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ORIGINAL ARTICLE

Study of Potato (*Solanum tuberosum* L. Agria Cultivar) Microtuberization and Physiological Properties in Salinity Stress via Tissue Culture Technique

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KEYWORDS

Physiological properties;
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ABSTRACT: One of the most important crop plants is potato. Plant tissue culture is a very important technology in plant cultivation. In this investigation, the biochemical characterizes are investigated of potatoes cultivated by the tissue culture method. The lateral and apical buds were separated from the stem. They were sterilized and cultured in Murashige and Skoog culture medium. The concentrations of salt (0, 25, 50, 75 and 100 mM) have been used for salinity. After the growth of buds, the leaves were sampled and the chlorophylls, proline, sugars, protein, and antioxidant enzymes activity such as catalase, superoxide dismutase, guaiacol peroxidase, glutathione reductase and ascorbate peroxidase were evaluated. The results indicate that salt decreased protein and chlorophylls content. Sugars content and fresh weight have also been reduced with salinity, but the content of proline, malondialdehyde, and hydrogen peroxide were increased under salinity stress. Phenolic compounds, anthocyanin content, and antioxidant enzymes activity increased up to 50 mM salinity, but they decreased above this concentration. According to these results, it can be suggested that Agria cultivar potatoes are not recommended in salinity higher than 50 mM.

INTRODUCTION

The Potato has an important role in the nutrition of the people of the world. One of the most important crops is potato, producing 388 million tons of crops, ranked fourth after rice, wheat, and corn in the world [1, 2]. The potato has protein, nutritious mineral elements and antioxidant substances such as vitamin E and C, phenolic, flavonoid carotenoid in potato. [3]. The best growth of this plant is in cold regions with full radiation and cool nights and moderate days. Potatoes are cultivated in many tropical and subtropical countries. Potatoes are primarily a temperate climate crop. One of the most important environmental stresses is salinity, which limits growth and yield of crops by decreasing the osmotic potential and impairing the absorption of certain nutrients [4]. Increasing sodium and chloride ions also reduces the absorption of essential ions, including potassium ions, calcium, ammonium, and nitrate, which also reduced the enzymes and it changes the flexibility of the membrane [5]. Salinity stress in plants causes the production of reactive oxygen species (ROS), including: H_2O_2, $^{\cdot}OH$, and $^{\cdot}O_2$ [6]. These ROS are reactive

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and can damage cell metabolism through oxidative damage of nucleic acids, proteins, and membranes (protein denaturation, DNA mutation, and lipid peroxidation). Potatoes tolerance to saline water is as moderately salt-sensitive. Many experiments to show the salinity sensitivity of potato have been done on several cultivars and varieties of potato in greenhouse (in pot) or field experiments in India, Tunisia, Pakistan and many countries [7]. Tolerance to salinity is different in varieties and species, salinity reduces the yield by reducing the tuber weight relative to the number of tubers. Some of the research evaluated the tolerance of potato varieties in salinity conditions. Sudhersan *et al*., show in the laboratory test of 25 potato cultivars, it was found that 7 cultivars were tolerant and the rest were sensitive [8]. Among the 8 cultivars that were screened for salt tolerance, it showed Kroda potato variety is salt tolerance [9]. Munira *et al*., to explore salinity tolerance among 10 potato cultivars, found that Diamant was slightly salt tolerant while, Felsina was slightly salt tolerant [10,11]. A large transport of Na^+ into the tonoplast (in exchange for H^+) is necessary to reduce Na^+ toxicity [12]. Jarsma and Boer show that high affinity for Na^+ absorption can be an important factor to explain the difference in salt tolerance of two potato cultivars [13].

Plant tissue culture is based on the totipotency property. It has a main role in biotechnology. The most important application of plant tissue culture is mass propagation of plants, production of haploid, virus-free, stress-resistant plants [14]. Tissue culture of potato plant helps in the production of varieties resistant to stresses and production of microtubers. Investigating biochemical and physiological characteristics in tissue culture seedlings is easier and more accurate because the conditions in plant tissue culture can be controlled [15].

Assessing the physiological characteristics and microtuberization of plant responses to salt stress helps us to discover biochemical pathways and use these results to improve agricultural products. Many agricultural areas are relatively salty in Damghan, Agira is one of the most potatoes cultivated in Damghan. However, the Agria variety was not examined for its tolerance to salinity in vitro compared with the other cultivated potato variety. For this purpose, the research was done to compare the physiological and biochemical characteristics of the Agria cultivar under salt stress in vitro.

MATERIALS AND METHODS

In this study, the lateral buds of the vegetative stems were used. After providing the lateral buds, they were sterilized in sodium hypochlorite (5%, 20 minutes) and after three times washing sterilized distilled water (15 minutes) under the laminar-flow hood. Murashige and Skoog medium (3% sucrose, 0.8% agar and pH=5.5) was used. Each side bud or node was transferred to a culture medium with salt concentrations (0, 25, 50, 75 & 100 mM NaCl) with three replications. After 4 weeks, to measure the physiological and biochemical characteristics, samples were taken from the leaves of the seedlings. For induce microtuberization were used MS media containing 10 milligrams per liter hormone 6-Benzylaminopurine (BAP) and 80 grams per liter sucrose with different concentrations of salt (0, 25, 50, 75 & 100 millimolar NaCl).

Photosynthetic pigments

One gram of leaf tissue was homogenized and it was centrifuged (acetone 80%, 5000 rpm). The absorbance of homogenate was identified according to the method described by Lichtenthaler [16].

Phenolic and hydrogen peroxide

Total phenolic compounds were identified by the Folin Ciocalteu's reagent and gallic acid use for standard (Gao *et al*., 2000). 500 µL of each extract or gallic acid was mixed with 5 ml of Folin Ciocalteu's reagent and 4 ml of aqueous Na_2CO_3. The absorbance of mixtures was identified at 765 nm after 15 minutes [17]. Hydrogen peroxide was determined by spectrophotometric method (Alexieva *et al*., 2001). Leaf tissues were mixed with 5cc of TCA. The obtained mixture was centrifuged. 0.5cc of the extract was reacted with 0.5^{cc} of KH_2PO_4 buffer and 2cc of reagent (1M KI). Then the absorbance was read at 390 nm after 1 hour in the dark [18].

Estimation of Anthocyanin, proline and sugars

The amount of anthocyanin was identified by the method of Wagner. 100 mg of tissue was mixed in acidic methanol and kept for 24 hours in the dark. Then the homogenates were centrifuged and finally the absorbance was read at 550 nm. The extinction coefficient for anthocyanin was considered to be 33000 M^{-1} cm^{-1} [19]. The method of Bates *et al.*, was used for measure the amount of proline. Leaf tissue (0.5g) was mixed with sulfosalicylic acid (3%), then the mixture was centrifuged. Glacial acetic acid and ninhydrin were added to the solution and placed in water (100ºC) for 1 hour. Then after cooling, 4^{CC} of toluene was added and mixed vigorously. Finally, the absorbance was read at a wavelength of 575 nm [20]. The amount of sugars was calculated by acid hydrolysis with sulfuric acid and creating furfural complex according to Roberts *et al.*, 1959. 0.5 gram of leaf tissues was mixed in water and centrifuged. Then phenol and sulfuric acid were added to the solution. The absorbance at 485 nm was read after 60 minutes [21].

MDA & Other aldehydes

The method of Heath and Packer was used for the malondialdehyde calculate. Leaf tissue(0.5g) was mixed in tri chloro acetic acid and centrifuged. The obtained solution was mixed with thiobarbituric acid and boiled in water for 25 minutes. Finally, the absorbance was read at 600, 532, and 455 nm. The extinction coefficient for MDA was considered to be 155 M^{-1} cm^{-1} [22]

Protein & enzyme extraction

The amount of protein amount was used by Bradford method. 0.5 grams of leaf tissue was mixed in 4^{cc} of extraction solution. The extraction solution consisted of Tris-HCl buffer (0.05 molar with pH=7.5), 3mM magnesium chloride, 1mM Ethylene diamine tetra acetic acid 1.5% W V^{-1} PVPP. The extraction solution used to measure ascorbate peroxidase contains 200 µM ascorbate. The mixtured solution is centrifuged for 20 minutes in 25000g and the resulting solution is used as a stock solution to measure the enzymes activity [23].

SOD activity

The method of Dhindsa *et al.*, (1981) was used to measure of SOD activity. The reaction solution (3mL), contains phosphate buffer (pH=7.8, 50mM), NBT, riboflavin, EDTA and 100 µl enzyme mixture. Finally, riboflavin was added and they were placed in a shaker under a fluorescence light source (30 cm) containing two 15-watt fluorescent lamps for 10 minutes and absorbed at 560 nm [24].

CAT activity

The method of Maehly and Chance (1959) was used to measure of the Catalase. The reaction solution consisted of phosphate buffer (50mM, pH=7.4), hydrogen peroxide, enzyme mixture, and reduced the absorption of 240 nm due to the removal of peroxide hydrogen was measured over a minute [25].

APX activity

The methods of Chen and Asada was used to measure of the APX activity. The reaction solution contains of a EDTA, phosphate buffer, ascorbate, hydrogen peroxide, and 50 mL of enzyme mixture. Then the reduction in absorbance was measured at 240 nm by oxidation of ascorbate during one minute [26].

GPX activity

The methods of Upadhaya et al., (1985) was used to measure of the GPX activity. The reaction solution consisted of H_2O_2 (1%), guaiacol (1%), phosphate buffer (pH=6.1, 50 mM) and of enzyme solution. Then the increased absorbation at 420 nm during One minute was measured [27].

Gr activity

The activity of glutathione reductase was measured by Klapheck *et al.*, and reaction solution consists of buffer (Tris-HCl, pH=8, 0.1 mM), NADPH (0.1mM), EDTA (1mM), GSSG (1 mM) and enzyme solution. Then the reduced (NADPH oxidation) absorbation (334 nm) was measured over a minute [28].

Statistical analysis

Each experiment was repeated three times. Then results were analyzed using ANOVA and reported as mean ± standard error. Duncan test was used for differences between means. Differences where $p \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Physiological properties

The results show that salinity reduces the content of carotenoids, chlorophylls (a, b, & total), protein, soluble sugars (Table1). Also their amount decreases more, with increasing salinity concentration. The maximum amount was measured in the 0mM (control) treatment and the minimum amount in the 100 mg treatment. Chloroplast organelles are most affected by salinity. Salt damages the chloroplast structure and colored protein compounds [2]. Carotenoids are also damaged. The salt stress is reduced the content of chlorophylls. Salinity has decreased the amount of protein [8]. In germinated rice seeds and during the next stages of plant growth, salinity damages protein synthesis and decreases Changes in the pattern of proteins under salt conditions are probably due to the effect of salt strss on protein metabolism [29]. Measuring the content of soluble sugars shows that there is a slight increase in concentrations of 25 and 50 mM, but this difference is not significant. In concentrations above 50 mM, the soluble sugar content decreases. Researchers have shown that the production of sugar in the cytoplasm is one of the best ways to protect the plant against osmotic stress. They also said that increasing carbohydrates, amino acids and polyamines inside the cell is one of the most effective ways to withstand stress in plants. It has also been reported that in tomatoes, sucrose value and sucrose phosphate synthase activity increased in leaves, but invertase activity decreased [30].

It has also been reported that the activity of sucrose phosphatase and the amount of sucrose in tomato leaves increased, but the activity of invertase decreased.

Table 1. The amount of chlorophylls, MDA & Other aldehydes, protein, Proline, soluble sugars, and Hydrogen Peroxide in potato under salinity. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

Salinity (mM)	Chlorophyll a & b (mg gFW^{-1})		Total Chlorophyll (TC.) & Cartenoeids (Car) (mg gFW^{-1})		Malondialdehyde (M) & Other aldehydes (O) (µM gFW^{-1})		Protein (mg gFW^{-1})	Proline (mg gFW^{-1})	Soluble sugars (mg gFW^{-1})	Hydrogen Peroxide (H_2O_2) (nM gFW^{-1})
0	a	0.809 ±0.007a	T.C.	1.117 ±0.003a	M	0.987 ±0.01 d	1.458 ±0.002a	0.485 ±0.007d	1.295 ±0.008a	12.51 ±0.10d
	b	0.496 ±0.008a	Car	0.485 ±0.003a	O	11.45 ±0.07 d				
25	a	0.696 ±0.006b	T.C.	1.117 ±0.003a	M	1.243 ±0.02 cd	1.383 ±0.003ab	0.534 ±0.005c	1.321 ±0.004a	12.78 ±0.11cd
	b	0.448 ±0.004b	Car.	0.485 ±0.003a	O	11.66 ±0.08 d				
50	a	0.598 ±0.009c	T.C.	0.845 ±0.008c	M	1.307 ±0.02 c	1.312 ±0.007b	0.562 ±0.006b	1.277 ±0.005a	13.25 ±0.85c
	b	0.394 ±0.006c	Car.	0.383 ±0.009c	O	12.49 ±0.09 c				
75	a	0.399 ±0.006d	T.C.	0.552 ±0.004d	M	1.860 ±0.06 b	1.077 ±0.005c	0.708 ±0.004a	1.106 ±0.004b	19.53 ±0.16b
	b	0.276 ±0.004d	Car.	0.254 ±0.006d	O	18.63 ±0.14 b				
100	a	0.295 ±0.008e	T.C.	0.410 ±0.002e	M	2.203 ±0.06 a	0.865 ±0.004d	0.694 ±0.007a	0.809 ±0.005c	29.14 ±0.20a
	b	0.195 ±0.005e	Car.	0.182 ±0.005e	O	23.12 ±0.15 a				

mg gFW^{-1}: Milligram per gram fresh weight. µM gFW^{-1}: Micromolar per gram fresh weight. nM gFW^{-1}: Nanomolar per gram fresh weight.

Changes in nitrogen metabolism can reduce the amount of chlorophyll. For example, the production of proline, which is used in osmotic regulation. [2]. Glutamate is the precursor of chlorophyll. Increasing proline production causes less glutamate to be produced. Glutamine kinase is an enzyme in the proline biosynthesis pathway. Salt has a stimulating effect on its activity [31]. The first chlorophyll biosynthesis enzyme is glutamate ligase, which prevents salt from its activity [32]. Therefore, in salinity conditions, the production of chlorophyll due to reduced activity of the glutamate ligase activity on the other side the consumption of more glutamate by the enzyme-activated glutamine lipase on the other is reduced [10,32]. Measuring the amount of proline shows that it has increased with concentrations of salinity, which is consistent with the results. However, there is no significant difference (75 mM and 100 mM groups) but a statistical difference is observed with the control. Sometimes, the amount of proline in increases to 100 times under salinity stress. Because its accumulation has a contractile role in the plant and is proposed as a carbon-nitrogen storage osmolyte [33]. The proline content was increased in salinity (Table1). Proline accumulation is one of the plant responses to stress conditions such as salt stress. The results show the amount of peroxide hydrogen, malondialdehyde (MDA), and other aldehydes increased in salinity stress, and the maximum value was measured at 100 mM and the minimum value was measured in the control group (Table1). Increasing MDA and other aldehydes at low concentrations of salinity (25 and 50 mM) is associated with a low slope but at high concentrations (75 and 100 mM) increased MAD significantly. Under salt stress conditions, electron transport is impaired in Cell organelles similar chloroplasts and mitochondria, it produces reactive oxygen species. Active oxygen species such as $^{\cdot}OH$, H_2O_2, O_2^- and are active forms of oxygen. They can react strongly with lipids, proteins, nucleic acids and cause lipid peroxidation, denaturation of proteins, and mutations in nucleic acids, which in acute conditions can disrupt the normal metabolism of the plant and ultimately lead to cell death [34].

Antioxidant compounds and antioxidant enzymes

The amount of anthocyanin, phenolic compounds and activity of antioxidant enzymes have been investigated in different variety of colored potatoes [35]. Although there have been various reports of this [36], Lee *et al*.'s research has shown that purple potatoes have more antioxidant properties than white and yellow potatoes. The difference in the number of polyphenols and radical scavenging activity may depend on the genotype and variety [37]. The results show that the amount of anthocyanin and phenolic compounds increased due to salinity from 0 to 50 mM concentrations. But the amount of anthocyanin and phenolic compounds decrease in concentrations of 75 and 10 mM salinity (Figures 1 & 2). Anthocyanin and phenolic compounds are the secondary metabolites that they observe in many plants. Salt is one of important parameters effective on production of secondary and plant growth [11, 38]. Soil or water salinity are the main factors that expanding in the world and especially in semi-arid and arid regions, which it can greatly reduce plant production. Based on the findings Sreenivasulu *et al*. [39], the harmful effects of salts on plant growth can be divided into three general categories: (1) it creates stress of water in plants by reducing the osmotic potential, (2) it reduces water permeability and soil aeration by changing the physical structure of the soil. and (3) plant metabolism is inhibited by increasing the concentration of some ions, and the balance of mineral nutrients is changed and deficiency is created. Results show that salinity up to 50 mM increased anthocyanin and phenolic compounds (Figures 3-7) but salinity 100mM and 75 mM decreased anthocyanin and phenolic contents.

Measuring the antioxidant enzymes activity similar APX, GR, CAT, GPX, and SOD show in Figures 3-7 respectively, and indicate salinity at low concentrations has increased their activity but decreased in activity at high concentrations (75 and 100 mM). The slope of activation in all measured enzymes is more or less similar, but in the high concentrations (75 and 100 mM) the slope decreases the activity differently, for example, in the catalase with a

slight slope, but in other enzymes, the activity decreases rapidly. SOD activity in salinity 0, 25 and 75 mM was not significant while salinity 50mM highest and 100mM lowest have activity. The CAT activity has the lowest at control and salinity 50mM has the highest. Remarkable that the CAT activity at 100mM salinity is higher than the control. APX activity in salinity 0, 25 and 75 mM were not significant while salinity 50mM highest and 100mM lowest have activity. The changes in GPX activity show that salt strss 25 mM and 50 mM increased GPX activity but salt stress 75 mM and 100 mM decreased GPX activity. GR activity in salinity 0 and 25 mM are not significant but the other concentration of salinity has significant changes.

Salinity reasons a sharp decrease in the yield of potato in the world [4]. Changes metabolism in plant, similar ion imbalances, toxicity and reduced water potential, and decreased levels of carbon assimilation result from salt stress. Although extensive changes in genetic adaptations to salt stress have been shown and there are several significant biochemical and physiological responses to induce stress tolerance in plants, but until now the basic mechanisms of salinity tolerance in plants are not well understood. The effects of various stresses similar salt stress are known to be mediated, by the production ROS including $^{\cdot}OH$, $^{\cdot}O_2$ and H_2O_2 [40]. The effect of salinity in the roots & leaves of salt-sensitive and salt-tolerant maize genotypes has been measured for the lipid peroxidation and antioxidant enzymes activity. [41].

Enhancement the activity of enzymes (antioxidant enzymes) and removing ROS can cause more tolerance to salt stress [42]. The interaction with increased antioxidant enzymes and salt tolerance has been shown in pea [4], *Arabidopsis*, rice [43], *Plantago*, soybean, tomato, and maize [40]. However, there is not enough information about defense response of potato cultivars to salinity [5, 44]. Furthermore, potato cultivars have statistical difference in salt resistance. SOD changes superoxide ions to hydrogen peroxide APX, GPX and CAT, analyze H_2O_2 to O_2 and H_2O, therefore, regulating H_2O_2 levels in plants. GR is one enzyme that protects many plants from AOS caused by salt stress. These study results show that antioxidant enzyme main role role in removing ROS at salinity in potato. Similar results for APX, CAT activity induced in salinity tolerance potato, tomato, Plantago, sugar beet and rice which is similar to our reported results [4,45]. A similar conclusion was observed for GR in maize. Rahnama and Ebrahimzadeh in a study of salinity on potato callus showed that the activity of superoxide dismutase in potato-resistant species was higher than salinity sensitive species [46]. Aghaei *et al*., by measuring of ascorbate peroxidase, glutathione reductase and catalase, showed that the activity of (antioxidant) enzymes in resistant species is higher than in sensitive species [47]. So the antioxidant enzyme activity of Agria can tolerant up to 50 mM NaCl concentration. If the reaction of other biological factors were similar to antioxidant enzymes. Then Agria can cultivate in salinity agriculture lands that have about 50 mM NaCl salt. Investigation of microtuberization in potato under salt stress is shown in Figure 8. The results show that salinity has reduced tuber formation, so salinity can play main function in reducing crop yield. The Figure 9 show the explant buds grown in 0, 25 mM and 100 mM salt.

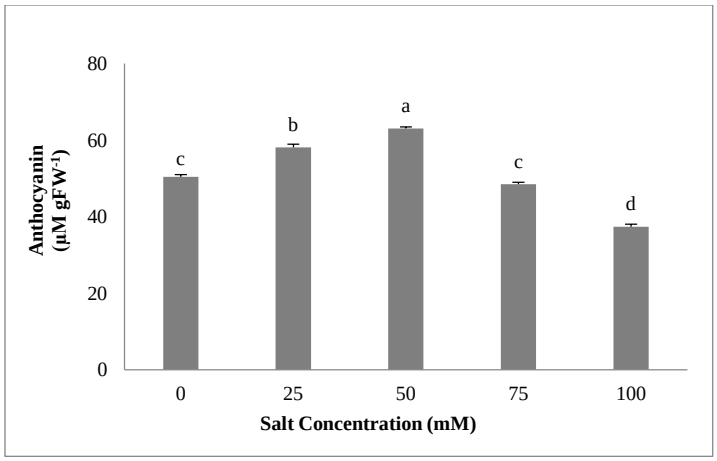

Figure 1. Effects of salinity on the anthocyanin. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

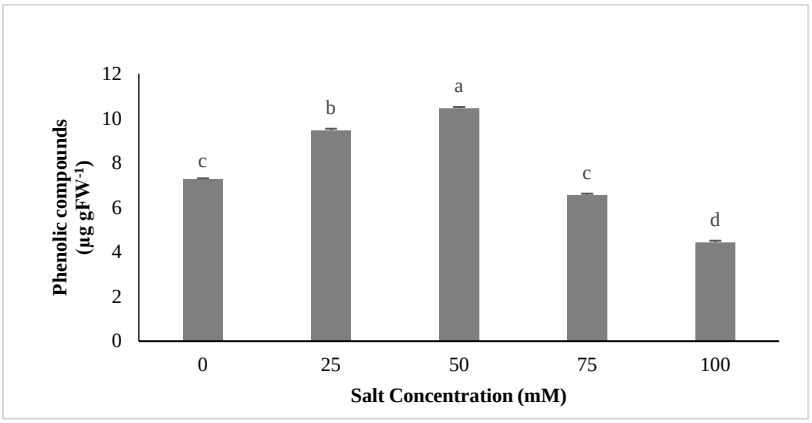

Figure 2. Effects of salinity on the phenolic compounds. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

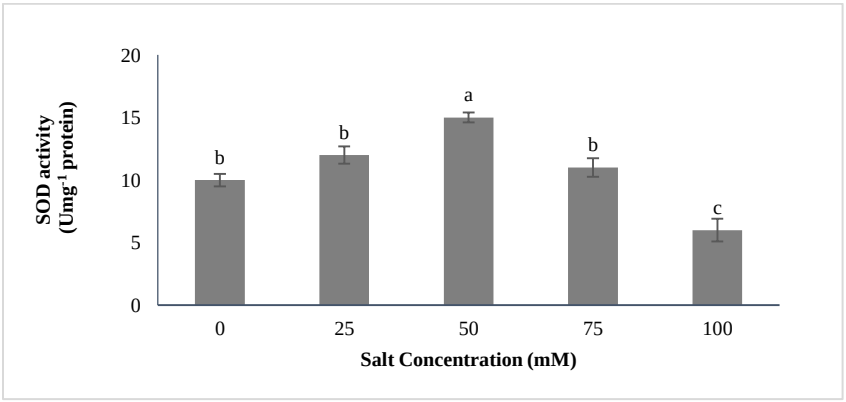

Figure 3. The activity of superoxide dismutase in salinity. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

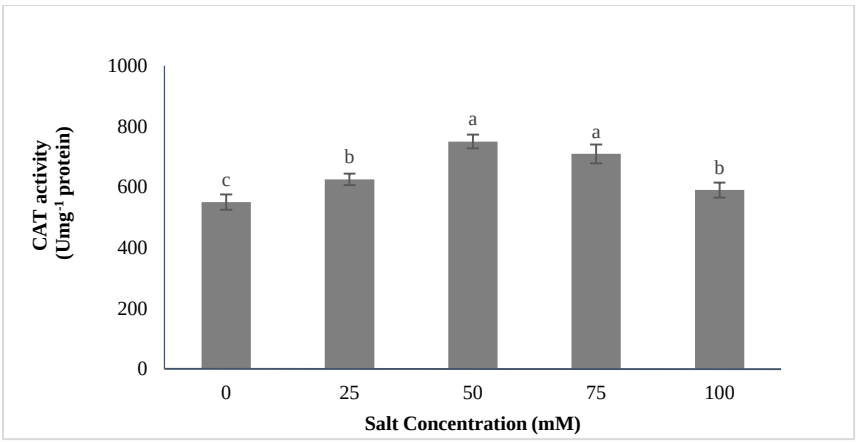

Figure 4. The activity of catalase in salinity. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

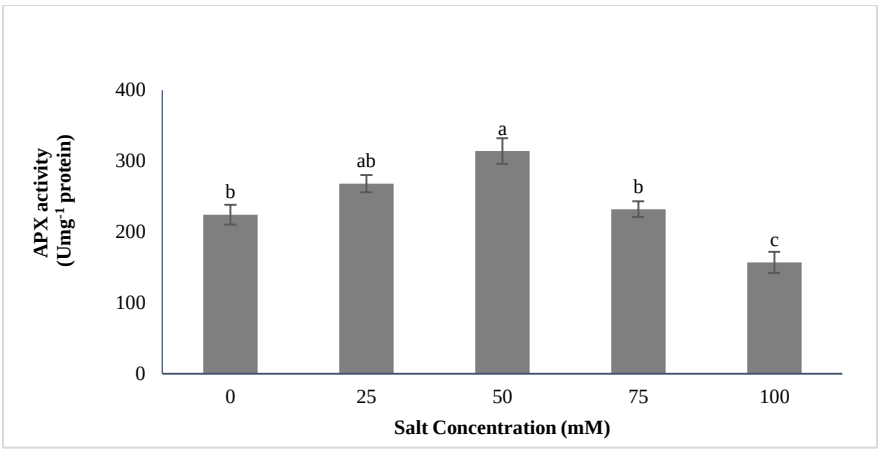

Figure 5. The activity of ascorbate peroxidase in salinity. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

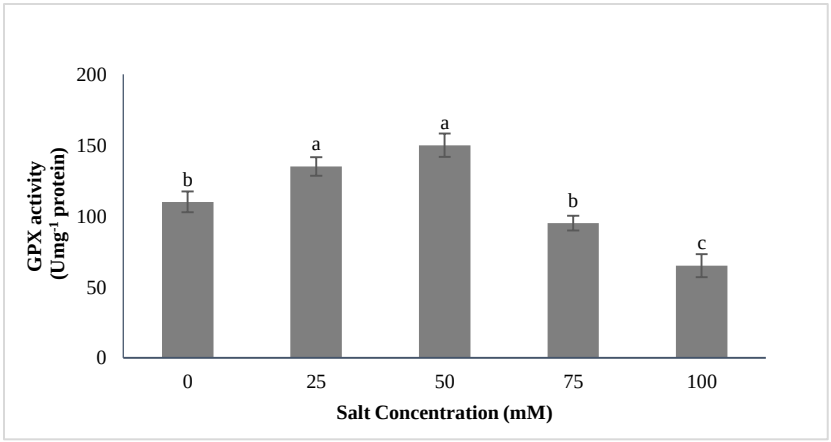

Figure 6. The activity of guaiacol peroxidase in salinity. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

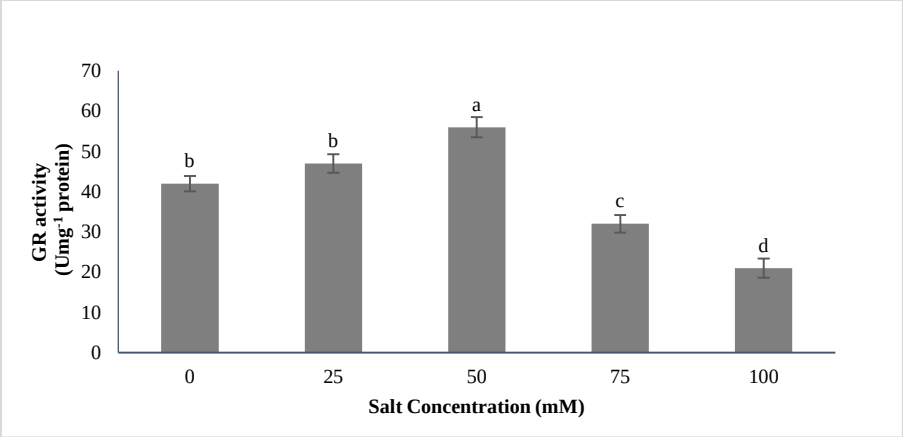

Figure 7. The activity of glutathione reductase in salinity. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

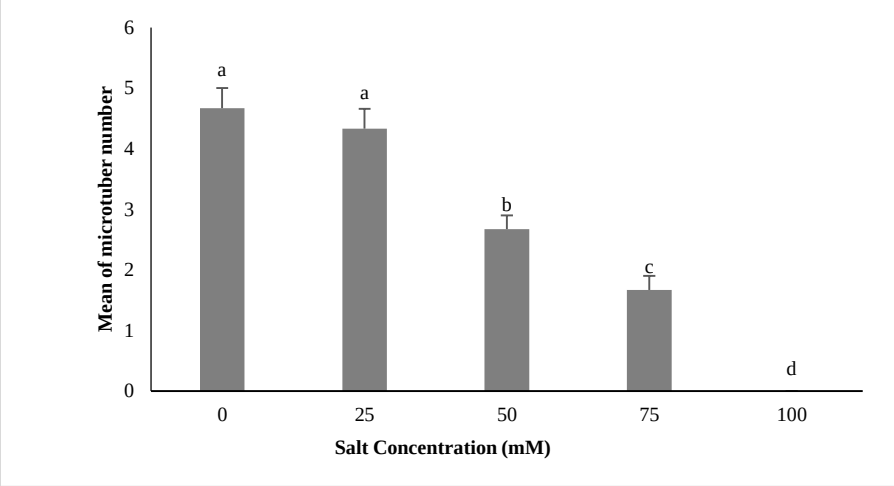

Figure 8. Effects of salinity on the microtuberization. The results (repeat three times) represent the mean±standard error. The similare letter wasn't significant (Duncan's test p≤0.05).

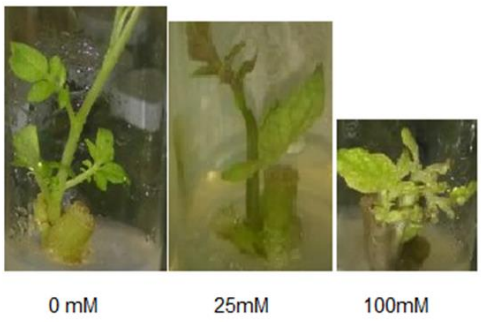

Figure 9. The explant buds grown in salt stress (0, 25mM and 100 mM). Growth and length of the bud and length were completely affected by salinity stress.

CONCLUSIONS

However, in today's agriculture, salinity is a limitation and salinity reduces the yield of potatoes, but scientific methods can identify the potential of plants and use them to deling with tensions. We examined the Biochemical parameters of

tissue culture potato in salinity stress. The results show that salt stress decreased protein and chlorophyll's content. The content of proline, H_2O_2 and MDA was increased under salinity. Phenolic compounds, anthocyanin content and (antioxidant) enzymes activity similar GPX, APX, GR, CAT and SOD increased up to 50 Mm salinity but decreased these factors at 75 and 100 Mm salinity. The results show that salt stress up to 50 mM salt concentration increases the amont of antioxidant enzymes but the higher concentration (such as 75 and 100 mM NaCl) has been decreased. It is strongly suggested that Potato Agria can tolerate a salinity of 50 mM NaCl. The results of this research can be used in potato planting in Damghan because Damghan has saline agricultural land.

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Conflict of interests

There is no conflict of interest.

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