PHYSIOLOGICAL AND BIOCHEMICAL RESPONSE OF *Phoenix dactylifera* L. HAYANI cv. EMBRYOGENIC CALLUS AND SOMATIC EMBRYOS TO SALT STRESS UNDER *IN VITRO* CONDITIONS

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ABSTRACT: Salinity stress as a major abiotic stress has influenced more than 6% of the world land which is increasing daily due to climate changes. The effect of salinity on callus regeneration and organogenesis of date palm was tested under *in vitro* conditions. This study was conducted to investigate the effect of salt stress on callus proliferation, somatic embryo formation of 'Hayani' date palm *in vitro*. The embryogenic callus was subjected to MS medium with BA at 0.05 mg/l and NAA at 0.1 mg/l supplied with different sodium chloride (NaCl) concentrations (0, 500, 1000, 1500 and 2000 ppm) for 2 subcultures. Salinity affects several physiological and biochemical processes. The investigated parameters include embryogenic callus growth, differentiation to form somatic embryos, fresh weight of both callus and embryos, biochemical analysis such as total carbohydrates, proline, protein content and minerals in embryogenic callus and somatic embryos. Low levels of abiotic stress factors can induce favorable responses in the growth and differentiation. However, high levels are detrimental resulting in reduced growth and some cell death. The callus fresh weight and the number of embryos were enhanced by adding 500 and 1000 ppm NaCl to the proliferation medium; higher salt concentrations affected the callus regeneration negatively, where at 2000 ppm NaCl the less somatic embryos were formed. High concentrations of NaCl (1500 and 2000 ppm) decreased total soluble carbohydrates in both embryogenic callus and somatic embryos, proline content increased gradually as the external concentration of NaCl increased and the protein content decreased in both embryogenic callus and somatic embryos with the increase of NaCl concentration in culture media to 2000 ppm. Salinity inhibits water uptake and increases the concentration of toxic ions such as Na^+ and reduced the K^+ content. Tissue culture with the use of this technique would allow selection for salt tolerance in the date palm. In fact, somatic embryos subjected to 500 and 1000 ppm NaCl concentrations were successfully regenerated into plantlets.

Key words: Date palm, Hayani cv., *in vitro*, salinity stress, physiological and biochemical analysis.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.), a monocotyledonous dioecious plant, is one of the most cultivated palms around the world (**Abass, 2013**). Date palm trees are cultivated in different regions worldwide, especially in the Middle East, North Africa, North and South America, Southern Europe, Pakistan and India (**Al-Shahib and Marshall, 2003; Al-Khayri *et al*., 2015**). Date palm grows very well under adverse conditions especially in arid and semiarid regions and produces highly valued date fruits. Date palm fruit is rich in carbohydrates and nutrients. It contains sugars in mostly inverted form, dietary fiber, high levels of essential amino acids and minerals (**El-Far *et al.,* 2016**).

Propagation through offshoots is slow technique, limited number of offshoots, survival of low life and the risk of disease transmission rate (**Al-Khalifah and Askari, 2011**). Propagation of date palm via *in vitro* culture using shoot tip explants provides effective substitution to traditional methods (**Quiroz-Figueroa *et al.,* 2006**). Somatic embryogenesis is the common method used for *in vitro* propagation of date palm (**Abahmane, 2013**) depending on the development of callus and

increases its ability to multiply and quickly differentiate into shoots; the somatic embryogenesis is widely used commercially for propagating date palm plantlets. **(McCubbin *et al.*, 2000 and Al-Khayri, 2013)**. Tissue culture techniques have become an ideal method to produce large number of date palm plantlets **(Jain, 2007)**. *In vitro* technology was used extensively in physiological studies of the mechanisms of stress tolerance and resistance of plants **(Jasim *et al.*, 2016)**.

Salinity stress is one of the most significant environmental stresses limiting agricultural production worldwide; and affects the anatomical, physiological and enzymatic features of plants **(Arslan *et al.*, 2016 and Nawaz *et al.*, 2016)**. Salinity affects several physiological and biochemical processes; there are two types of effects, first the adverse osmotic effect, which is the presence of high concentrations of salts in the soil solution making it harder for roots to extract water and reducing the ability of the plant to take it up, leading to slower growth **(Munns and Tester, 2008)**. Osmotic stress delays the growth of plant and affects cell division and elongation. The division of cells is crucial processes which determine the meristem activity and the overall plant growth rate **(Bartels and Sunkar, 2005)**. Secondly, the toxicity effects, which are the presence of high concentrations of salt in the plant which can be toxic and lead to cellular damage **(Munns, 2005)**. The salinity stress causes a negative impact on plant physiology including reduced photosynthetic capacity, impaired signaling and alterations to cellular metabolism **(Munns and Tester, 2008).** To understand the effect of salinity on the behavior of date palm *in vitro* culture, several studies have been conducted **(Al-Khayri and Al-Bahrany, 2004; Al-Bahrany and Al-Khayri, 2012; Al-Khayri and Ibraheem, 2014)**. Responses of date palm *in vitro* to salt stress by using different concentrations of NaCl and the positive effect of NaCl at low concentration (25 µM) on the proliferation of shoot tip derived callus was examined**.** Accumulation of proline in callus tissue date palm cultured under salt stress has been noted with stress tolerance **(Al-Khayri, 2002)**. Under stress the proline acts as an alternative resource for carbon and nitrogen, helps reducing oxidative damages and stabilizing DNA and membrane protein **(Szabados and Savoure, 2010)**.

The aim of this study was to determine the effect of different concentrations of NaCl on growth, development and differentiation of *in vitro* embryogenic callus of Date Palm Hayani cv. and find out the effect of salt stress on the biochemical content of embryogenic callus and somatic embryos of date palm as well as, their content from total carbohydrate, total proline, total protein and minerals.

MATERIALS AND METHODS

This study was conducted in The Central Lab of Date Palm for Researches and Development - Agricultural Research Center, Egypt during the period from 2017 to 2018.

Offshoot preparation

The date palm offshoots Hayani cv. about 3-4 years old and weighing 5-7 kg were separated from healthy mother trees from date palm orchard in (Sakara), Giza governorate, Egypt. The shoot tips were washed with distilled water to eliminate any organic ruins then soaked in Benlate 1 g/l fungicide solution for 10 min and rinsed three times with sterile distilled water. The offshoots were thoroughly cleaned and the outer leaves were removed to expose the shoot tip under aseptic condition, the shoot tips separated and immersed in antioxidant solution (150 mg citric acid and 100 mg ascorbic acid). Shoot tips were surface sterilized with 70% ethanol for 1 min followed by sodium hypochlorite treatment (60 % v/v Clorox solution) containing 2 drops of tween 20 for 25 min after that washed three times with sterile distilled water, soaked in 0.2% $HgCl_2$ solution for 5 min. The shoot tips were then rinsed thoroughly with sterile distilled water, as shown in **Fig. 1 (A)**. All leaf primordial were removed except 2 pairs surrounding the apical meristems and shoot tip was cut into 6 equal segments, then instantly moved to a prepared culture medium.

Initiation stage

For callus culture initiation, the pieces of shoot tips were cultured into callus induction medium consisting of MS salts **(Murashige and Skooge, 1962)** supplemented with $Na_2H_2PO_4$ (170.0 mg/l), myo-inositol (100.0 mg/l), glutamine (200.0 mg/l), nicotinic acid (0.5 mg/l), pyridoxine-HCl (0.5 mg/l), thiamine (1.0 mg/l), sucrose (40.0 mg/l), agar (6.0 g/l) and 0.5 g/l of activated charcoal. Plant growth regulators were added as per requirement for each *in vitro* stage.

For initiation stage, MS growth medium supplemented with 10.0 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D), 3.0 mg/l of isopentenyl adenine (2ip) and the medium was adjusted to a pH of 5.7 and autoclaved. Explants were cultured on this medium and incubated in dark culture room at 25 ± 2°C for 4 months (four subcultures) to improve the initiation of callus during this period explants were re-cultured on the same medium each 4 weeks. Explants swell during the second subculture. After 4 months from culture the callus was initiated and the resultant callus

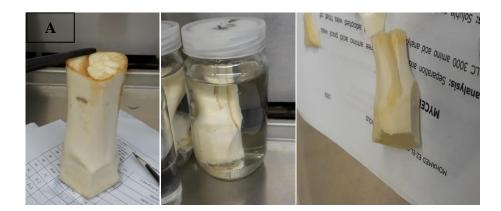was transferred to callus proliferation medium, as shown in **Fig. 1 (B, C)**.

Callus formation stage

The concentration of 2,4-D was reduced gradually to enhance the proliferation callus as shown in **Table 1**. The explants were cultured on MS medium having 2,4-D at 5.0 mg/l mixed with 2ip at 3.0 mg/l for three subcultures. After that the callus was transferred to embryogenic callus medium which consist of MS basal medium supplemented with 2,4-D at 3.0 mg/l with 2ip at 1.0

mg/l to produce the embryogenic callus, as shown in **Fig. 1 (D)**.

All cultures were incubated in the growth room at 25±2°C for 3 months (three subcultures) at total darkness. Explants or calli were recultured on the same medium each for 4 weeks. Thereafter, embryogenic callus was maintained on callus maintenance medium which consists of MS basal medium supplemented with BA at 0.05 mg/l + NAA at 0.1 mg/l. These cultures served as a callus source for the salt induced stress study.

Table 1. Plant growth regulators were requirement for date palm *in vitro* culture stages

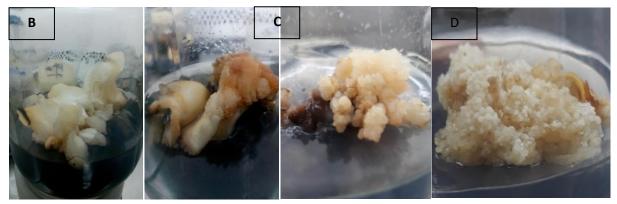| Plant growth regulators	Callus induction			
	Callus initiation	Callus proliferation	Embryogenic callus	Callus maintained
2,4-D	10 mg/l	5 mg/l	3 mg/l	-
2iP	3 mg/l	3 mg/l	1 mg/l	-
BA	-	-	-	0.05 mg/l
NAA	-	-	-	0.1 mg/l

Fig. 1. Different stage of *in vitro* date palm Hayani cv., A: Offshoot preparation, B: swelling stage, C: callus formation stage, D: Embryogenic callus formation

Salinity stress

The embryogenic callus was used to begin *in vitro* salt tolerance experiments. For that we used MS basal nutrient medium supplemented with BA at 0.05 mg/l + NAA at 0.1 mg/l with the addition of different concentrations of NaCl (0, 500, 1000, 1500 and 2000 ppm) with five replicates. Cultures were incubated in complete darkness at 25 ± 2ºC for 8 weeks (two subcultures). Each treatment consisted of 8 cultures containing about 0.5 g embryogenic callus/jar.

a. Physiological traits

After the incubation period, all exposed callus was analyzed to determine their responses to salt stresses, the fresh weight of embryogenic callus was measured after 8 weeks from culture and also the number of embryos induction on callus was counted.

b. Biochemical analyses

Chemical analyses were done for embryogenic callus and somatic embryos including the following:

1. Determination of total carbohydrates

The total carbohydrates were determined in the embryogenic callus and somatic embryos of date palm according to the method of **Watanabe *et al.* (2000)** using a spectrophotometer which measure absorption spectrum at wave length of 620 nm and standard curve of glucose was made for calculation.

2. Determination of proline

Proline content was determined by a spectrophotometric assay as described by **Bates *et al.* (1973).**

3. Determination of total soluble protein

The method of **Bradford (1976)** was followed to measure the total soluble protein, Albumin was used to make a standard curve and absorbance measured at 595 nm.

4. Mineral analysis

The Na^+ and K^+ concentration in the treated calli were measured based on the method described by **Skoog *et al.* (2007)** using Jenway Model PEP7 flame photometer.

Embryos culture

After the experiment of salt stress, the embryo clusters were derived from callus cultures and transferred to fresh medium without NaCl consists of ¾ MS basal medium supplemented with 30 g/l sucrose, 0.1 mg/l NAA and 0.05 mg/l BA (**Ibrahim**

***et al.*, 2009**) for continued embryo proliferation. The somatic embryo cultures were incubated in growth room at 26 ± 2°C with light conditions 16-h photoperiods, and recultured to the same fresh medium for two subcultures (8 weeks) to increase mass production of somatic embryos and germination.

Shoot elongation and rooting stage

Regeneration shoots were transferred to the ¾ MS medium supplemented with 0.5 mg/l gibberellic acid (GA_3) + 1.0 mg/l NAA to obtain shoot elongation and root formation (**AL-Mayahi, 2015**). The cultured jars were incubated at 25 ± 2°C with light intensity 3000 lux for 16h photoperiod in the growth room for 8 weeks and each 4 weeks the shoots were transferred to the same fresh medium. To enhance root formation, for two month (two subculture) the healthy regenerated plantlets were cultured on ½ MS medium supplemented with 0.1 mg/l IBA, 1.5 g/l activated charcoal and 30 g/l sucrose (**Eke *et al.*, 2005**) and incubated under the same conditions as described above.

Statistical analysis

This experiment was designed as a randomized complete block design as described by (**Gomez and Gomez, 1984**). The obtained data were statistically analyzed using MSTAT Computer Program (**MSTAT Development Team, 1989**). To verify differences among means of various treatments, means were compared using Duncan's Multiple Range Test as described by (**Duncan, 1955**).

RESULTS AND DISCUSSION

Effect of salinity on callus fresh weight (g)

The embryogenic callus continued to grow normally without any sign of cell dehydration or salt induced damage showed tolerance to the lower concentration of NaCl added to the culture medium. This may point to the suitability of the osmotic potential of the medium for date palm callus growth and the best proliferation (**Al-Mansoori *et al.*, 2007**).

Results presented in **Table 2 and Fig. 2** show the effect of NaCl concentration on fresh weight of embryogenic callus. Callus fresh weight was determined after 8 weeks in salt treatments. The results showed a gradual decrease in fresh weights with an increase in salt concentration over 1000 ppm of NaCl as compared to the control treatment. Salt concentrations at 500 and 1000 ppm gave the highest fresh weight reached 4.84 and 4.79 g,

respectively compared with control treatment 3.22 g and significant difference with all treatments. Also, there was a significant decrease in fresh weight at concentrations of 1500 and 2000 ppm and the lowest fresh weight was recorded with concentration of 2000 ppm reached 1.82 g which differed significantly with control and all other treatments.

Perhaps low NaCl concentrations (500 and 1000 ppm) are optimal for the growth of the embryogenic callus and somatic embryos. It may also be the reason behind the significant enhancement of its fresh weight due to the maximum possible absorption of water and essential mineral elements by cells as a means of resistance to salt stress conditions **(Rains *et al.*, 1986)**. It was found that using NaCl at concentrations of 25-50 µM caused a significant increase in callus fresh weight of other date palm cultivars Barhee and Helali **(Al-Khayri, 2002)**.

But the reduction in callus fresh weight at high salt stress 2000 ppm may be attributed to the negative effect of salt on cells grown at high salt concentrations. This finding was similar with previous reports under *in vitro* salinity stress **(Al-Khayri, 2002; Queiros *et al.*, 2007; Rafiq *et al.*, 2008; Lokhande *et al.*, 2011 and Golkar *et al.*, 2017)**.

There was a significant decrease in fresh weight of callus with high concentration of NaCl at 320 µM. The effect on growth is probably due to osmotic effect and ion cytotoxicity, due to the accumulation of Na^+ and Cl^- leading to growth inhibition. It has been suggested that the reduction of growth in response to salinity is the result of great portion of respiratory energy being transferred to processes resulting in salt tolerance rather than growth **(Mass, 1986)**. Data indicated the passive effect of NaCl treatments on callus growth and frequency of somatic embryo germination percentage after 12

weeks. The Highest value of callus growth (1.93 and 2.00 g) was observed at 50 µM NaCl compared to control treatment (1.98 and 2.05 g), following by 100 µM (1.47 and 1.90 g), 200 µM (1.25 and 1.49 g), 300 µM (0.24 and 0.45 g) for cv. Barhi and cv. Khalas, respectively although callus of cv. Khalas recorded the minimum value of callus growth (0.13 g) under 400 µM NaCl **(Al-Dhebiani *et al.,* 2018)**. Adding NaCl to callus culture of *N. sativa* results in reducing the callus growth from control (0.17 mm) to (0.068 mm) significantly in the treatment with 250 µM NaCl under *in vitro* salt stress. In addition, salinity stress represents a negative osmotic stress, leading to a decrease in the water available in the callus cells, which consumes energy leading to the reduced growth of callus **(Golkar *et al.,* 2019)**. Salinity stress could reduce the plant productivity by unbalancing cellular ions **(Tester and Davenport, 2003)**. It had been previously observed by many workers that the presence of salt in the medium generally reduced or even completely inhibited the plant regeneration **(Lutts *et al.*, 1999).**

Effect of salinity on somatic embryos induction

There was a significant difference between different concentrations of NaCl and the number of somatic embryos. The number of somatic embryos of Hayani cv. was enhanced in response to low level of salinity as NaCl at 500 and 1000 ppm which resulted in the increase in the number of somatic embryos 12.45 and 11.60 embryo/jar, respectively compared with the control treatment which recorded low number of embryos 5.13 embryo/jar. However, increasing the concentration of NaCl over 1000 ppm decreased the number of embryos gradually; it was reduced from 6.29 to 4.03 embryos/jar with the treatments 1500 and 2000 ppm NaCl, respectively as shown in **Table 2 and Fig. 2**.

Table 2. Effect of NaCl concentrations on fresh weight of embryogenic callus and number of somatic embryos of the date palm Hayani cv. after 8 weeks from culture

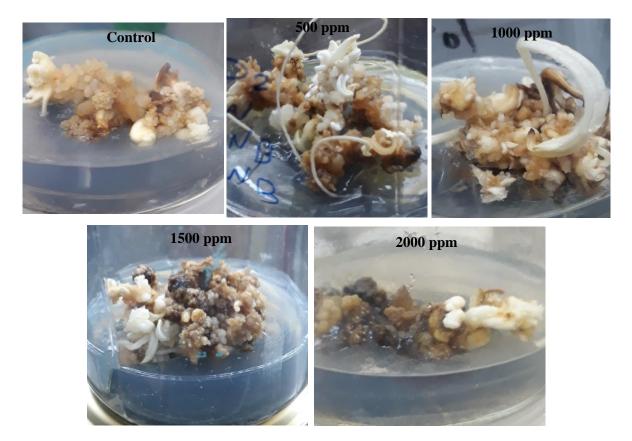| NaCl concentration (ppm)	Fresh weight (g) of embryogenic callus	Number of somatic embryos
0	3.22 b	5.13 b
500	4.84 a	12.45 a
1000	4.79 a	11.60 a
1500	2.47 c	6.29 c
2000	1.82 d	4.03 d

Fig. 2. The growth of embryogenic callus and somatic embryos of date palm Hayani cv. under salinity conditions by treatment with different concentrations of NaCl

These results were in agreement with several studies, low levels of salinity can enhance growth and development of *in vitro* date palm callus culture (**Al-Khayri, 2002**). Moreover, the number of somatic embryos of cv. Zagloul was enhanced in response to low concentration (25 µM) of NaCl. However, higher concentrations of NaCl reduced callus growth and the number of somatic embryos (**Ibraheem *et al.*, 2012**). In addition, using of seawater in all *in vitro* culture stages, proved to be highly effective for enhancing somatic embryogenesis of cv. Malkaby (**Taha, 2014**). According to **Ibraheem *et al.* (2012)** the number of somatic embryos of cv. Zaghloul was enhanced with adding 25 µM NaCl to the regeneration medium; however, at 75 µM NaCl the number of resultant somatic embryos was reduced and no embryo formed at 175 µM NaCl.

The results showed that increasing NaCl concentration significantly decreased the fresh weight of both the embryogenic callus and somatic embryos (**Abbas *et al.*, 2012**). The addition of NaCl to the media caused a significant effect on the reduction of somatic embryo germination percentage (**Al-Zubaidi *et al.*, 2013 and Ibraheem, 2013**). Adverse effect was reported by **Jasim *et al.* (2010)** where 0.5-2.0% NaCl inhibited callus growth and

somatic embryogenesis of cv. Ashkar. The presence of salt in culture medium resulting in the loss of regeneration in organogenic callus and shoots were obtained from culture media with 42.7 mm and 85.57 mm NaCl (**Sharry and Teixeira da Silva, 2006**). Date palm regeneration through somatic embryogenesis is affected by the salt concentration of the culture medium. A low concentration of salt (0.4% NaCl) observed to increase the length of *in vitro* shoots of cvs. Bartamuda, Sewy and Samani. However, shoot growth reduction was noticed at 0.8% and 1.2% NaCl (**El-Sharabasy *et al.*, 2008**).

Chemical analysis

Plant acclimation under abiotic stresses such as salinity has been examined through biochemical analysis. There is much studies of embryos subjected to salt treatment (**Munns, 2002 and Santos *et al.*, 2011**). The tolerant cells make osmotic adjustments in response to abiotic stress; such as, highly soluble compounds like sugars, amino acids and proline accumulate in higher plants under salinity stress (**Ashraf, 1994**). They serve as organic compatible solutes, which cause osmotic adjustments and play a role in stabilization of proteins, protection of membrane and help in regulating ionic sequestration (**Patade *et al.*, 2011**).

Determination of total carbohydrates

Data in **Fig. 3** revealed that, there was a significant difference between different concentrations of NaCl and total carbohydrate content in the embryogenic callus. Added NaCl to culture medium at concentration 500 ppm increased the carbohydrate content (21.80 mg/g fw) in the embryogenic callus compared with the control treatment (20.16 mg/g fw). Increasing NaCl concentration to 1000 ppm increased total carbohydrate content to (22.5 mg/g fw); however, increasing the concentration of salinity stress over 1000 ppm to 1500 ppm was resulted reduced the total carbohydrate content to (20.00 mg/g fw).

While, the lowest carbohydrate content (17.30 mg/g fw) was recorded at the highest concentration of NaCl at 2000 ppm.

There was significant difference between different concentrations of NaCl and total carbohydrate content in the somatic embryos. The highest total carbohydrate content (20.76 and 21.60 mg/g fw) was recorded with the concentration of NaCl at 500 and 1000 ppm compared with the control treatment which was (19.20 mg/g fw). The content of carbohydrate was reduced due to increasing the salinity concentration to 1500 and 2000 ppm and recorded (18.62 and 16.80 mg/g fw), respectively.
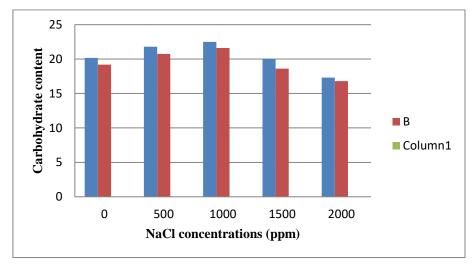

Fig. 3. Effect of different concentrations of NaCl on carbohydrate content (mg/g fw) in embryogenesis callus (A) and somatic embryos (B) after 8 weeks in culture

These results were in agreement with **(Mehr, 2013)** who reported the increase in the amount of production of some compounds in plants as exposure to salt stress and this is one of the mechanisms by which plants resist salt stress examples of such compounds are carbohydrates. **Jasim *et al.* (2010)** also found that an increase of NaCl concentrations in the medium led to a decrease of total soluble carbohydrates and proteins of embryogenic callus and somatic embryos of date palm, but it increased free proline.

The decrease in carbohydrates under salt stress condition might be related to the salt stress that make cells spend more energy for osmoregulation to endure the turbulence caused by salt accumulation inside cells, also high salt condition increase respiration rate through the effect of sodium ions on respiration cycle that led to the decrease in carbohydrates (**Wang *et al.*, 1999 and Huang and Liu, 2002**). Salinity induced soluble sugar accumulation has also been observed in *P. euphratica* (**Watanabe *et al.* 2000; Zhang et al.**

2004). The best characterized biochemical response of plant cells to osmotic stress is accumulation of some compatible organic solutes like soluble sugars **(Flowers and Colmer, 2008).** The results showed clearly that salinity significantly inhibited fresh weight of the callus of *Citrus sinensis* and decreased the concentration of carbohydrates (**Abbas *et al.*, 2013**). The carbohydrate concentration of *Salsola arbuscula* callus increased with used 300 µM of NaCl, but decreased significantly in the 400 µM of NaCl (**Amini *et al.*, 2017**).

Salinity also induces oxidative stress, which influences enzyme activity causing a reduction in carbohydrate metabolism (**Huang and Liu, 2002**). It was observed that low salinity caused by NaCl at 10 µM significantly increased total soluble carbohydrate in plantlet leaves as reported by **Al-Kabi (2004)** for date palm. As the concentration of NaCl increased, the concentration of soluble carbohydrates was significantly decreased. The salt tolerant callus of sugarcane accumulated more soluble sugars under NaCl stress (**Gandonou *et al.*,**

2006). However, the soluble sugars did not accumulate in calli from salt-resistant wheat cv. Belikh (**Lutts *et al.*, 2004).** Increasing seawater levels increased total soluble sugars content compared with the control. Total soluble sugars in leaves of two young Iranian commercial olive cultivars increased with an increase in salinity up to 80 µM but decreased with additional increase in salinity (**Mousavi *et al.*, 2008**). In addition, soluble carbohydrate concentration of two sugar beet cultivars significantly increased with increasing salt stress. **Palma *et al.* (2009)** suggested that the response to salinity (100 µM NaCl) was improved by increasing plant dry weight and decreasing the contents of organic solutes like total soluble sugar contents in *Phaseolus vulgaris* L. **Dhanapackiam and Ilyas (2010)** reported that the increase of total carbohydrates in the shoot is considered to be playing an important role in the osmotic adjustment. To evaluate osmotic adjustment of the callus, the contents of soluble sugar were determined. The soluble sugar contents were increased by 1.6-fold under 50 µM NaCl and 1.8-fold under 150 µM NaCl treatments. The soluble sugar content was induced in the alkaligrass calli and leaves under stresses of NaCl (**Yu *et al.*, 2011**). The decrease in the photosynthesis rate occurred at high salinity, also inhibited cell expansion and cell division stomatal conductance and closure in *Bruguiera parviflora*

followed by the decrease in chlorophyll and carbohydrates content (**Sofy and Fouda, 2013).**

Determination of proline

The data indicate that salt stress had a significant effect on proline content in embryogenic callus cultured in MS medium with different concentration of NaCl. An increase in NaCl concentration cause a significant increase in proline content to 2.06 mg/g fw with adding 2000 ppm of NaCl in culture medium compared with control treatment which recorded (1.03 mg/g fw). Whereas low concentration of salt stress at 500 ppm gave less proline content (1.22 mg/g fw) and differed significantly with control treatment and with all treatments as showed in **Fig. 4.**

There was a significant difference between different concentration of NaCl and the proline content in somatic embryos. The increase in proline content was found to be gradual with the increase of NaCl concentration in the culture medium. The somatic embryos grown on MS medium supplemented with 1500 and 2000 ppm NaCl showed the highest proline content (1.93 and 2.20 mg/g fw, respectively) in comparison to control treatment which recorded (1.20 mg/g fw), as shown in **Fig. 4.** The low proline content (1.34 mg/g fw) resulted with low level of salt stress at 500 ppm compared to control.
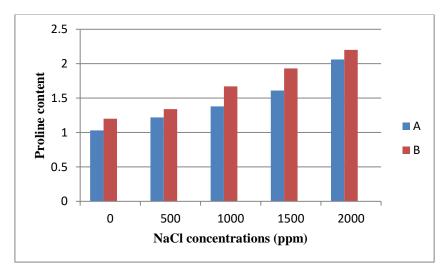

Fig. 4. Effect of different concentrations of NaCl on Proline content (mg/g fw) in embryogenesis callus (A) and somatic embryos (B) after 8 weeks in culture

These results agree with the results of **Watanabe *et al.* (2000)** in which salinity stress showed to increase the proline content in grape var. Khoshnaw. The highest content of free proline was obtained at the 100 µM salinity level, while the lowest was recorded in the control treatment. The calli of *Sesuvium portulacastrum* grown on MS medium supplemented with 200 and 400 µM salt

showed significantly the highest proline content in comparison to control calli (**Lokhande *et al.*, 2010).**

Physiological studies on callus exposed to salt stress have shown an increase in proline accumulation in response to increased salinity. Proline accumulation was correlated to callus growth inhibition (**Al-Khayri, 2002**) and hence is useful as a biochemical marker to understand how

plant tolerates the abiotic stresses (**El-Hadrami *et al.*, 2011**). The role of proline accumulation in tolerance to salt stress is thought to play role in osmotic phase of salt stress at early stages of salt treatment or at mild salt stress conditions (**Arzani, 2008**). Proline acts as osmotica that protects the cytosol from dehydration as symptomatic salt stress damage (**Ashraf and Foolad, 2007**). proline may act as a regulatory molecule able to activate multiple responses that are component of the adaptation process (**Ashraf and Harris, 2004**) and act as a osmolyte, stabilizer of the proteins structure, thereby protecting cells from damage caused by stress (**Szabados and Savoure, 2010**). Under salinity stress, higher levels of glutamate as a preproduct for chlorophyll and proline were consumed to generate proline (**Ahmad, 2014**). Proline can adjust the osmotic pressure of cells under various stresses. Additionally, free proline can remove and detoxify the reactive oxygen species (ROS) generated as a result of stresses, thus protecting cell membranes against these radicals (**Saed-Moucheshi *et al.*, 2014**). The response of proline and carbohydrates to salt stress may be due to these osmoprotectants that play an important role in decreasing stress-induced cellular acidification and osmotic adjustments, stabilizes sub-cellular structures for recovery (**Tan *et al.*, 2008**). In *Plantago ovata* callus, the proline content averaged over all the genotypes was found to increase with increasing salt from 0.1 mg/l FW in control to 1.1 mg/l FW in 200 µM NaCl (**Golkar *et al.*, 2017**).

Determination of total soluble protein

There was a significant difference between different levels of NaCl and protein content in both of embryogenic callus and somatic embryos. Results in **Fig. 5** indicated that increase in NaCl concentration in culture medium led to decrease in protein content in both embryogenic callus and somatic embryos especially under high level of salt stress 2000 ppm where the protein content reached to (0.72 mg/g fw) compared to (2.10 mg/g fw) under control treatment for embryogenic callus. While, in somatic embryos resulted (0.65 mg/g fw) at 2000 ppm salt stress compared to (1.8 mg/g fw) under control treatment.

The decrease in protein content under salt stress might be related to the inhibition of protein synthesis by high salty condition (**Kaouthar *et al.*, 2001**), also high salts in the culture media might inhibit absorption of necessary elements for protein synthesis such as nitrogen, besides the negative effect of salinity on the biosynthesis of mRNA which lead to the disturbance of protein synthesis (**Whitting and Wilson, 2003**) whereas, the increase in protein content at 20, 40, 60 and 80 µM may be due to stress induced synthesis of proteins (**Cherian and Reddy, 2003**). Proteins are a potential biochemical useful in salt resistant of plant cells and protect high plants against stresses by adjusting osmotic pressure by many compatible solutes (**Shonjani, 2002**).
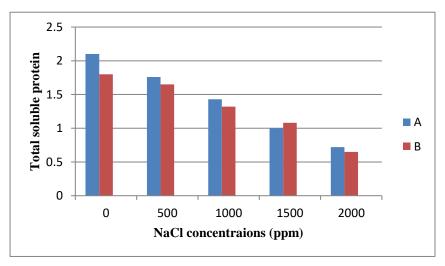

Fig. 5. Effect of different concentrations of NaCl on total soluble protein (mg/g fw) in embryogenesis callus (A) and somatic embryos (B) after 8 weeks in culture

Rahnama and Ebrahimzadeh (2004) also observed a decrease in protein in shoots and callus of potato with increasing NaCl concentrations. The effects of NaCl treatments (100 - 200 µM) on protein contents in potato leaves were studied by **Fidalgo *et al.* (2004)**. They observed a significant decrease in protein content under salt stress.

Shankhdhar *et al.* (2000) indicated that total protein contents of callus cultures decreased with an increase in salt concentration after 4 weeks of culture in six cultivars of rice callus. **Agastian *et al.* (2000)** reported that soluble proteins increased at low salinity but decreased at high salinity in mulberry. Similarly, **Khedr *et al.* (2003)** also

reported a decrease in growth and protein contents due to salt stress in the desert plant *Pancratium maritimum* L. Soluble protein contents of leaves were shown to have decreased in response to salinity in *Oryza sativa* L. (**Alamgir and Ali, 1999**), *Raphanus sativus* (**Muthukumarasamy et al., 2000**) and *Bruguiera parviflora* (**Parida et al., 2002**). Total protein content was significantly the highest in callus of *Sesuvium portulacastrum* stressed with 100 µM NaCl compared to control and further decreased with increased salt concentrations (**Lokhande et al., 2010**). Protein synthesis and lipid metabolism are affected under salt stress, positive antioxidant response might be responsible for a higher tolerance to stress in *Carrizo citrange*. The photosynthesis, respiration rate and total protein content of plants decreased under salt stress (**Yaish, 2015**).

In contrast to above, the increase in protein contents under salinity stress was observed with **Bekheet et al. (2000)** who selected two cultivars of *Asparagus officinalis* by culture shoot segments on callus induction medium supplemented with salt mixture. The cultivars showed better growth, high protein content, fresh and dry weight as salt concentration increased up to 6000 ppm.

Minerals analysis

In the current study, the effect of different concentrations of salinity on mineral contents in Hayani cv. was investigated. The results indicated a positive relationship between salinity levels and Na^+ ion content, while on the other side there was an inverse relation between salinity level and K^+ ion content.
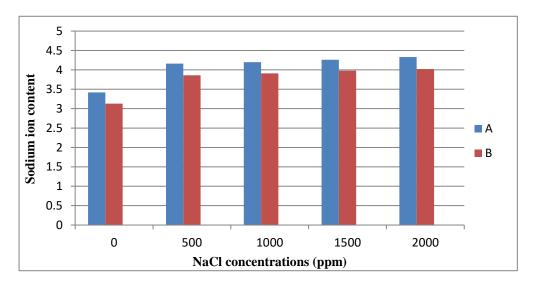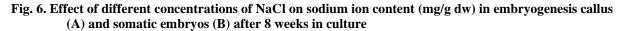
Sodium ion content

In the embryogenic callus, the Na^+ concentration increased significantly with increasing the concentration of NaCl in the culture medium, especially at the concentrations of 1500 and 2000 ppm NaCl which recorded (4.26 and 4.33 mg/g dw, respectievely) compared with the control treatment (3.42 mg/g dw) as shown in **Fig. 6**.

Na^+ concentration in the somatic embryos increased significantly with increasing salt concentration in culture medium, as shown in **Fig. 6**. The increase in Na^+ concentration was observed when the embryos were cultured on a medium containing high level of salt stress at 2000 ppm NaCl which resulted (4.02 mg/g dw) compared with the control treatment (3.13 mg/g dw).

This increase in Na^+ is due to its high concentration in the culture medium, which increases its absorption over other essential elements, such as K^+. This study was confirmed with previous reports on different plant species as safflower (**Golkar and Taghizadeh, 2018**), sunflower (**Alvarez et al., 2003**) and *P. ovata* (**Golkar et al., 2017**) subjected to *in vitro* salinity stress. The increase in Na^+ concentration in other date palm cultivars has been reported by **Al-Khayri (2002)** and also for tomato and grapes under salt stress (**El-Hammady et al., 1999**). Salinity has a negative effect on growth and development of plants through the osmotic stress due to the toxic effect of sodium ion, besides the ionic imbalance induced by accumulation of this ion (**Sairam and Tyagi, 2004**). The callus Na^+ content increased with increasing salt level, whereas callus K^+ content decreased under the same conditions. The increase in Na^+ contents is possibly an osmotic adjustment mechanism, which enable the tissues to adapt to the low water potential of the external environment (**Sabbah and Tal, 1990**).

Fig. 6. Effect of different concentrations of NaCl on sodium ion content (mg/g dw) in embryogenesis callus (A) and somatic embryos (B) after 8 weeks in culture

Sotiropoulos *et al.* **(2006)** reported that explants are under stress in two ways under *in vitro* salinity by the increase of culture media osmotic potential as a result of high Na^+ content and by Na toxic effects. Also, **Rus *et al*. (2000)** studied the rising of Na^+ concentration in *N. sativa* calli under salinity stress. High concentrations of sodium ions decreased osmotic balance and membrane structure, reduced growth and inhibited cell division. In addition, high concentration of Na^+ ion in nutritional solutions destroy hydraulic conductivity and tissue permeability related to water, thus decreasing plant survival **(Abadı ´a *et al.,* 1999)**. Such effect on growth is probably due to the effects of salinity, include osmotic effect and ion cytotoxicity; the accumulation of Na^+ and Cl^- in culture cell resulted in the growth inhibition. Furthermore, it has been suggested that the reduction of growth in response to salinity is the result of great portion of respiratory energy being diverted to processes which resulted in salt tolerance **(Mass, 1986)**.

Potassium ion content

K^+ has an important role in enzyme activation in plant cells **(Tester and Davenport, 2003)**. **Fig. 7** shows the effect of NaCl on the potassium concentration in the embryogenic callus and somatic embryos of date palm Hayani cv. It is clear that different concentrations of NaCl significantly decreased the level of K^+.

The highest concentration of K^+ (4.35 mg/g dw) recorded in the control treatment; however, with added NaCl at different concentration (500, 1000, 1500 and 2000 ppm) the level of K^+ decreased gradually (4.22, 4.18, 4.10 and 4.02 mg/g dw, respectively) in embryogenic callus. On the other hand, in the somatic embryos, as shown in **Fig. 7** the increase of NaCl concentration at 1500 and 2000 ppm led to a significant decrease in K^+ concentration ranged from (4.52 mg/g dw) under the control treatment to (4.31 and 4.28 mg/g dw, respectively) under the highest level of salinity.
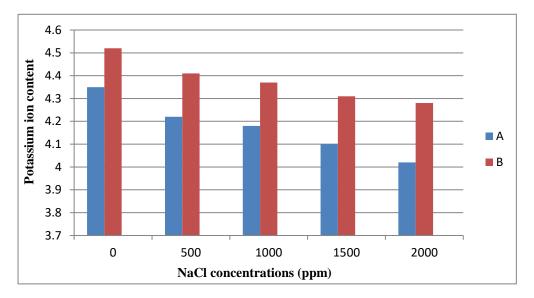

Fig. 7. Effect of different concentrations of NaCl on potassium ion content (mg/g dw) in embryogenesis callus (A) and somatic embryos (B) after 8 weeks in culture

This reduction in levels of K^+ is due to excessive accumulation of Na^+ and Cl^-, which reduces the accumulation of K^+. Furthermore, osmotic stress induced by salinity also reduces water uptake and hence accumulation of potassium **(Abbas *et al.,* 2012)**. There is a significant reduction of the concentration of K^+ in the callus tissues of *Citrus sinensis* (L.) and increasing the concentration of NaCl in the culture medium. This decrease in K^+ concentration is due to the presence of Na^+ at high concentration, which competes with K^+ uptake and may also block the K^+ specific transporters of the

callus cells under salinity **(Zhu, 2003)** resulting in toxic levels of Na^+ as well as insufficient concentration of K^+ for enzymatic reactions and osmotic adjustment.

The reduction in K^+ concentration in callus cells under salt stress could be explained by the change in expression and function of transporter as well as the ion channels especially those related to K^+ **(Arzani and Ashraf, 2016)**. The increase nutritional uptake of both Na^+ and K^+ might have led to the retention of water in the callus **(Chaudhary *et al*., 1997)**.

Increasing the concentration of NaCl in the culture medium generally resulted in increase the Na^+ and reduction in K^+ concentrations. However, at 25 µM NaCl, the only level at which callus growth was significantly enhanced; an increase in K^+ content was noted, in comparison to the NaCl free control **(Al-Khayri, 2002)**.

Na^+ accumulation in tissues under salinity stress is generally considered as a major factor behind the adverse effect of salinity on nutrient uptake and growth **(Shibli *et al*., 2001)**. The increase in Na^+ content of cells was accompanied by a decrease in K^+ accumulation and differences in Na/K ratio under saline conditions **(Cherian and Reddy, 2003)**.

The somatic embryos cultures after treatment with different level of salinity stress for two months were transferred to optimized regeneration medium without NaCl. The recovery percentage decreased with increase in NaCl concentration. It was observed that regeneration of embryo cultures without salt or with low concentrations of NaCl was better than treated with high concentrations of NaCl (1500 and 2000 ppm). The shoots produced from the experiment of salinity with using low concentration of NaCl were the strongest in growth and ability to continue to rooting stage, as shown in **Fig. 8.**
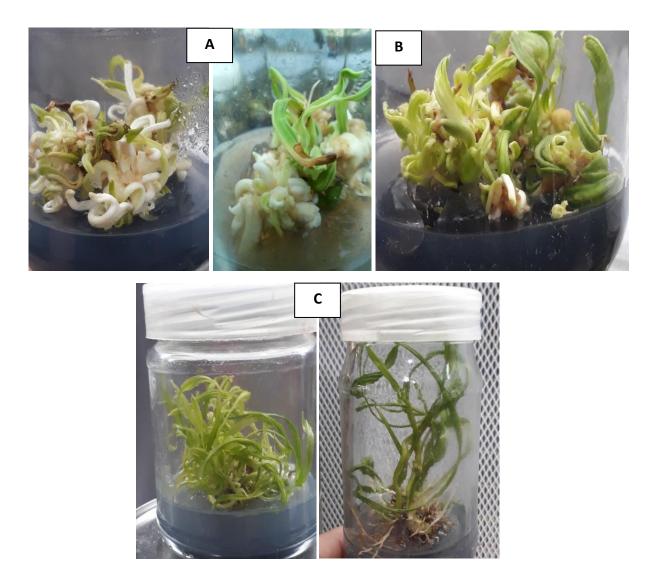

Fig. 8. Different stages of somatic embryos to produce the shoots. A: Embryos culture, B: Shoots proliferation from embryos, C: Shoot elongation and rooting stage

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