SALT TOLERANT CELLS SELECTION OF POTATO CALLUS FROM CULTURING AT DIFFERENT CONCENTRATIONS OF SODIUM CHLORIDE BY *in vitro* CULTURE TECHNIQUE

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ABSTRACT

The study was carried out in the laboratory of Plant Tissue Culture at the Faculty of Agriculture, University of Basra. Tubers of three certified Dutch cultivars of potato plant (Lizita, Arnova and Safari) were brought from the Horticulture Station for Potato Seed Production Project, Babel city, Iraq. The aim was to produce salinity- tolerant cells from the callus of three cultivars of potato Lizita, Arnova and Safari cultured on MS medium containing different concentrations of sodium chloride. The results of the study showed that callus of Lizita cultivar grew when cultured on MS media supplemented with 0, 80 and 100 mmol.L^{-1} NaCl and did not grow at 120, 140 and 160 mmol.L^{-1} NaCl after four weeks of culture. The Lizita and Arnova cultivars gave the highest fresh weight of callus compared with Safari cultivar after four weeks of culture. The control and 0.250 mmol.L^{-1} salicylic acid + 120 mmol.L^{-1} NaCl treatments improved the callus growth of Lizita cultivar after six weeks from culturing. However, when the callus of Lizita cultivar was subjected to NaCl at a concentration of 80 mmol.L^{-1}, only one additional protein band appeared on the gel. This treatment also indicated the appearance of four proteins with high molecular weight in the callus of Lizita cultivar, reached 225.000, 150.000, 100.000 and 75.000 kDa.

Keywords: Abiotic stress; cultivar; dry weight; protein pattern; salicylic acid.

INTRODUCTION

The potato (*Solanum tuberosum* L.) belongs to the Solanaceae family [1]. Potato is one of the most important vegetable crops of high economic importance in terms of production and consumption [2]. The potato crop production in Iraq was 58,000 tons since 2013. The total

cultivated area of the potato crop is 14,490 ha [3]. Iraqi agricultural land suffers from problems of salinity of the soil. As 75% of the land planted with potato crop is affected by salinity [4]. Potato is a plant of medium sensitivity to salinity. It has the ability to tolerate salinity in the range of 1.6 – 2.5 $dS.m^{-1}$ [5]. Plant tissue culture technique was used to improve the ability of the potato plant to tolerate salinity. Saline-tolerant cells were selected from the growing callus tissue in media supplemented with different concentrations of sodium chloride. After that, the tolerant plant cells for the salinity are produced through multiplication by *In vitro* culture [6,7]. Salicylic acid is a phenolic endogenous growth regulator that contributes to the protection of cells from biotic and abiotic stresses, such as salt stress, as well as regulating physiological processes in the plant [8]. Salicylic acid promotes the process of gene expression in plant cells exposed to saline stress to help it adapt to and tolerate salt [9]. Sajid & Aftab [10] noticed that salicylic acid stimulated growth of callus on explants when they had cultured single nodes of two potato cultivars (Cardinal and Desiree) on MS medium supplemented with 60 $mmol.L^{-1}$ NaCl and different concentrations of Salicylic acid (0.0, 0.125, 0.500 and 0.750 $mmol.L^{-1}$. In the studies of potato plant cells, many researchers observed that the fresh and dry weight of the callus tissue decreased with the increased concentrations of sodium chloride salt added to MS medium [11-13]. Many changes occur in the protein pattern when plant cells are under conditions of saline stress. Many studies have shown that new proteins called shock or stress proteins are produced as a reaction to the saline stress [7,14,15,16]. The present study aimed to produce salt tolerant cells from the callus of three potato cultivars cultured on MS medium containing different concentrations of sodium chloride.

MATERIALS AND METHODS

The study was carried out in the laboratory of Plant Tissue Culture at the Faculty of Agriculture, University of Basra. Tubers of three certified Dutch cultivars of potato plant (Lizita, Arnova and Safari) were brought from the Horticulture Station for Potato Seed Production Project, Babel Government in Iraq. The tubers were washed with tap water to remove the dust and then left to dry. They were incubated at a temperature of 20-27°C in the dark for two weeks to break the dormancy and promote vegetative growth of sprouts. The sprouts grew to a length of 2-3 cm. These sprouts were excised from the tubers and put in sterilization solution (Caravan G Insecticide/fungicide, Greencast is a trademark of a Syngenta Group Company) at 10% for 10 minutes. Then sprouts were kept in the antibiotic solution containing 100 mg. L^{-1} Tetracycline and Rifampicin for 10 minutes. They were rinsed with sterile distilled water 3 times. After that they were sterilized with 20% commercial chlorax solution containing 1.05% sodium hypochlorite, and a drop of Polysorbate 20 (Tween 20) for 15 minutes. They were rinsed in sterile distilled water 3 times after that.

The Medium Preparation for Callus Induction

Using full strength MS basal medium [17] supplied with sucrose at 30000 $mg.L^{-1}$, Thiamine-HCl at 0.4 $mg.L^{-1}$, Adenine sulphates at 40 $mg.L^{-1}$, Nicotinic acid, Biotin, Pyridoxine-HCl at 0.5 $mg.L^{-1}$, Benzyl adenine at $mg.L^{-1}$ and Naphthalene acetic acid at $mg.L^{-1}$. The pH of the media was adjusted to 5.7 with 0.1 N NaOH or HCl after adding 6% agar, and before autoclaving at 1.04 $Kg.cm^{-2}$ for 15 minutes. All media were dispensed in culture tubes containing 15 ml medium cultures. After sprouts reached length of 5-7 cms, they were cut into several nodal segments. Those nodal segments were cultured in the medium mentioned above. The cultures were incubated at a temperature of 27 ± 1 ˚C and in the darkness. Callus was formed after four weeks of culture.

Effect of Salt Stress on Callus Multiplication

100 mg of callus was cultured on the same of MS medium components supplemented with NAA at 3.0 $mg.L^{-1}$, BA at 1.0 $mg.L^{-1}$, sodium chloride with different concentrations (0, 80, 100, 120, 140 and 160 $mmol.L^{-1}$). The cultures were incubated at a temperature of 27 ± 1 ˚C under darkness. The fresh and dry weights were recorded after four weeks from culture.

Effect of Salicylic Acid and Salt Stress on Callus Multiplication

100 mg of potato callus of Lizita cultivar was cultured on the same MS medium components

supplemented with combinations of salicylic acid at 0.125, 0.250, 0.500 and 0.750 mmol.L^{-1} and sodium chloride with different concentrations (120 and 140 mmol.L^{-1}). The cultures incubated at a temperature of 27 ± 1˚C under darkness. The dry weight was recorded after six weeks from culture.

Effect of Salt Stress on Protein Pattern

Protein extraction and SDS- PAGE

Primary callus for three cultivars (Lizita, Arnova and Safari) obtained from the previous step were frozen immediately and freeze-dried. The freeze-dried samples were grounded just before extraction. 300 mg of the ground tissues were mixed with 1ml of extraction buffer (1.5 M Tris-HCl, pH 6.8). The extract was centrifuged at 10000 rpm for 6 minutes. Characterization of proteins was carried out using one-dimensional sodium dodecyl sulphate polyacrylamide as described by Bavi et al. [18]. Protein samples were prepared by mixing clear super ant with sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS). Protein bands were separated at constant current, 2.5 mA for 120 minutes and the current increased up to 5 mA for 120 minutes too, until the tracking dye, reached the end of the gel. Protein bands were visualized by staining the gels with 0.1% Coomassie Brilliant blue R-20. The used marker is produced by Promega (Broad Range Protein Molecular Weight Markers). Molecular weights of proteins were calculated and plotted according to a special computer program (Photo Capt Mw, Version 17).

Statistical Design and Analysis

The complete randomized design was used with ten replicates. The data were subjected to the analysis of variance and mean values were compared using revised LSD at 5% [19].

RESULTS AND DISCUSSION

Effect of Salt Stress on Callus Multiplication

The results of the study showed that callus did not grow when cultured on MS media supplemented with 120, 140 and 160 mmol.L^{-1} NaCl after four weeks of culture (Plate 1).

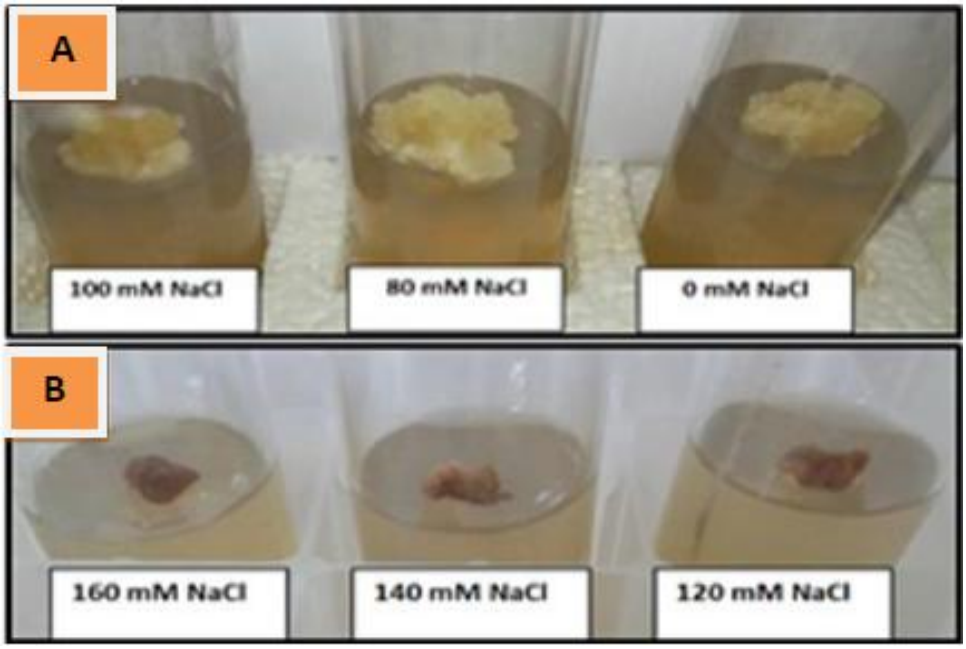

Plate 1. A- Multiplication and growth of callus of Lizita cultivar when cultured on MS medium supplemented with 0, 80 and 100 mmol.L^{-1} NaCl, B- Failure of callus growth of Lizita cultivar when cultured on MS medium supplemented with 120, 140 and 160 mmol. L^{-1} NaCl after four weeks from culture

The main effect of cultivar showed that Lizita cultivar had significantly higher fresh weight of callus reached 186.25 mg compared to Arnova and Safari cultivars (Table 1). Safari cultivar gave the lowest fresh weight of callus reached 154.90 mg.

The main effect of control treatment was significantly better than NaCl treatment at 80 mmol.L^{-1} in fresh weight of callus reached 199.13 and 148.10 mg, respectively (Table 1). Table 1, also shows significant interactions in fresh weight of callus. The interaction treatment between Lizita cultivar and NaCl at 0.0 mmol.L^{-1} gave the highest significant superior on other interaction treatments in fresh weight of callus reached 217.30 mg. While the interaction treatment between Safari cultivar and NaCl at 80 mmol.L^{-1} NaCl gave the lowest value in fresh weight of callus reached 133.30 mg (Table 1). Fig. 1 shows that Lizita cultivar gave the highest significant superior on Arnova cultivar in fresh weight of callus when cultured on MS medium supplemented with 100 mmol.L^{-1} NaCl after four weeks from culture (120.60 and 111.90 mg, respectively). While the callus tissue of Safari cultivar failed to grow when cultured on 100 mmol.L^{-1} NaCl after four weeks from culture.

Table 2, indicates that there are no significant differences in the main effect of the cultivar in dry weight of callus. The control treatment has significantly exceeded in dry weight of callus compared to the treatment at 80 mmol.L^{-1} sodium chloride concentration, reached 21.43 and 13.39 mg, respectively (Table 2). The interaction treatment between Arnova cultivar and 0.0 mmol.L^{-1} NaCl gave the highest significant superior in dry weight of callus compared with the other interaction treatments reached 22.34 mg. While the interaction treatment Lizita cultivar with NaCl at 80 mmol.L^{-1}, gave the lowest dry weight of callus reached 12.65 mg (Table 2). Callus of Lizita cultivar did not show a significant difference with Arnova cultivar in dry weight when they were cultured on MS medium supplemented with 100 mmol.L^{-1} NaCl, reached 13.88 and 12.21 mg, respectively (Fig. 2).

Table 1. Effect of the cultivar, concentration of sodium chloride and interaction between them on the fresh weight (mg) of potato callus after four weeks of culturing

Cultivar	Concentrations of sodium chloride (mmol. L^{-1})		
	0	80	Mean of Cultivar
Lizita	217.30	155.20	186.25
Arnova	203.60	155.80	179.70
Safari	176.50	133.30	154.90
Mean of NaCl concentration	199.13	148.10	
R-L.S.D P≥0.05	Cultivar	Concentration of NaCl	Interaction (Cultivar + NaCl)
	4.30	3.80	7.30

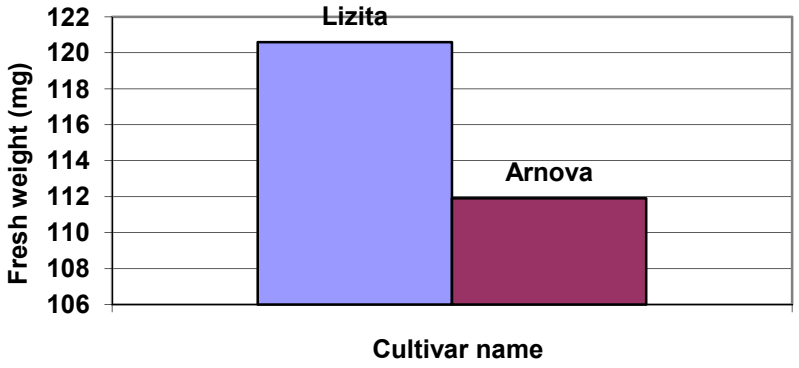

Fig. 1. Effect of 100 mmol.L^{-1} NaCl on fresh weight of callus after four weeks from culture (RLSD0.05= 5.22)

That the failure of the growth of the callus in high saline concentrations is due to the increased salinity cause imbalance in the ionic, damage to the nucleic acid, decrease in the activity of antioxidant enzymes and decrease in the proteins biosynthesis, that negatively affect the growth of callus [20].

The reason for the difference in fresh and dry weight between the three cultivars is due to the variation in their genetic information. The reason for the low fresh and dry weight with the increased concentration of sodium chloride salt is the toxic effects of ions in salt that negatively affect cell division and growth which in turn causes fresh and dry weight loss [7]. The present study results agreed with the results of many researchers [11,12,21].

Effect of Salicylic Acid and Salt Stress on Callus Multiplication

Callus did not grow when cultured on all the treatment combinations between Salicylic acid and Sodium chloride except for the control and 0.250 mmol.L^{-1} salicylic acid + 120 mmol.L^{-1} NaCl treatments (Plate 2). This is due to the fact that the levels of salicylic acid are lower or higher than the optimal level and therefore did not improve the growth of callus under salt stress conditions [22]. The treatment combination between 0.250 mmol.L^{-1} salicylic acid and 120 mmol.L^{-1} NaCl gave 16.20 mg dry weight of callus. The reason for the increase in the dry weight of callus could be due to the role of salicylic acid in stimulating cell division in salt stress conditions which led to increased accumulation of dry matter. It is also the optimum concentration to stimulate the growth of callus and thus increase its quantity. [22]. This experiment shows that adding the optimum concentration of salicylic acid to MS medium with high concentrations of sodium chloride has improved the growth of potato callus (Plate 2).

Effect of Salt Stress on Protein Pattern

The Table 3 and Fig. 3, show that the control treatment (without salicylic acid and NaCl), showed that the presence of four protein bands in all three potato cultivars (Lizita, Arnova and Safari). However, when the callus of Lizita cultivar was subjected to NaCl at the concentration of 80 mmol.L^{-1}, only one additional protein band appeared on the gel with the molecular weight of 50.000 kDa (Table 3). This treatment also indicates that the other four protein bands that appeared with high molecular weight, reached 225.000, 150.000, 100.000 and 75.000 kDa. The appearance of new proteins by adding new protein bands or increasing their molecular weights indicates significant changes in the process of gene expression, thus increasing the tolerance of callus cells to salt stress [7,15]. As for the callus of the two cultivars (Lizita and Arnova), which were cultured on MS media supplemented with 100 mmol.L^{-1} NaCl and Safari cultivar at 80 mmol.L^{-1} NaCl, led to the disappearance of one protein band and the emergence of three protein bands that differ in their molecular weights in comparison with the 0.0 mmol.L^{-1} NaCl. But the four protein bands appeared with a slight decrease in their molecular weights when callus of Arnova cultivar was cultured on MS medium supplemented with 80 mmol.L^{-1} NaCl. The reason for the change in the pattern of protein is due to the emergence or disappearance of proteins or changes in molecular weights [7,15]. Because the callus cells have increased the gene expression

Table 2. Effect of the cultivar, concentration of sodium chloride and interaction between them on the dry weight (mg) of potato callus after four weeks of culturing

Cultivar	Concentrations of sodium chloride (mmol. L^{-1})		
	0	80	Mean of Cultivar
Lizita	20.73	12.65	16.69
Arnova	22.34	13.92	18.13
Safari	21.21	13.60	17.41
Mean of NaCl concentration	21.43	13.39	
R-L.S.D P≥0.05	Cultivar	Concentration of NaCl	Interaction (Cultivar + NaCl)
	N.S*	1.82	2.68

*Non-significant

that has led to the formation of proteins have tolerance to the salt stress [7]. As the callus of Lizita cultivar cultured on the MS medium supplemented with 0.250 mmol.L^{-1} Salicylic acid and 120 mmol.L^{-1} NaCl, appeared four of protein bands with molecular weights almost similar to the Lizita callus proteins of control treatment (Table 3). The increase of callus cells tolerance to high concentrations under salt stress could be due to the physiological role of salicylic acid. [23]. Salicylic acid leads to increased calcium ions in the cytoplasm, which positively affects the process of gene expression and proteins synthesis that help the plant adapt to salt stress [9].

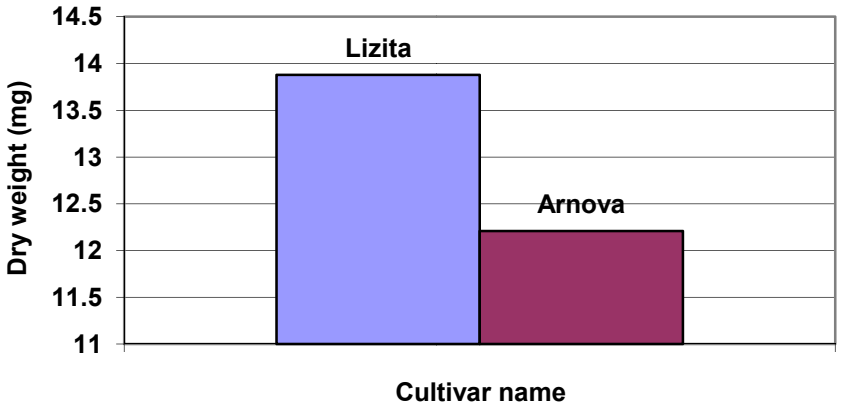

Fig. 2. Effect of 100 mmol.L^{-1} NaCl on the dry weight of callus after four weeks from culture (RLSD0.05= Non-significant)

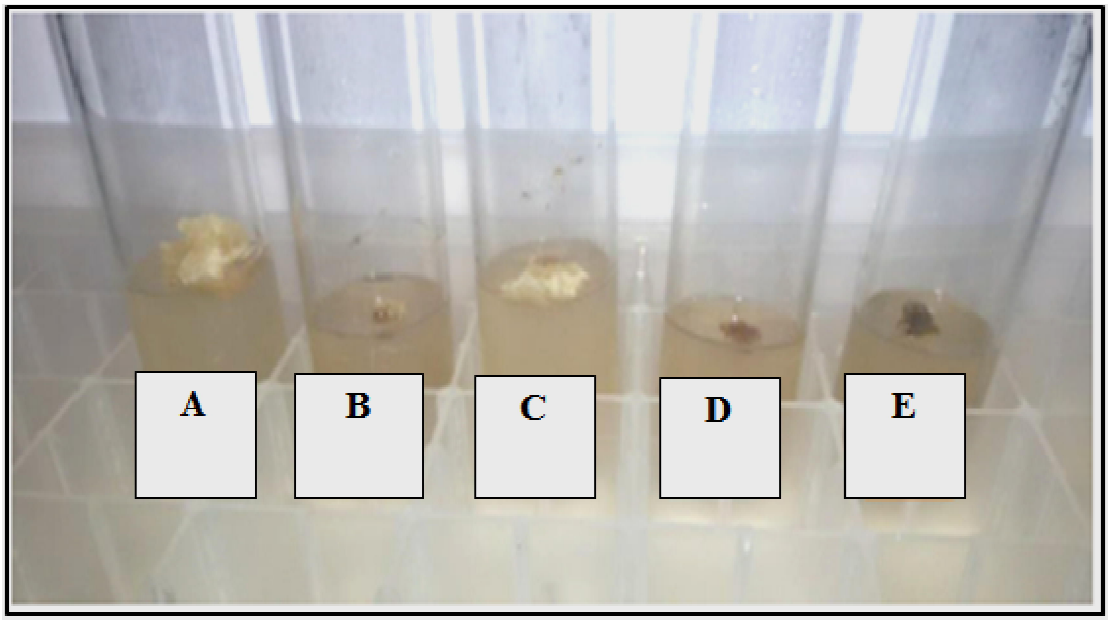

Plate 2. Multiplication and growth of callus of Lizita cultivar when cultured on MS medium supplemented with A- 0+0, B- 120+0.125, C- 120+0.250, D- 120+0.500 and E- 120+0.750 mmol.L^{-1} (NaCl + Salicylic acid), after six weeks from culture

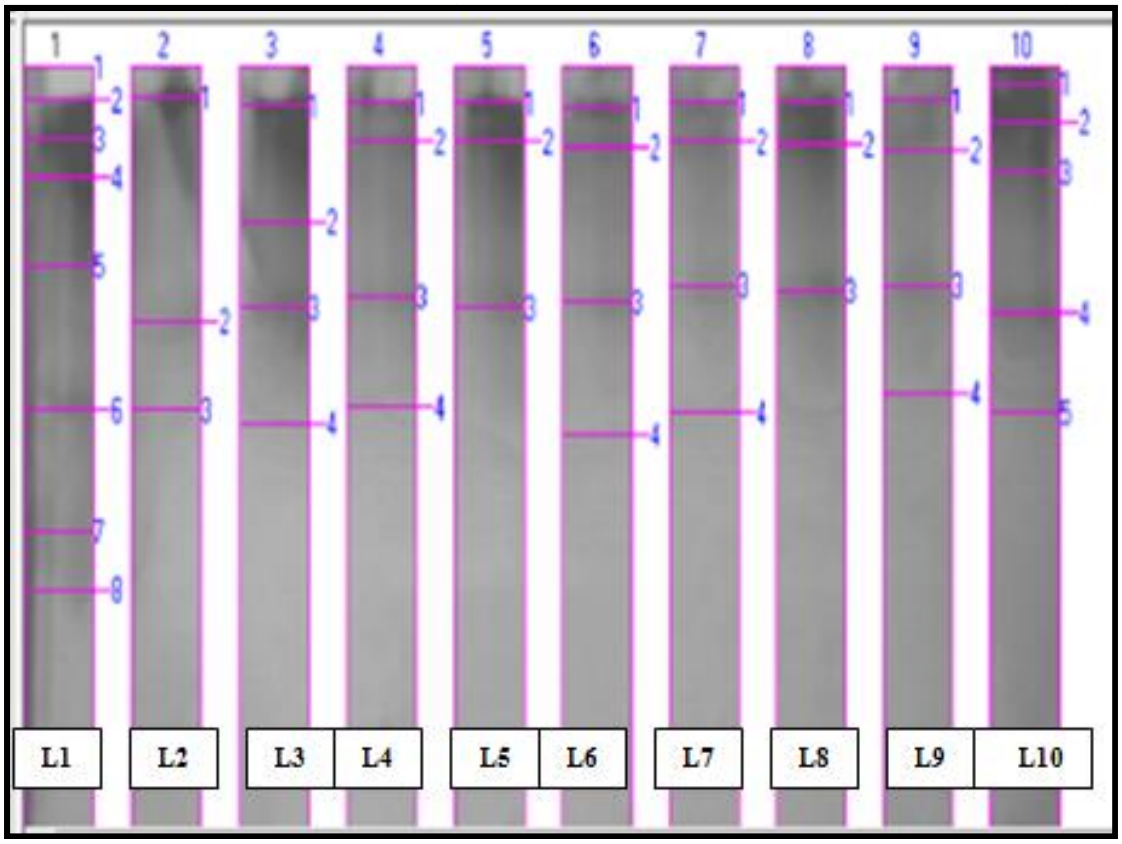

Fig. 3. Specifications of novel proteins for study treatments

L1-Marker
L2- Safari cultivar + 80 mmol.L^{-1} NaCl
L3- Safari cultivar + control
L4- Lizita cultivar + 0.250 salicylic acid + 120 mmol.L^{-1} NaCl
L5- Arnova cultivar + 100 mmol.L^{-1} NaCl
L6- Arnova cultivar + 80 mmol.L^{-1} NaCl
L7- Arnova cultivar + control
L8- Lizita cultivar + 100 mmol.L^{-1} NaCl
L9- Lizita cultivar + control
L10- Lizita cultivar + 80 mmol.L^{-1} NaCl

Table 3. Number of novel proteins and their molecular weights for study treatments

Type of treatment	Novel proteins							
Marker	257.143	190.320	131.502	96.043	75.149	50.938	20.139	5.556
Safari+80 mmol.L^{-1} NaCl	195.955	74.200	50.938	-	-	-	-	-
Safari+ control	179.352	77.949	75.338	47.222	-	-	-	-
Lizita+0.250 Salicylic acid+ 120 mmol.L^{-1} NaCl	184.781	128.515	75.623	51.874	-	-	-	-
Arnova+ 100 mmol.L^{-1} NaCl	184.781	128.515	75.338	-	-	-	-	-
Arnova+ 80 mmol.L^{-1} NaCl	174.051	122.682	75.537	44.444	-	-	-	-
Arnova+ control	184.781	128.515	75.550	50.000	-	-	-	-
Lizita+ 100 mmol.L^{-1} NaCl	184.781	125.573	75.619	-	-	-	-	-
Lizita+ control	190.320	119.848	75.550	56.502	-	-	-	-
Lizita+ 80 mmol.L^{-1} NaCl	225.000	150.000	100.000	75.000	50.000	-	-	-

CONCLUSIONS

The three cultivars (Lizita, Arnova and Safari) differed significantly in the callus growth when cultured in MS medium containing different concentrations of sodium chloride and Lizita was the best. Callus tissue grew within low NaCl concentrations (0-100 $mmol.L^{-1}$) and did not grow in high concentrations (120-160 $mmol.L^{-1}$). The addition of salicylic acid at a concentration of 0.250 $mmol.L^{-1}$ to the MS medium with sodium chloride at 120 $mmol.L^{-1}$ has stimulated the growth of callus of Lizita cultivar. The protein pattern of potato callus was differed when cultured on the MS medium containing different concentrations of sodium chloride.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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