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In Vitro Multiplication of *Chrysanthemum morifolium* Ramat and it is Responses to NaCl Induced Salinity

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Abstract

A micropropgation method by multiple shoot formation of *Chrysanthemum morifolium* has been developed. Explants growing in greenhouse were used to establish cultures of *C. morifolium*. Shoot tips were surface sterilized and cultured on Murashige and Skoog (MS) media. Successful *in vitro* multiplication of chrysanthemum was achieved on MS medium supplemented with benzyl amino purine (BAP) at 0.3 mg l^{-1} . *In vitro* rooting was successfully achieved on MS media supplemented with different concentration of auxins. The *in vitro* response to salinity stress (0 to 300 mM NaCl) was also tested. Shoot proliferation was gradually reduced at higher NaCl concentrations. Shoot length, number of leaves, fresh and dry weight, chlorophyll, and carotenoid decreased with elevated salinity concentration. Proline and sodium contents increased with elevated salinity, whereas potassium, nitrogen and protein content decreased. Plantlets grown *in vitro* presented tolerance and their growth was negatively affected at high salt concentrations. Elevated salinity significantly reduced microshoot protein. It is concluded that in *C. morifolium* response to *in vitro* salinity stress may provide a system for production under field conditions.

الملخص

لقد تم تطوير طريقة للاكثار بواسطة تكوين الاغصان المضاعفة لنبات غريب (الكريزنثم). ان الاغصان النامية من النسيج قد تم زراعتها في البيت الزجاجي لاجل تاسيس مزرعة نسيجية من الغريب. لقد تم اجراء التعقيم السطحي الاغصان وثم زراعتها في وسط حار موراشيج وسكوج (MS) ان التضاعف الناجح داخل الانابيب لنبات غريب قد تم الحصول علية في وسط (MS) والمزود بمنظم النمو ينزل امينوبيورين (BAP) بتركيز 0.3 مغم/لتر. ان التجديد داخل الانابيب قد تم التوصل اليها في وسط (MS) وباستخدام تراكيز مختلفة من الاوكسجين. بالاضافة لذلك، فقد تم اختبار الاستجابة داخل الانابيب الى الاجهاد الملحي (بتراكيز من ملح الطعام من صفر الى 300 ملمول) . ان توالد الاغصان قد نقص تدريجياً في التراكيز العالية من ملح الطعام، ان التركيز العالي من ملح الطعام قد نتج عنه نقصاً في طول الغصن ، عدد الاوراق الورق الطري والجاف المحتوى من الكلورفيل والكاروتين . ان المحتوي من البلورين والصوديوم قد ازداد مع زيادة تركيز ملح الطعام في حيث قل مثل المحتوى من البوتاسيوم والنتروجين. ان النبيتات النامية داخل الانابيب قد اظهرت مقاومة وان نموها قد تأثر سلبياً في تراكيز الملح العالية. ان التركيز العالي من الملح قد خفضً معنوياً نمو الاغصان الصغيرة من البروتين. نستنتج من ذلك بان نبات الغريب الذي يستجيب للاجهاد الملحي يجب ان يزود بنظام للانتاج تحت الظروف الحقلية. اخيراً فقد تم الحصول على النبيتات الكاملة النمو المنتجة داخل الانابيب وتحت مستويات مختلفة من المزراع الملحية .

Keywords: *Chrysanthemum morifolium*, salinity, shoot multiplication, root formation.

1. Introduction

Simple screening of plantlets by tissue culture provides a unique opportunity for studying many aspects of plant growth and development under well defined conditions (Shatnawi, 2006). Moreover, tissue culture provides an important tool for studying the physiological effects of salt at the cellular level under controlled environment (Olmos *et al*., 1994). Cell and tissue culture systems have been considered for selection of plant tolerance to salinity, drought, and other stresses (Luttus *et al*., 1999). Salinity affects crop production and agricultural sustainability as it reduces productivity of the effected land (Al-Karaki, 2000). Plants frequently respond to water deficit, salt, or osmotic stress with identical or similar physiological and growth adaptations (McCue and Hanson, 1990).

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Chrysanthemum morifolium belongs to the family *Composaetae* (*Asteraceae*) (Arora, 1990). Chrysanthemum is one of the largest cut-flower among the ornamental plants traded in the global flower market. Chrysanthemums are important not only for their outstanding aesthetic beauty and long lasting as marketing cut flowers. They can be propagated vegetatively either through root suckers or terminal cuttings; this conventional process of shoot cutting is very slow (Nhut *et al*., 2005). However, clonal propagation through *in vitro* culture can enhance the multiplication rates (Sauvaire and Galgy, 1978).

In vitro culture allows monitoring plant responses to salinity at biochemical and physiological level (Shibli *et al*., 2000; 2007). Thus, it is crucial to establish a relationship between *in vitro* and *in vivo* responses (Lutus *et al*., 1999). Response of cultures to *in vitro* and *in vivo* induced stress was similar (Sawwan *et al*., 2000). Artificial salt stress on plant tissue grown *in vitro* is imposed by adding sodium chloride to the media (Shibli *et al*., 2007) because NaCl is the most predominant salt in saline condition.

Salinity is one of the most significant abiotic stresses for plant agriculture. It causes a serious problem as it reduces the value and productivity of the effected land (Al-Karaki, 2000). Salinity disrupts physiological processes in plants, leading to reduction in growth and yield (Cordovilla *et al*., 1995). Salt destroys the vital physiological process in plants by slowing cell division, cell enlargement, or both in the growing region (Ali *et al*., 1994), and increasing salt concentration and ions composition cause growth restriction or even lead to plant death (Cavagnaro *et al*., 2006). The mechanisms to determine plant responses to salinity involve complex interactions, since plants grow under saline conditions subjected to different types of stress including water stress caused by osmoticum (Schwarz and Kuchenbuch, 1998), mineral toxicity (Shannon 1985), and disturbance in mineral nutrition of the plants (Franco *et al*., 1999).

In vitro culture constitutes a useful tool for rapidly and economically evaluating tolerance in plants, especially for species that have long reproductive cycles. Shoots are easy to propagate *in vitro* and plant material selected from drought or salt stressed cultures can be used to establish plantations in dry or saline soils. Therefore, the aim of this study was, to initially establish an effective way for *in vitro* proliferation method for *C. morifolium* Ramat and furthermore to investigate the *in vitro* response of *C. morifolium* when being subject to NaCl stress.

2. Materials and methods

2.1. Establishment of in vitro culture

Microshoots of *C. morifolium* Ramat, (Balady) shoot tips were washed under running tap water and then sterilized with 70% ethanol for 30 seconds, then dipped in 3.5% sodium hypochlorite for 15 minutes. Finally, excess detergent was removed by rinsing in sterile distilled water four times each for five minute, under the laminar air-flow cabinet.

Medium was solidified using 8.0 g l^{-1} agar agar, then dispensed in test tubes (8 ml each), and autoclaved at 121 ºC. Shoot tips were grown on solid half strength MS medium (Murashige and Skoog, 1962). Cultures were maintained in the growth chamber at 24 ± 2 ºC and 16 h light/8 h dark. Microshoots were then subcultured on full strength MS medium for six times to have enough mother stock prior to experiments initiation. The pH of MS medium was adjusted to 5.8. Medium was solidifying 8.0 g l^{-1} agar agar, dispensed in flasks (60 ml), and autoclaved at 121 ºC. Cultures were maintained in the growth chamber at 23 ± 2 ºC and 16 h lights (50 µmol $m^{-2}s^{-1}$) / 8 dark.

2.2. In vitro propagation

Microshoots were subcultured to hormone-free MS medium for two weeks to eliminate any carry-over effects of the basic cytokinin. For shoot proliferation, microshoots (15 mm in length) were subcultured to MS medium supplemented with either, benzyl amino purine (BAP) or kinetin, at 0.0, 0.3, 0.6, 0.9, 1.2 and 1.5 mg l^{-1}. The pH of MS medium was adjusted to 5.8. Medium was solidified using containing 8 g l^{-1} agar and 30 g l^{-1} sucrose supplemented to the medium. 60 ml / flask of the MS medium was dispensed into 250 ml Erlenmeyer flasks and autoclaved at 121 ºC. Explants were incubated in a growth room under 16 h light/ 8 h dark (50 µmol $m^{-2}s^{-1}$). After six weeks growth periods, data were collected on shoot length and number of shoot per explants.

2.3. In vitro root formation

Microshoots were subcultured to hormone-free MS medium for 2 weeks to eliminate any carry-over effects of the cytokinin. Microshoots (15 mm in length) were subcultured on MS medium supplemented with (0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 mg l^{-1}) IBA (indole-3-butyric-acid), IAA (indole-3-acetic-acid) or NAA (1- naphthalene acetic acid containing 8 g l^{-1} agar and 30 g l^{-1} sucrose. Explants were incubated in a growth room under 16 h light/8 h dark (50 µmol $m^{-2}s^{-1}$). Data were collected after six weeks growth period on number of roots, roots length, and number of leaves.

2.4. Effect of salinity on microshoots physiological responses

2.4.1. Plant growth

Microshoots 15 mm in length with two leaves were subcultured on MS medium on proliferation medium containing 0.3 mg l^{-1} BAP, and supplemented with different concentration of NaCl (0, 20, 40, 60, 80, 100, 150, 300 mM). Data were collected after six weeks growth period on shoot length, number of shoot per explants, number of new leaves, fresh weight, and dry weights.

2.4.2. Chlorophyll and carotenoid content

A fresh weight of 0.1 g of leaves was sampled per replicate, with 5 replicates per treatment. Samples were extracted by acetone, following the method of De Filippis *et al*. (1981). Pigments assay was prepared after six weeks exposure to salts. A fresh weight of 0.1 g shoot material was homogenized in 2.0 ml of 80% acetone using a pestle and mortar. The extract was pipetted into 2.0 ml microtubes and centrifuged at 15000 g for 2 min Helitch Microfuge. The clear green supernatant was collected using a Pasteur pipette, and made up to 3.0 ml in a 10 ml measuring cylinder with extra 80 % acetone. The

absorption spectra were measured by a spectrophotometer (Pye Unicam Sp6) at 480, 510, 626, 645, 649, 663 and 665 nm absorbance, 80 % acetone was used as a reference. Chlorophyll *a*, and Chlorophyll *b* contents were calculated according to Anderson and Boardman (1964), whereas carotenoids content was calculated according to Duxbury and Yentsch (1956).

2.4.3. Proline content

After six weeks growth period, free proline was extracted from leaves and measured colorimetrically. About 0.5 g of fresh weight plant was homogenized in 10 ml of 3% aqueous 5- sulfosalicylic acid and the extract was filtered through Whatman # 2 filter paper. Two ml of the filtrate and 2 ml of both acidic ninhydrin and glacial acetic acid were mixed in a test tube and placed in a boiling water bath (100 ºC) for 1 h to allow color development. The reaction was stopped by placing the tubes in an ice bath. To extract the chromophore, 4 ml of toluene were added to the tubes and mixed vigorously for 15-20 sec. and left at room temperature until the aqueous phase separated from toluene. The extract was quantitatively transferred to a clean test tube. Absorbance of the tested extract was compared with toluene blank by Milton Roy spectrophotometer 1001 at 520 nm. Stock solution of 250 mg praline ml l^{-1}, in 3% aqueous 5-sulfosalicylic acid, was used and a linear standard curve was constructed over a rang of 30 to 250 mg l^{-1}.

2.4.4. Mineral composition

Plant samples were dried at 80 ºC for 24 h and grounded to analyzed nitrogen, sodium and potassium contents. Total nitrogen was determined using Micro-Kjeldahl digestion procedure. A 0.5 g of dry weight plant samples were placed in large test tubes. 10 g of digestion mixture (1M K_2SO_4 + 1M $CuSO_4.5H_2O$) and 20 ml of concentrated H_2SO_4 was added to each test tube. Test tubes were placed in the digester (Buchi Digest Automat K-438) for 135 min at 400 ºC. After cooling down, samples were placed in the distilater (Buchi Autokjeldahl Unit K-370) to determine nitrogen concentration (Bremner *et al*., 1992). Crude protein content in the microshoots was determined by multiplying the total nitrogen by a factor of 6.25 (Balman and Smith, 1993). Na and K concentration were determined by using flame photometer. Plant samples were ashed at 500-550 ºC for about 20-24 h using Thermolyne muffle furnace (6000 Furnace). 10 ml of 2N HCl was added to the samples and gently heated 7-10 min on 75-80 ºC. The solution was filtered using Whatman # 42 filter paper, the solution was filtered and diluted to 50 ml with distilled water then mixed gently. Using Flame photometer 410, Na and K concentration were determined after calibration with different concentration of either Na or K solutions (Chapman and Pratt, 1961).

2.4.5. Experimental design and statistical analysis

Experimental design was performed as a completely randomized design. Each experiment consisted of 5 treatments and was replicated 4 times; each experiment was repeated at least twice. Data were subjected to ANOVA; differences between individual means were determined by Fishers' Least significant difference (LSD) at the 0.05 probability level. Data were analyzed using STATISTICA (StatSoft, Inc 1995).

3. Results

3.1. In vitro shoot formation

Increasing BAP from 0.0 to 0.3 mg l^{-1} increased the number of proliferated shoots from 1.98 to 4.35 (P=0.05). Number of proliferated shoots at 1.5 mg l^{-1} BAP was lower compared to those at 0.3 mg l^{-1} BAP (Table 1). Maximum shoot production was obtained on a medium containing 0.3 mg l^{-1} BAP (Table 1). Similarly, kinetin increased average number of shoots/explants. Increasing concentration of BAP or kinetin decreased shoot length.

3.2. In vitro root formation

Microshoots were successfully rooted *in vitro* on MS medium supplemented with 0.0, 0.2, 0.4, 0.6, 0.8 or 1.0 mg l^{-1} of IBA, IAA or NAA. Root formation start after 14 days growing period. Rooting occurred in bases of shoots growth on solid media supplemented with IBA, IAA, or NAA (Table 2). No callus formation appeared at the bases of the cuttings. Increasing IBA, IAA or NAA concentrations resulted in a significant effect on root length (Table 2). Maximum root number was obtained with the addition of IBA at 0.2 mg l^{-1}, with an average of 18.75 roots per microshoot. Root length was significantly decreased with the use of IBA, IAA and NAA at 1.0 mg l^{-1} (Table 2). Increasing IBA concentrations significantly increased number new leaves formation (Table 2). Maximum number (17.07) of leaves was obtained at 0.4 mg l^{-1} IAA, followed by 14.75 at 0.4 mg l^{-1} IBA (Table 2).

Table 1; Influence of BAP or kinetin on shoot length, number of shoots after six weeks growth period of *in vitro* grown *C. morifolium*

Growth regulators (mg l^{-1})	Shoot length (cm)	Number of new shoot
	BAP	
0.00	2.51	1.98
0.30	3.79	4.35
0.60	2.12	4.15
0.90	2.04	4.13
1.20	1.98	3.90
1.50	1.90	3.45
	Kinetin	
0.30	3.11	1.98
0.60	3.26	1.65
0.90	3.00	2.10
1.20	2.97	3.00
1.50	2.44	1.95
Means	2.88	1.45
LSD at 0.05	0.287	2.02

Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. Differences between individual treatments were determined with the least significant difference (LSD) at 0.05 level of probability.

3.3. Physiological responses of microshoots to salinity levels

3.3.1. Growth

Effect of NaCl on shoot length, number of new shoots, number of new leaves, fresh weight, and dry weight after six weeks growth periods on MS medium supplemented with 0.3 mg l^{-1} BAP were reported (Table 3). After six weeks growth periods on MS medium supplemented with 0.3 mg l^{-1} BAP, shoot length and number were decreased with the increased salt concentration in the medium (P=0.05). Number of shoots, number of new leaves, fresh weight, and dry weight decreased with increasing salt in the medium. The highest number of shoots was 2.80 at 20 mM NaCl whereas the lowest number of shoots was 1.00 at 300 mM NaCl (P=0.05). Whereas the highest number of new leaves was produced (26.15) at 40 mM NaCl and then it was decreased with increasing NaCl and it was ceased at 300 mM NaCl. Control treatment produced 13.79 new leaves. Fresh weight was increased with the increase in NaCl concentration (P=0.05). Maximum fresh weight (1.68 g) was obtained at 60 mM NaCl, whereas the minimum fresh weight (0.24 g) was obtained at 300 mM NaCl (Table 3). Similar to fresh weight, dry weight was decreased with increasing salt in the medium, and the highest dry weight was obtained at 60 mM NaCl (Table 3).

Table 2: Effect of IBA, IAA or NAA on roots number, root length, number of new leaves after six weeks growth periods of *in vitro* grown *C. morifolium*

Growth regulator (mg l^{-1})	Root number	Root length (cm)	Number of new leaves
0.0	5.65	3.23	9.33
IBA			
0.2	18.75	4.94	13.32
0.4	16.68	4.63	14.75
0.6	15.52	3.82	13.35
0.8	15.35	3.61	11.95
1.0	14.18	3.20	9.41
IAA			
0.2	10.68	4.59	14.36
0.4	10.26	3.89	17.07
0.6	9.62	3.48	16.99
0.8	8.32	3.01	16.25
1.0	7.20	2.44	13.60
NAA			
0.2	14.82	4.94	11.20
0.4	14.58	4.70	13.31
0.6	13.72	4.20	13.05
0.8	13.52	3.93	12.32
1.0	12.45	1.94	9.70
LSD at 0.05	0.950	0.484	0.958

Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. Differences between individual treatments were determined with the least significant difference (LSD) at 0.05 level of probability.

3.3.2. Chlorophyll and carotenoid content

Chlorophyll content decreased as NaCl concentration increased in the media (Table 4). Chlorophyll a and chlorophyll b contents were declined with elevated salinity. Maximum value for chlorophyll a was 122.56 (µg g^{-1} Fw) at 0.0 NaCl (Table 4). Chlorophyll b content decreased with increasing salt in the medium (P=0.05). However, the chlorophyll a/b ratio was not affected by NaCl concentration. Carotenoid content decreased with increasing salts in the medium. Carotenoid content was the highest (39.61 µg g^{-1} Fw) at 0.0 mM NaCl concentration, whereas the lowest content (13.306 µg g^{-1} Fw) was obtained at 300 mM. Maximum carotenoid-chlorophyl ratio (0.2005 µg g^{-1}) was obtained at 300 mM NaCl which was lower than the ratio (0.2001 µg g^{-1}) obtained at the control (Table 4).

Table 3: Effect of NaCl on shoot length, number of new shoots, number of new leaves, fresh weight, and dry weight of *in vitro* grown *C. morifolium* after six weeks growth period on MS medium supplemented with 0.3 mg l^{-1} BAP.

NaCl (mM)	Shoot length	Shoot number	Leaves number	Fresh weight (g)	Dry weight (g)
0	2.70	1.94	13.79	0.31	0.01
20	2.98	2.80	21.90	0.57	0.04
40.0	2.08	2.20	26.15	0.75	0.07
60.0	1.95	2.15	25.30	1.68	0.17
80.0	1.94	2.14	24.90	1.17	0.15
100.0	1.88	1.89	21.16	0.97	0.13
150.0	1.76	1.40	16.88	0.67	0.10
300.0	1.59	1.00	0.00	0.24	0.07
LSD at 0.05	0.146	0.153	0.613	0.055	0.003

Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. Differences between individual treatments were determined with the least significant difference (LSD) at 0.05 level of probability.

3.3.3. Mineral composition

Table 5 represents the Na, K, N contents in leaf tissue of *C. morifolium* after six weeks growth periods. As NaCl increased, nitrogen content decreased. Maximum value 7.33% was obtained at control, and then it started to decline dramatically until it reached its minimum value (3.61) at 300 mM NaCl (Table 5). Na content in leaf tissue of *C. morifolium was* significantly increased as NaCl increased.

As NaCl in the medium increased, sodium content increased in plant tissue after six weeks growth periods (Table 5). Na concentration reached maximum value 7.21% at 300 mM. Maximum value was significantly different as compared to minimum value (0.30) which was obtained at the control treatment. Potassium content in leaf tissue of *C. morifolium* was significantly decreased as NaCl level increased in the medium (Table 5). Maximum value (6.02) occurred at control (0.0 mM NaCl) and then it

decreased with salinity elevated salinity reaching a minimum value of 1.28 at 300 mM NaCl. There are significant differences among all values (Table 5).

Table 4: Effect of NaCl on chlorophyll and carotenoid content of *in vitro* grwon *C. morifolium* after six weeks growth periods on MS medium supplemented with 0.3 mg l^{-1} BAP.

Concentration (mM)	Chl a (µg g^{-1} Fw)	Chl b (µg g^{-1} Fw)	Chl a/b ratio	Total Chl (µg g^{-1} Fw)	Carotenoid (µg g^{-1} Fw)	Carot-Chl ratio (µg g^{-1})
0	122.56	75.41	1.63	197.96	39.61	0.2001
20	111.10	68.79	1.61	179.89	36.02	0.2002
40	100.83	62.51	1.61	163.34	32.71	0.2002
60	88.34	54.81	1.61	143.15	28.67	0.2002
80	78.93	49.05	1.61	127.97	25.62	0.2002
100	65.17	40.57	1.61	105.74	21.18	0.2003
150	52.87	32.95	1.60	85.83	17.19	0.2003
300	40.87	25.51	1.60	66.37	13.31	0.2005
LSD at 0.05	0.046	0.033	0.001	0.074	0.017	0.001

Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. Differences between individual treatments were determined with the least significant difference (LSD) at 0.05 level of probability.

Table 5: Effect of different concentrations of NaCl on sodium, potassium and nitrogen contents of *in vitro* micoshoots of *C. morifolium* grown on medium supplemented with 0.3 mg l^{-1} BAP after six weeks growth periods

Concentreation mM	**Na%**	**K%**	**N%**
0	0.30	6.02	7.33
20	1.13	5.11	6.83
40	1.96	4.62	6.67
60	3.18	4.24	6.20
80	4.44	3.81	5.63
100	4.76	3.46	5.34
150	6.30	2.81	4.22
300	7.21	1.28	3.61
LSD at 0.05	0.130	0.504	0.277

Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. Differences between individual treatments were determined with the least significant difference (LSD) at 0.05 level of probability**.**

As NaCl in the medium increased, sodium content increased in plant tissue after six weeks growth periods (Table 5). Na concentration reached maximum value 7.21% at 300 mM. Maximum value was significantly different as compared to minimum value (0.30) which was obtained at the control treatment. Potassium content in leaf tissue of *C. morifolium* was significantly decreased as NaCl level increased in the medium (Table 5). Maximum value (6.02) occurred at control (0.0 mM NaCl) and then it decreased with salinity elevated salinity reaching a minimum value of 1.28 at 300 mM NaCl. There ere significant differences among all values (Table 5).

3.3.4. Proline and protein content

Proline content increased with the increases in NaCl (Figure 1). The lowest proline value was obtained at 0.0

mM NaCl, whereas the highest proline content was obtained at 300 mM NaCl. Protein contents declined with the increases in NaCl concentration (Figuer. 2). Protein content was 40.41 and 22.58 mg g l^{-1} . FW when the medium was supplemented with 20 and 300 mM NaCl, respectively.

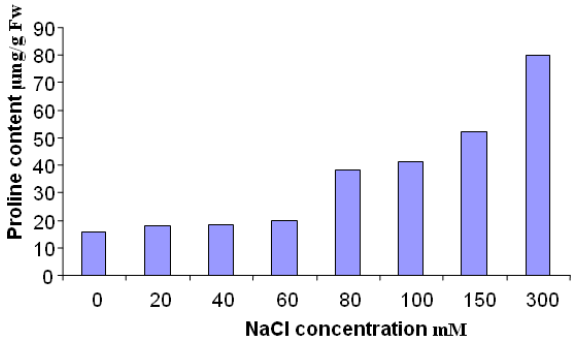

Figure 1: Effects of different level of NaCl on proline content of *in vitro* grwon *C. morifolium* after six weeks growth period on MS medium supplemented with 0.3 mg l^{-1} BAP. Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. *LSD at 0.05=0.2325*

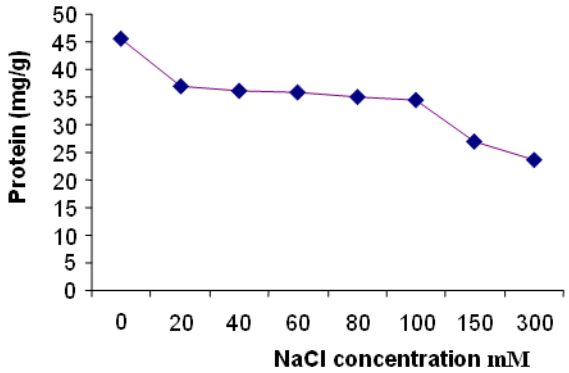

Figure 2: Effect of different level of NaCl on protein content of *in vitro* grwon *C. morifolium* after six weeks growth periods. Values represented means; each experiment consists of five treatments, each treatment consisted of 4 replicates and each experiment was repeated twice. ***LSD at 0.05= 3.199.***

4. Discussion

4.1. In vitro propagation

The study aimed at identifying the best media for shoot proliferation and studying the effect of NaCl on *in vitro grown C. morifolium*. The results of the investigations indicated that an efficient *in vitro* propagation method, with high levels of survival and reproducibility, could be achieved for *C. mirifolium.* BAP and Kinetin stimulate cell division (Table 1). Increased BAP to 0.3 mg l^{-1} produce maximum microshoot. Optimum shoot proliferation of juvenile and adult chrysanthemum plants was obtained on MS medium containing 2.22 µM BAP (Long *et al*., 2006). However, proliferation was inhibited at 4.4 µM BAP, which is similar to our finding in *C. morifolium* where proliferation rate decreases at higher BAP concentration (Table 1).

Kinetin has been used for shoot proliferation on cotton (Meloni *et al*., 2001), and chrysanthemum (Karim *et al*., 2002; Long *et al*., 2006); thus the effect of kinetin was varied with concentration (Table 1). The effectiveness of BAP was proved to be superior to kinetin. MS media supplemented with BAP have been satisfactory for many species and cultivars (Hutchinson, 1981). Superiority of BAP over other growth regulators in producing *in vitro* shoots has also been confirmed in other plants like *Prunus amygdalus* (Shatnawi, 2006), *Arachis hypogaea* (Mhatre *et al*., 1985) and *Atropa beladona* (Benjamin *et al*., 1987). On the other hand, Karim *et al.* (2002) approved the superiority of BAP over Kinetin in regeneration of shoots from chrysanthemum explants. Karim *et al*. (2003) reported that, among different concentrations used, best response towards shoot proliferation from nodal and shoot tip explants was obtained on MS supplements with 1.0 mg l^{-1} BAP.

Root induction was undertaken using three growth regulators IBA, IAA or NAA. Percentage of root induction and number of roots per shoots were highly influenced by concentration and types of auxin (Table 2). IBA confirmed its superiority over other auxins regarding number of new leaves, number of roots and root length (Table 2). Similar results on chrysanthemum were obtained by Long *et al*. (2006) and Karim *et al*. (2002). Similar to Hoque *et al*. (1995) on C. *morifolium*, maximum number of roots was obtained on MS media supplemented with 0.2 mg/L IBA (Table 2). Whereas Long *et al*. (2006) reported that highest number of roots where obtained when chrysanthemum microshoot cuttings where treated with IBA. In our study maximum root length (4.94) was obtained by using 0.2 mg l^{-1} IBA or NAA (Table 2). This result is similar to previous finding by Karim *et al.* (2002), who reported that 0.2 mg l^{-1} IBA produced the highest root length. IAA produces the highest number of leaves, whereas NAA produce the highest fresh weight and dry weight at 0.2 and 0.4 mg l^{-1} concentration (Table 2).

4.2. Physiological responses of microshoots to salinity

Increasing salinity reduced growth rate of chrysanthemum microshoots. Number and length of new shoots were declined with increasing salt in the growth media (Table 3). Similar results were obtained in microshoots of *Citrus aurantium* (Shiyab *et al*., 2003). Carvaja *et al*. (1998) found that muskmelon shoot growth decreased as salt concentration increased. Similar results were obtained on olive (Shibli and Al-Juboory, 2002). Number of new leaves was reduced at 60 mM NaCl, generating zero number of new leaves at 300 mM NaCl (Table 3). This reduction could be caused by toxicity associated with excessive uptake of Na (Yong *et al*., 2004) and nutrition imbalance (Cabanero *et al*., 2004).

Increased salt concentration caused an increase in fresh weight, up to 80m M NaCl) (Table 3). This agreed with the result obtained by Shibli *et al.* (2000) on apple microshoot. On the other hand, dry weight showed a similar pattern in which it increased until reaching the maximum at 80 mM NaCl (Table 3). Thus the reduction in fresh weight and dry weight could be due to salinity induced water deficit which reduced translocation of assimilates. Plant responds differently to alternation in the medium and this causes change in physiological responses

through it metabolic pathways (Harrak *et al*., 1999). Different stress conditions can cause changes in the physiological process of the plants. Chlorophyll and caretenoid content were decreased with progressive increasing in NaCl concentration (Table 4).

Mineral nutrients and uptack is adversely affected by high salinity levels (Al-Karaki *et al*., 1995). In this study nitrogen and potassium responded in a similar pattern. As salinity increased, N and K contents were decreased whereas Na contents were increased (Table 4). A similar result was obtained in hydroponic tomato (Al-Karaki, 2000; Alian *et al*., 2000; Flores *et al*., 2001.), cucumber microshoot (Abu-Romman and Suwwan, 2008) and lettuce (Irigoyen *et al*., 1992; Tarakcioglu and Inal, 2002), sour orange (Shiyab *et al*., 2003). Reduction of K content in plant leaves adversely affect metabolic function and eventually reduce plant growth (Greenway and Munns 1980).

Proline contents in chrysanthemum leaves increased significantly with increasing salt concentration (Figure 1). A similar result was obtained in tomato leaves (Bolarin *et al*., 1995). Proline, an osmotically active substance, is usually released from the cell due to salt and osmotic shocks (Gangopdhyay *et al*., 1997). Proline accumulation may be increased in plants tissue; this may be due to hyperosmotic stresses, primarily drought (Balibrea *et al*., 1997), and salt stress (Guerrier, 1998; Aziz *et al*., 1999). Proline accumulation could be used as a salt sensitive trait because it inhibits callus growth (Bolarin *et al.,* 1995; Cano *et al*., 1996).

Salinity reduced protein content of chrysanthemum microshoots (Figure 2). The reduction in the protein level in stressed tissues appeared to be due to more degradation of proteins as well as overall inhibition of protein synthesis under stress (Kumar and Singh, 1991).

The results of this study indicated that an efficient *in vitro* propagation method, with high levels of survival and reproducibility, could be achieved. BAP at 0.3 mg l^{-1} produced the highest number of new shoots, with the highest proliferation. Growth of *C. morfolium* microshoots was adversely affected by elevated NaCl levels. Plant growth at 20 mM NaCl was significantly improved. Furthermore, the production of adventitious microshoots was reduced by using higher concentrations of NaCl (Ben Amor *et al*., 2005). Chlorophyll and caretenoid content were decreased with progressive increase in medium NaCl supplements.

Mineral content in *C. morifolium* tissue were significantly affected by increasing NaCl levels. The effectiveness of this system, however, should be further tested on a greater number of genotypes with known performance for root characteristics related to drought tolerance under field conditions. In such comparisons, data from field evaluations must be based on well-conducted trials repeated over years. Unfortunately, such evaluations for root characteristics are available for only a few genotypes. Nevertheless, the results of the present study clearly showed that screening of *C. morifolium* simulate the *in vivo* conditions which might provide a high efficacy *in vitro* screening method for abiotic stresses. This might identify promising cultivars recommended for growers in salt-affected areas of the world. Further physiological and molecular studies are still needed to understand many physiological issues of *C. morifolium*, to salinity tolerance**.**

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