

Changes in organic and inorganic solutes of *in vitro* tomato cultivars under NaCl stress

Abdalmajid N Mohamed^{1*}, Mohd Razi Ismail²

¹Institute Tropical of Agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

²faculty of agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

*Corresponding author: mohamedabdalmajid@yahoo.com

Abstract

Osmotic adjustment plays an important role in plants to address the growing conditions under different environmental stresses and particularly saline conditions. Changes of carbohydrate, soluble protein, proline and ions content (Na^+ , K^+ , Ca^{+2} , and Mg^{+2}) were studied under salt stress in shoots of Pearl and Beril *in vitro* cultivars induced from hypocotyls and cotyledons explants. The explants were cultured in MS + 2mg BAP, supplemented with different levels of NaCl (0, 25, 50 and 75 mM) for eight weeks and the carbohydrates, protein, proline and elements were determined. The carbohydrates, proline contents and Na^+ were increased in tomato cultivars with increasing NaCl levels, whereas the protein, K^+ , Ca^{+2} and Mg^{+2} were decrease with rising of the NaCl level. Significant differences were observed between tomato varieties for various salt tolerance-associated traits under salinity stress. Regarding physiological parameters, higher accumulation in organic and inorganic solutes was determined in Beril cv. compared to Pearl cultivar. The type of explant showed a difference in their content of organic and inorganic solutes, in which the cotyledons was superior than hypocotyl in physiological traits. This might be an indication of higher salt tolerance of cotyledons.

Keywords: carbohydrate, *in vitro*, proline, protein, salt stress, tomato.

Abbreviations: MS: Murashige and Skoog, BAP: 6-Benzylaminopurine.

Introduction

Salinity is one of the biggest environmental constraints that limits agricultural production, especially in areas suffering lack of rain (Yokoi et al., 2002). Use of groundwater for the irrigation of agricultural crops causes the accumulation of salts, and with the passage of time, the land becomes unfit for cultivation in arid and semi-arid areas (Singh, 2004). Continuous degradation of agricultural lands and scarcity of fresh water are very important factors to stimulate thinking of possible selection and development of plants tolerant to salt stress. Plants under salt stress are normally affected by different constraints such as ions toxicity and osmotic and nutrition imbalance (Lauchli and Epstein, 1990). Multiple biochemical pathways determine the tolerance to salt stress. These pathways play the role in protection of protoplast functions, maintenance of ion homeostasis and control of ion and water flux. These methods include synthesis of osmotic adjustment, specific protein and free radical enzymes (Parida and Dos, 2005). Sugars represent up to 50% of the total of osmotic potential in glycophyte plants under salt stress (Carm, 1976). Accumulation of carbohydrates enhances the plant salt tolerance (Kerepesi and Galiba, 2000), and might be a useful character to select drought and/or salt-tolerant genotypes. The accumulation of soluble carbohydrates depends on the type of plant and genotype. Some researchers reported that tolerant genotypes have the ability to accumulate more soluble carbohydrates (Kerepesi and Galiba, 2000; Almodares et al., 2008), whereas some others reported the different response of soluble carbohydrate accumulation in different tomato cultivars (Amini and Ehsanpour, 2005). In barley varieties, the increase of salinity induced carbohydrates accumulation (Khosravinejad et al.,

2009). Proteins also may play role in osmotic adjustment. The plant storage protein is generally in the form of nitrogen under saline stress conditions, and reused when the effects of stress goes away (Singh et al., 1987). The response of plants to accumulation of proteins under salt stress depends on plant species and cultivars. Hurkman (1989) reported that accumulation of soluble proteins is higher in salt tolerant barley than sensitive individuals. Conversely, Ashraf and O'Leary (1999) were observed that the content of soluble proteins is higher in sensitive wheat cultivar than tolerant line. Shibli et al. (2007) found that protein content decreased in both sweet sorghum species, in response to salinity stress. A different response was reported in tomato cultivars in accumulation of soluble proteins. Amini Ehsanpour (2005) reported that increase of salinity in growth media has accompanied by the increase of soluble protein accumulation in Isfahani cv. but adversely decreasing in Shirazy cv. Khosravinejad et al., 2009 also recognized the decrease in protein content in barely varieties under salt stress. Proline is the most common organic compatible solute in the cytoplasm and organelles to keep stability of osmotic pressure of ions in the vacuole. The high-level proline may improve the osmotic adaptation and protect the plants against the salt or drought-induced injuries. Proline is significantly accumulated under salt stress and performs the positive role in the adaptation of cells to salt and water stress (Kaviani, 2008). Proline directly or indirectly plays an important role in protein accumulation and in cell adaptation to salinity stress (El-Enany, 1995) and accumulation of proline in plant may be related to osmotic and saline stress tolerance (Watanabe et al., 2000). Increase

Table 1. Accumulation in carbohydrate, protein and proline in tomato cultivars under NaCl levels after eight weeks of treatments, means \pm SE

NaCl mM	Cultivars					
	Pearl			Beril		
	Carbohydrates (mg/g)	Soluble protein (mg/g)	Proline (μ moles/g)	Carbohydrates (mg/g)	Soluble protein (mg/g)	Proline (μ moles/g)
Hypocotyls						
0	2.80 \pm 0.173	6.10 \pm 0.115	2.09 \pm 0.074	8.80 \pm 0.17	5.27 \pm 0.617	2.33 \pm 0.055
25	5.17 \pm 0.088	4.63 \pm 0.23	2.88 \pm 0.348	9.37 \pm 0.12	4.90 \pm 0.115	4.52 \pm 0.20
50	6.03 \pm 0.328	3.23 \pm 0.12	3.92 \pm 0.01	13.07 \pm 0.317	4.43 \pm 0.07	5.24 \pm 0.51
75	11.5 \pm 0.52	2.70 \pm 0.10	4.92 \pm 0.64	16.53 \pm 0.67	4.03 \pm 0.088	6.92 \pm 0.146
Cotyledons						
0	3.73 \pm 0.088	6.23 \pm 1.28	2.44 \pm 0.13	5.7 \pm 0.40	5.63 \pm 0.27	3.99 \pm 0.166
25	6.1 \pm 0.35	4.5 \pm 0.057	3.63 \pm 0.18	7.57 \pm 0.409	5.50 \pm 0.057	4.67 \pm 0.33
50	8.75 \pm 0.475	3.93 \pm 0.49	4.70 \pm 0.60	12.06 \pm 0.58	4.10 \pm 0.208	5.46 \pm 0.155
75	10.3 \pm 1.40	3.33 \pm 0.145	7.53 \pm 0.33	20.20 \pm 0.17	3.67 \pm 0.088	13.69 \pm 0.97

Table 2. Accumulation in Na⁺ and K⁺ content in tomato cultivars under NaCl levels after eight weeks of treatments, means's \pm SE

NaCl mM	Cultivars			
	Pearl		Beril	
	Na ⁺ (mg/g)	K ⁺ (mg/g)	Na ⁺ (mg/g)	K ⁺ (mg/g)
Hypocotyls				
0	0.18 \pm 0.007	3.78 \pm 0.11	0.54 \pm 0.01	4.15 \pm 0.17
25	3.96 \pm 0.04	3.12 \pm 0.06	3.03 \pm 0.16	1.70 \pm 0.047
50	4.24 \pm .06	2.51 \pm 0.095	4.71 \pm 0.09	2.65 \pm 0.06
75	4.91 \pm 0.11	1.78 \pm 0.05	5.48 \pm 0.13	0.98 \pm 0.05
Cotyledons				
0	0.30 \pm 0.01	4.41 \pm 0.11	0.62 \pm 0.05	4.79 \pm 0.07
25	3.92 \pm 0.07	4 \pm 0.04	3.16 \pm 0.05	4.15 \pm 0.1
50	4.88 \pm 0.07	2.79 \pm 0.07	5.12 \pm 0.12	3.22 \pm 0.14
75	5.34 \pm 0.06	2.43 \pm 0.06	5.76 \pm 0.11	2.46 \pm 0.06

of salinity level in the growth medium led to low osmotic potential by accumulation of specific Na⁺ and Cl⁻ stress. Increased level of Na⁺ associated with producing nutrient imbalance, and so led to causing ion toxicity, in addition to imbalance in the distribution of osmoregulation (Ashraf and Harris, 2004). Increasing Na⁺ ion concentration leads to repression of absorption of K⁺, Ca⁺² and Mg⁺² ions (Benlloch et al., 1994). This study was designed with the objective to investigate the chemical and biochemical characteristics in shoots resulting from different explants in two varieties under sodium chloride conditions.

Results

Total soluble carbohydrates

The interaction between cultivar explants and salt stress has shown in (Table 1). The high level of carbohydrate content was observed in higher levels of salinity (75Mm). Different response of tomato cultivars and explants for carbohydrate accumulation was shown, where the average carbohydrate in Beril cv. was better than Pearl cv. (Fig 1). The cotyledon explants achieved better accumulation compared with hypocotyl explants (Fig 2). Salinity caused a significant increase in carbohydrate content in shoots with increase of salinity levels, and the maximum carbohydrate was produced in shoots that grew in high salinity levels (Fig 3).

Total soluble protein

Performance of Tomato cultivars and explants were different under salinity levels (Table 1), where the average of protein content in Beril cv. and cotyledon explants were better than

Pearl cv. and hypocotyl explants (Fig 1 and 2). Salinity caused a significant reduction in protein content in shoots compared with control and the maximum reduction observed in shoots that grew in high salinity levels (Fig 3).

Proline content

Proline was increased with increase of salinity level in tomato cultivars and explants (Table 1). The effect of salt stress on proline accumulations in shoots was positively with the salt level in the growing medium, where the higher levels of proline content were observed under salinity treatment compared with control. The proline content was three times higher in 75 Mm compared to control (Fig 3). Beril cv. was better than Pearl cv. in proline accumulation and the cotyledons explant superior than hypocotyls explant (Fig. 1, 2).

Ions contents

High levels of Na⁺ and K⁺ ions in plant tissues accompanied with a small amount from Ca⁺² and Mg⁺² ions (Table 2 and 3). The cultivars and explants varied in Na⁺ and K⁺ ion content, where the Beril cv. was better than Pearl cv. (Fig 1). The hypocotyls had lower capability to accumulate Na⁺ and K⁺ ions than cotyledons (Fig 2). A positive relation between Na⁺ accumulation and salt level in the growth media was observed, whereas a negative relation noticed between salt stress and K⁺ level (Fig 3). A great difference in content of Na⁺ was showed in 75 mM of salinity level compared to control. The behavior of Beril cv. in accumulation of Mg⁺² and Ca⁺² ions was better than Pearl cv., especially in cotyledons (Fig 1 and 2). The change in Mg⁺² and Ca⁺²

Table 3. Accumulation of Mg⁺² and Ca⁺² ions in tomato cultivars under NaCl levels after eight weeks of treatments, means's ±SE

NaCl mM	Cultivars			
	Pearl		Beril	
	Mg ⁺² (mg/g)	Ca ⁺² (mg/g)	Mg ⁺² (mg/g)	Ca ⁺² (mg/g)
Hypocotyls				
0	0.13± 0.002	0.22± 0.005	0.12± 0.003	0.22± 0.003
25	0.11± 0.003	0.17± 0.004	0.11± 0.002	0.19± 0.003
50	0.09± 0.003	0.12± 0.003	0.09± 0.004	0.16± 0.003
75	0.062± 0.002	0.11± 0.004	0.07± 0.002	0.14± 0.005
Cotyledons				
0	0.14± 0.002	0.22± 0.007	0.14± 0.002	0.27± 0.001
25	0.12± 0.003	0.20± 0.004	0.12± 0.001	0.19± 0.004
50	0.10± 0.003	0.13± 0.003	0.11± 0.002	0.16± 0.002
75	0.08± 0.002	0.10± 0.002	0.10± 0.002	0.15± 0.003

ions under salt stress were similar to the K⁺, where the relation was negative with salinity levels (Fig 3). It was also shown that the amount of Mg⁺² and Ca⁺² ions in tissue was lower in comparison with Na⁺ and K⁺.

Discussion

The *in vitro* culture techniques are appropriate tools to study physiological and biochemical indicators of salinity tolerance in the plants (Perez-Clemente et al., 2008). Thus, salt tolerance seems to be related with efficiency of tissues to modulate the level of inorganic and organic solutes in response to salt stress. Metabolic pathway involving the synthesis of metabolites such as carbohydrates, and proline and protein and inorganic has been shown to be associated with stress tolerance (Rajam et al., 1998). The plant species can adapt to salt stress in the growth medium by increasing the accumulation of organic solutes such as the total soluble sugar, proline (Zhu, 2002) and inorganic solutes in tissues like Na⁺ and K⁺ (Asch et al., 2000). Sugars represent up to 50% of the total osmotic potential in glycophyte plants under salt stress (Carm, 1976). Accumulation of carbohydrate in plant may enhance the ability of plant to salt tolerance (Watanabe et al., 2000; Kerepesi and Galiba, 2000). This might be a useful trait to select drought and/or salt-tolerant. The results indicated that under NaCl condition carbohydrate content is increased. Similar results reported by Amini and Ehsanpour (2005), where the increasing salinity in growth medium led to higher carbohydrates in Shirazy tomato cultivar, whereas a negative effect observed in Isfahani cv. Moreover, Shibli et al. (2007) reported increase in sugar content in higher salinity levels in tomato Roma and Patio cv. On the other hand, Potluri and Devi Prasad, 1994 found a difference in potato cultivars in carbohydrates content under salt stress, in which the carbohydrates increased in almost all cultivars in higher salinity levels. Dumet and Singh (1999) found increase in sugar content in rice cultivars with increase of salinity and the sensitive cultivars produced more sugars than tolerate cultivars. Similar results reported by Khosravinejad et al. 2009 that found the salinity increase enhanced the carbohydrate levels in barely cultivars. Protein accumulation under salt stress conditions depends to the genotypic nature of plants and cultivars. Ashraf and Fatima, 1995 not found any difference among sensitive sunflower accessions for salt tolerance. The degradation of protein content with salinity increase might be the result of breakdown of protein molecules, which are used as substrate for biosynthesis of proline (Stewart, 1981). Pareek et al. (1997) suggested the possibility of using proteins as molecular marker to improve the salt stress. In the present study, degradation in protein content was observed in shoots

with salinity increasing. The similar results were reported by Shibli et al (2007) in tomato cultivars and Abed Alrahman et al (2005) in cucumber. The content of soluble protein in leaves of (*Lens culinaris* Medic.) plants was decreased with salinity increase (Ashraf and Waheed, 1993). On the other hand, Potluri and Devi Prasad (1994) found difference in protein content of potato cultivars under salt stress. Some cultivars showed decrease in protein content, whereas other cultivars showed positive relation in salt stress conditions. Similar results reported by Khosravinejad et al. (2009) in barely varieties. Proline is very important compatible solute, which plays important role to enhance plant growth under stresses, especially salt stress. The high level of proline may improve the osmotic adaptation and protect the plants against the salt induced damages. Proline is generally accumulated under salt stress condition and plays positive role in adaptation of cells to salt and water stress (Kaviani, 2008). In present study, proline content increased with salinity intensity on growth medium. Many authors were reported the increase of proline accumulation under salt stress in different plants such as tomato (Amini and Ehsanpour, 2005; Mohamed et al., 2007), potato (Potluri and Devi Prasad, 1994), green gram (Misra and Gupta, 2005) and Jojoba plant (Fayek et al., 2010). In this study, positive relation has been shown between Na⁺ content and salinity treatments, whereas the negative relation was observed with the K⁺, Ca⁺² and Mg⁺² ions. This result was supported by several authors in tomato cultivars (Shibli et al., 2007), cucumber (Abed Alrahman et al., 2005), potato (Potluri and Devi Prasad, 1994) and jojoba (Fayek et al., 2010). The content of Ca⁺² and Mg⁺² was less than K⁺ content. Increase of salinity intensity in growth medium led to nutrition imbalance and competition between ions where the Na⁺ ion was the strongest to compete with other ions, especially K⁺ in the membrane system (Hu et al., 2007; Ashraf, 2004). This also led to increase of Na⁺ ion and adversely reduction of K⁺ accumulation in plant. The enhancement of Na⁺ ion also leads to repression of absorption of Ca⁺² and Mg⁺² ions (Benlloch et al., 1994). Decrease of K⁺ in competition with the Na⁺ also leads to reduce the osmotic adjustment capacity and turgor maintenance and reflex on the metabolic functions (Ashraf and McNeilly, 2004).

Materials and methods

Two tomato cultivars of Pearl and Beril were used in this experiment. Seeds of cultivars were obtained from local companies in Malaysia.

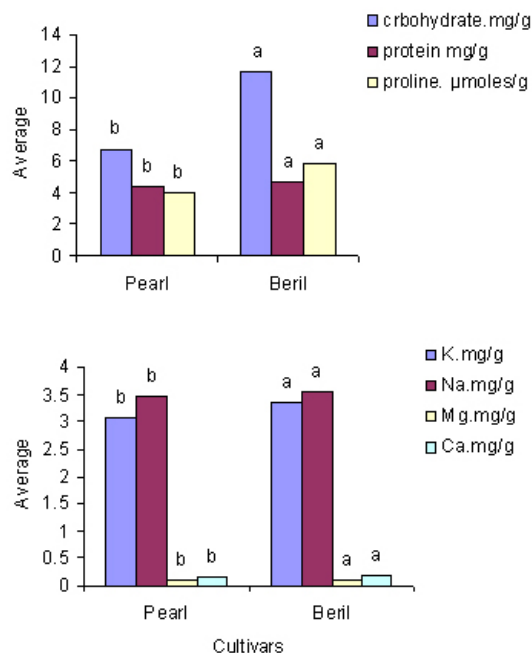


Fig1. Response of tomato cultivars to accumulation of organic and inorganic solutes under NaCl stress

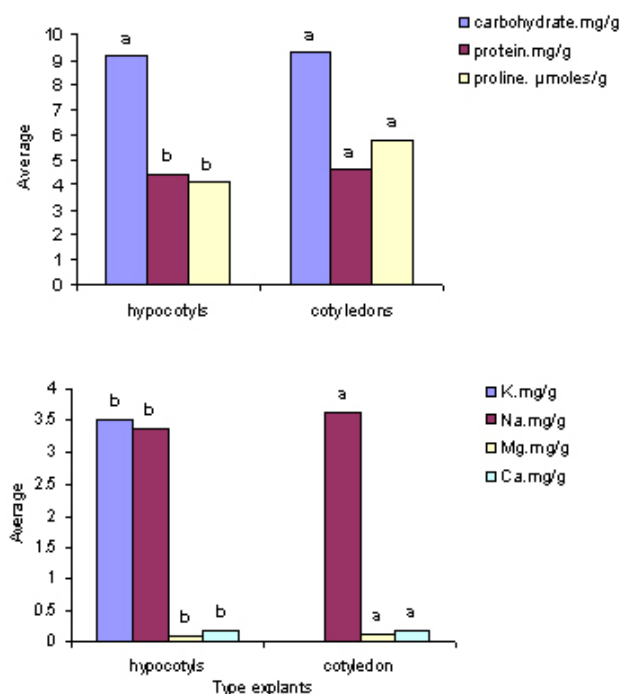


Fig2. Response of explants to accumulation of organic and inorganic solutes *in vitro* under NaCl stress

Sterilization and germination of seeds

The collected seeds were subjected to sterilization by 8% Clorox (Sodium hypochlorite) for 10 min followed by washing, three times by double distilled water. The seeds were cultured in MS basal medium (Murashige and Skoog, 1962) and incubated in dark for four days and after that transferred to the growth room and maintained 16/8 light/dark condition for ten days.

Explant isolation and culture

After two weeks of germination *in vitro*, the hypocotyls and cotyledons explants were excised (approximately 1 cm) and cultured in MS media, supplemented with 2.0 mg/l BAP, 3% sucrose, 0.7% agar with a range of different NaCl salinities (0, 25, 50 and 75 mM) treatments. The 50 ml of medium was dispensed in each of the culture vessel. The pH of the media was adjusted to 5.8±1 and autoclaved at 121°C for 20 min. The cultures were incubated at 25±1°C with 16/8 light/dark cycle at 15 μmol m⁻² s⁻¹ light intensity. Five culture vessels in each replication and four replications of each cultivar at each salinity level were employed. The experiment ended up on eighth week and explants harvested and cleaned by tap water to remove agar remains and dried by tissue paper to remove the surface moisture.

Organic content determination

Total soluble carbohydrates were estimated by phenol-sulphuric acid method (Dubois et al., 1956). One gram of plant fresh materials weighted and digested by hot ethanol 80% two times, each time by 5 ml ethanol and then filtered by whatman No 2. filter paper and the extracts diluted by distilled water to the volume of 50 ml. One ml from each sample placed in the test tube and then 1 ml p phenol solution added. The procedure was followed by adding 5 ml of sulphuric acid and well shaking. The yellow-orange colour was pipetted off and the wave length was read in 490 nm by spectrophotometer machine (Pharmaspec UV-1700 model) The amount of carbohydrates was presented from the glucose standard curve. Total soluble protein was determinate by the method (Lowry et al., 1951) using Folin-Ciocalteau reagent. 1g of fresh plant materials weighted and digested by hot ethanol 80% two times, each on 10 ml and the extract diluted to 50 ml by double distilled water. The blue color read in 660 nm by spectrophotometer machine (Pharmaspec UV-1700 model). The amount of soluble protein calculates from bovine serum albumin standard curve. Proline content was estimated by Bates et al. (1973) method. 0.5 gr of plant fresh material weighted and homogenized. Then 10 ml of 3% aqueous sulphosalicylic acid added and filtered through whatman No 2 filter paper. 2ml of filtered extracts was taken into test tube and 2 ml of glacial acetic acid and finally 2 ml of ninhydrin acid was added. The test tubes were heated in boiling water in warm bath for one hour and the reaction was terminated by placing the tubes in ice bath. 4 ml of toluene was added to the test tube and stirred. The toluene layer separated in room temperature, and the purple colour was read at 520 nm by spectrophotometer machine (Pharmaspec model UV-1700) and the length waves were written from the proline standard curve. Express of proline content in fresh weight was calculated as follows:

$$\mu\text{moles proline/g fresh weight tissue} = \left[\left(\mu\text{g proline/ml} \times \text{ml toluene} \right) \div 115.5 \right] \times 5 \div \text{g sample}$$

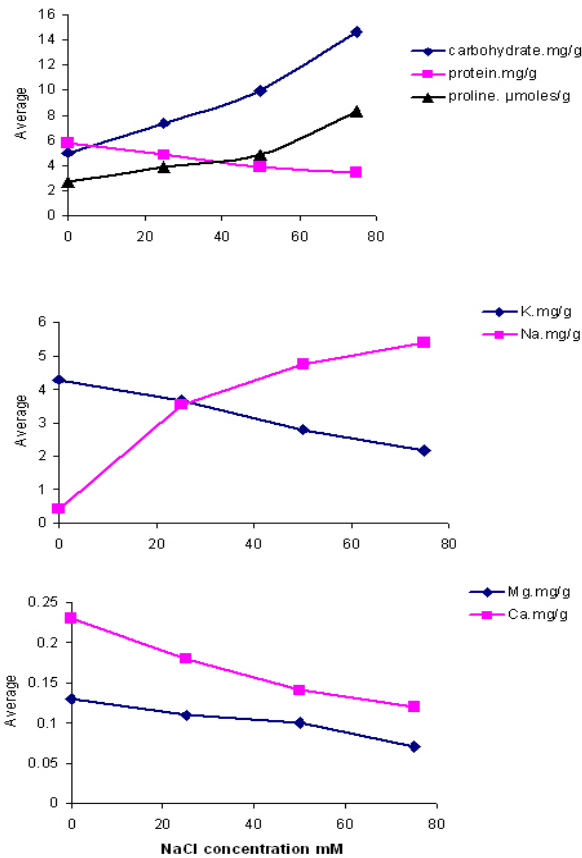


Fig 3. Organic and inorganic solutes accumulation *in vitro* under different level NaCl stress

Inorganic contents determination

Plant samples dried in oven at 70 °C for 48 hours. 0.3 g of dried sample ground to fine powder, transferred to 50 cm³ digestion flasks, and supplemented by 1 ml perchloric acid 80% and 5ml of concentrated H₂SO₄ and the flasks heated gently over a hot plate until the solution become colorless. Digested material diluted by double distilled water to 100 ml. Na⁺, K⁺, Mg²⁺ and Ca²⁺ estimated in extract solution by atomic absorption spectrophotometer model Perkin Elmer 3110 USA.

Statistical analysis

The factorial experimental design with two varieties, two explants and four salinity levels were arranged in a completely randomized design (CRD) with four replications and the data were analyzed using the software package, SAS windows and the mean separation by LSD_{0.05}.

Conclusion

The carbohydrate contents, proline and Na⁺ increased by salinity level. Adversely, the protein content, K⁺, Ca²⁺ and Mg²⁺ decreased in plant tissues under salinity stress. The cultivar Beril was better than Pearl in organic and inorganic solutes contents, and this may be an indicator for salt tolerance. In addition, the cotyledons showed better

performance of physiological traits under salt stress conditions than hypocotyls.

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