick and economical tool for evaluating the salt and drought tolerances via shoot organogenesis from leaf explants. Adding NaCl to medium changes growth of callus which is due to change in photosynthesis activity of tissue and osmotic condition. Salinity causes ion-speciûc stresses resulting from altered K^+/Na^+ ratios leads to build up in Na^+ and Cl^- concentrations that are detrimental to plants[27]. The percentage of reduction of the dry weight, generally considered as a sensitivity index. Reduced growth rate started in 2 g L^{-1} NaCl, this concentration was critical level for tolerant of tissue in *Punica granatum* ½Nana¼ In fact, during the application of salt stress, some calli showed NaCl-tolerance (lack of browning and keeping a continuous growth). These calli had potential to be used for evaluating enzyme activity

Reduction in growth rate under drought condition was observed in 6 and 8% PEG treatments. These reductions in growth rate were significant and probably were related to water content of tissue. This result is in agreement with many reports in tomato[1] and palm[2]. Moderate level of PEG (less than 4%) had positive effects in fresh and dry weights and also shoot regeneration of callus (data not shown). in some reports, positive effects of low level of PEG in growth and embryogenesis of callus are shown[11,25]. Scavening of H_2O_2 by CAT induced organogenesis. CAT is known to play a role in growth and organogenesis[9]. Increasing drought and salinity increased proline content of callus[2, 3]. Higher plants accumulate free proline in response to external salt and drought stresses. Proline can act as an osmotic

regulating agent and protect enzyme and cellular structure, also as storage compound to reduce nitrogen during the process of rapid re-growth after stresses. In this study, an increase in proline content was observed in both conditions. The results of this investigation are in agreement with earlier reports on proline accumulation under stress conditions in seedlings, as well as in fully grown plants[7,8]. Under normal growth conditions, the production of reactive oxygen species (ROS) in the plant cell is generally at low level. However, under salt stress, cellular homeostasis is disrupted and leads to the production of relatively high levels of ROS[13,17,22]. These radicals can damage vital cellular macromolecules (example via denaturation of proteins, peroxidation of lipids). In this study, ROS activity increased in both drought and saline conditions. The SOD enzyme destroys the superoxide radical; however, as a result of that it creates hydrogen peroxide, which also has high toxic properties. It was stated by other researchers that SOD activity increases with salt application[10,15]. CAT and POD eliminated H_2O_2 by decomposing it directly to water and oxygen[1,29]. In this study, Salt and drought treatments increased CAT and POD activities under stress conditions. In high level of salinity (5 g L^{-1}), POD activity decreased and CAT activity increased. But in higher level of PEG, the activity of POD and CAT was reversed so that, CAT activity in the highest level of PEG decreased and POD activity increased. These differences in POD and CAT may be related to the kind of homeostasis ion that produced under drought and saline conditions. Under saline conditions high level of Na ions were produced and affected enzyme activity and ion ratio. However, under drought conditions, water deficit occurred and mainly affected water potential and increased concentration of solute within cell. Therefore, differences in antioxidant enzyme activity under drought and saline conditions mainly related to changes in ions and osmotic condition. Measuring antioxidant enzyme of callus generally showed a similarity to the results obtained from experiments conducted with the intact plants[18,23,28,29]. But the callus culture gave more useful parameters to screen the plant for salt-tolerance. For example, SOD activities were increased in callus tissues of all of the melon genotypes, except salt-sensitive ones[14]. In conclusion, the antioxidative enzyme activities play a protective role against salt and drought-stress, and that antioxidative defense mechanism was effective in providing tolerance to salt and drought stress. Callus with high levels of antioxidant activity has potential to produce salt and drought tolerance cultivars

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