MORPHOLOGICAL CHARACTERIZATION OF *IN VITRO* SALT TOLERANT CELL LINES AND REGENERATED PLANTS OF *DAUCUS CAROTA* VAR. *SATIVUS*

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ABSTRACT: The *in vitro* growth of the plants is affected by different chemical and environmental factors in addition to their genetics. Therefore, the basic requirements of the present study was to standardize the different physical and chemical factors to evaluate the *in vitro* growth potential of *Daucus carota* var. *sativus* under different concentrations of NaCl. For this purpose, *Daucus carota was* cultured on 1.0 mg/l BAP +0.2 mg/l 2,4-D in MS basal medium. Different concentrations of NaCl were used to evaluate the maximum tolerance of salt stress for carrot in terms of its *in vitro* growth. It was observed that 0.1 mg/l NaCl stress gave the maximum, i:e 70% growth of carrot *in vitro* cell lines, at 25±2ºC in 16 hr photoperiod. The data were analyzed statistically through ANOVA technique using SPSS programme.

Key Words: Plant tissue culture, Phyto Growth Regulators (PGR), *Daucus carota*. Salinity, Abiotic stress.

INTRODUCTION

Soil salinity is widely reported to be a major agricultural problem, particularly for irrigated agriculture, but a few cultivars, resistant to saline soils can be developed. Approximately 850 million (6% of the total world area) hectares of land throughout the world are considered as salt affected (FAO., 2011),so soil salinity is considered as one of the major problems that imbalances the sustainability of the system over a vast area (Flowers, 2004). Salinity is now taken as one of the major abiotic stresses for the agriculture (Mahajan and Tutea, 2005).

The soils having electrical conductivity of lesser than 4 dS m/l are generally considered as normal soils, where almost all of the crops can be grown. Improper irrigation and poor drainage have led to the deposition of salts in the soil up to the harmful concentrations. The efficiency of crops to grow on saline soils varies from species to species and depends on the concentration of salts present in the root zone and on various environmental and culture conditions (Maas, 1990).

Different chemical and environmental factors in addition to the genetics affect the *in vitro* growth specially the micropropagation which is now considered as major and widely accepted practical application of plant tissue culture. There is plenty of work on micropropagation and regeneration of Carrot. Gorecka *et al*. (2005) studied the regeneration of plants from embryos obtained from anther cultures of seven carrot cultivars. Embryogenesis occurred on four of the tested media: B5 and MS without hormones, MS with charcoal, and MS with 1mg dm^{-3} BA and 0.001 mg dm^{-3} NAA. Similarly Taveres *et al.* (2009) and Jimenez *et al* (2005) carried out the experiments to evaluate the potential of *in vitro* propagation and embryogenesis of carrot and observed that shoot tips of *in vitro* germinated seeds were able to proliferate in the presence of benzyladenine, with the best results being achieve using 4.4µM, both in the first and second cultures.

Kumar *et al*. (2004) stated that salinity is one of the major factors that limits the geographical distribution of plants and adversely effects the crop productivity and quality of high level expression of betaine aldehyde dehydrogenase (BADH) in culture cell, roots and leaves of *Daucus carota* via plastid genetic engineering.

The effects of salinity on vacuolar pH in carrot cells grown in liquid suspension cultures was recorded by using the pH data both in the absence or presence of 150mM NaCl by Reuveni (1992). Gibberd *et al.* (2002) reported that 7% growth decrease occurred for every mM increment in salinity above 20 mM and salt stress resulted in reduction of apparent photosynthetic capacity in cultivated carrot crops and classified it as salt sensitive plant.Similar results were observed by Kiyosue *et al.,* (1989), who observed that cotyledons and root explants of carrot turned yellow with 0.1 M to 0.4 M NaCl and no somatic embryo was formed. Work on salt cell lines is abundantly reported for other cultivars too e:g Ennay *et al.*(2002) found that NaCl inhibited the shoot regeneration at markedly 100 mM and 150 mM concentration in tomato.

The present research was carried out for the optimization of physical and chemical factors for the *in vitro* growth of *Daucus carota* var. *sativus* during an induced salt stress of varying degrees and also to study the morphological characterization of salt cell lines of this cultivar.

MATERIALS AND METHODS

Certified Surface Sterilized seeds of *Daucus carota* var *sativus* were grown on MS (Murashige and Skoog, 1962) basal medium to get sterilized plantlet for in *vitro* growth. For the purpose of carrot tissue culturing, different plant parts were used as explants i.e. cotyledon, leaf, node, internode and primary root etc. MS basal medium was used along with different Phyto growth regulators i.e. BAP and 2, 4-D. Seeds were surface sterilized by dipping them in the small amount of alcohol for 5-10 seconds and rinsed with distilled water three times.

The explants taken from the field grown plants were washed with running tap water and commercial detergent, several times, carefully without damaging the delicate and young tissues. The explants were rinsed with double distilled water three times and then were transferred to laminar air flow cabinet and treated with few drops of Tween 20, then were surface sterilized with 2 % (w/v) Mercuric Chloride solution for 3-4 minutes, followed by 3 to 4 times washing with double distilled water and were kept to carry out the inoculation. Stock solution for culture medium including growth hormones (BAP and 2-4,D), MS(Murashige and Skoog, 1962) basal medias's inorganic salts and vitamins were prepared in double distilled water in 20X to 200X concentrations and were stored in amber glass bottles at 4°C(Annex:1). The nodal region of approximately 3-6mm long of already surface sterilized stems and 3mm long leaves from both *in vivo* (sterilized) and *in vitro*, plants were removed carefully and inoculated on Murashige and Skoog's (MS) medium supplemented with different plant growth regulators and additives, either singly or in combination and kept in controlled growth room. The optimum temperature required for culture environment was maintained at 25 ^{o}C. The culture were incubated at 16 -18 hours photoperiod in cool fluorescent light (2000-3000 lux).The pH of medium was adjusted at 5.7-5.8. Thirty g/l sugar was used in the inoculation medium. Three subcultures were grown to get the calli under different concentrations of NaCl (0.1mg/l, 0.2mg/l, 0.3mg/l & 0.4mg/l) using 30 flasks for each concentration. Replica of each experiment was repeated for three times to get the average. The data were also recorded for the cultures without NaCl(control).

Following Morphological characterizations were observed during the *in vitro* growth of *Daucus carota* for different concentrations of NaCl added, in the culture media.

a. a Effect of salt stress on morphological characteristics(Type and colour) of callus cultures.

b. Effect of salt stress on callus browning and necrosis in callus cultures.

c. Effect of salt stress on regeneration potential including, shoot formation, root formation and conversion (both root and shoot formation) of callus cultures.

The data for regeneration frequency, number of shoots per culture tube and number of roots per culture tube were recorded.

The Experiment was laid out according to completely randomized design(CRD) and data collected were analysed through Analysis of Variance Technique FOLLOWING Steel *etal.,*(1997) using SPSS soft ware(Levesque,2007)and means were compared through Duncan's Multiple Range Test(Duncan,1955).

RESULTS AND DISCUSSION

The basic requirements of the present study was to standardize the different physical and chemical factors for *in vitro* growth of *Daucus carota* var. *sativus* in the presence of varying degrees of salt (NaCl).

As far as, physical factors are concerned, for *Daucus carota,* maximum *in vitro* growth was 90% and & 70% (in the absence and presence of NaCl respectively), Table: 2, with solidified medium where as liquid medium showed only 15% growth. The Phytagel solidified media gave maximum *in vitro* growth during the present investigation which is in agreement with finding of Jayasankar *et al*., (2003) who reported that *Vitis vinifera* embryos produced on solid medium had large cotyledons, whereas those derived from liquid-medium produced smaller cotyledons.

Temperature range of 25±2ºC showed 90% and 70% (in the absence and presence of NaCl respectively) *in vitro* growth, which was recorded at a temperature of 25±2°C (Table: 2). The temperature range of 15-25^{o}C was also used by Hussey and Stacey (1981) for *in vitro* propagation of potato shoots. The optimum pH in *Daucus carota* was 5.75 which gave 90% *in vitro* growth. Mohsen and Ibrahim (2000) investigated that medium pH of 5.7 resulted in the maximum multiplication rate in *Maranta leuconeura.*

For *Daucus carota in vitro* growth the best sucrose concentration was found to be 30g/l giving highest growth rate i.e 90% and 70% as given in Table :2 (in the absence and presence of NaCl respectively). A majority of researchers have mentioned the use of the similar concentrations of sucrose in their media. (Suboti *et al*., 2009; Pant and Manandhar., 2007) With regard to photoperiod, the highest growth rate 90% and 70% (in the absence and presence of NaCl respectively) was observed in 16 hrs light period. Hussey and Stacey (1981) also investigated the maximum *in vitro* propagation at 8-24 hrs light period in *Solanum tuberosum. Daucus carota in vitro* growth has also been reported to vary along with variations in different physical and chemical factors e:g composition of MS basal medium, concentrations and combinations of plant growth regulators, temperature, pH, photoperiod, and explants related factors (Bhatia *et*

al., 2004). Many members of Apiaceae family are reported to be propagated by using different explants and different propagated methods. (Joshi *et al*.,2003).

It was observed during the present work that all the explants showed maximum shoot formation in MS media containing 0.2 mg/l 2, 4- D + 0.1 BAPmg/l under the stress of 0.1 mg/l NaCl(Table:1,Fig:2). The excellent shoot formation (70 %) was observed with leaf explants calli but these multiple shoots were yellow in colour because the higher concentration of NaCl effects the availability of nutrients and the reduction of the photosynthesis ability occurs. This result is in arrangement with Gibberd *et al.,* (2002) who reported that 7% growth reduction occurred for every mM increment in salinity above 20 mM and salt stress resulted in reduction of apparent photosynthetic capacity in cultivated carrot crops and classified it as salt sensitive plant. Ennay *et al.,* (2002) also reported that NaCl inhibited the shoot regeneration at markedly 100 mM and 150 mM concentration in tomato. The same results were observed by Kiyosue *et al.,* (1989) who reported that cotyledons and root explants of carrot turned yellow with 0.1 M to 0.4 M NaCl and no somatic embryo was formed.

During the present piece of work, Maximum *in vitro* growth was 90%(in the absence of NaCl) with 1.0 mg/l BAP + 0.2 mg/l 2,4-D, while 0.1 mg/l NaCl in the same media combination gave 70% growth decreasing the growth rate up to 20%(Table 1:Fig:1). Present study has shown the multiple shoot formation (Table :4) but these shoots were yellowish in colour and this colour change increased with the increase of NaCl concentration in the culture medium. Similar results were obtained by Pant and Manandhar (2007) who used carrot leaves as explants but they obtained these results by using 2mg/l NAA instead of 2,4-D.

The regeneration frequency was found to be lesser in the plants regenerated from NaCl-treated callus cultures as compared to control (calluses grown on 0 mg/l NaCl concentration). Pant *et al*., (1996) also reported the formation of multiple shoot in *Cnidium officinale* by shoot tip culture. However, for the present investigation, the excellent shoot formation in 0.1mg/l NaCl (70%) was observed with leaf explants but they were yellow in colour(Table :4). Gibberd *et al*., (2002) showed the similar results who studied that 7% growth reduction occurred for every 10 mM increment in salinity above 20 mM and salt stress resulted in reduction of apparent photosynthetic capacity in cultivated carrot crops and classified it as salt sensitive plant.

It was found that callus cultures of *Daucus carrota* retained 70% regeneration with 0.1mg/l NaCl and this frequency decreased sharply at 0.2mg/l salt concentration and only 10 % callus cultures retained regeneration potential but the shoots formed were yellow in colour and could not survive after 2nd subculture, where as 0.3mg/l salt showed necrosis of 5% formed, calli after 1st subculture and 0.4 mg/l NaCl concentration in the culture medium did not give rise any callus at all(Table:3)

It was also observed during the present study that the number of regenerated shoots from callus cultures treated with various NaCl concentrations was greater as compared to the control calli maintained at 0 mg/lNaCl.

Table 1. Effect of different concentrations of NaCl in MS medium on *in vitro* growth of *Daucus carota* var *sativus* using leaf explants.

Concentration of PGRs & NaCl used	%age *in vitro* growth (Mean)
1.0mg/lBAP+0.1mg/l 2,4-D+0.00mg/l NaCl	90^{a}± 0.81 (90%)
1.0mg/lBAP+0.1mg/l 2,4-D+0.1mg/l NaCl	70^{a} ± 1.24 (70%)
1.0mg/lBAP+0.1mg/l 2,4-D+0.2mg/l NaCl	10^{b} ± 2.09 (10%)
1.0mg/lBAP+0.1mg/l 2,4-D+0.3mg/l NaCl	05^{b} ± 1.05 (05%)
1.0mg/lBAP+0.1mg/l 2,4-D+0.4mg/l NaCl	00^{c} ± 0.00(00%)

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table2. Optimization of physical factors for maximum *in vitro* growth of *Daucus carota* var *sativus* with 0.1mg/l NaCl.

Physical Factors	Optimum Physical state	Maximum *in vitro* growth mean %age
State of Medium	Phytagel solidified Medium	70 ± 1.24 (70%)
Temperature Range (°C)	25 ± 2	70 ± 1.24 (70%)
Photoperiod (3000 lux)	16 hrs	70 ± 1.24 (70%)
Sucrose concentration g/l	30	70 ± 1.24 (70%)
pH of the Medium	5.8	70 ± 1.24 (70%)

Table3. Effect of salt stress on Morphological Characteristics of *Daucus Carota*.var *sativus* callus cultures.

Cultures	NaCl Conc. mg/l	Callus Induction %age(Mean)	Colour of callus	Type of callus	Necrotic state of callus
Main culture	0.00	$90^{a} \pm 0.81$	Yellowish	Compact	No necrosis
	0.1	$70^{b} \pm 1.24$	Yellowish Brown	Compact	No necrosis
	0.2	$10^{c} \pm 2.09$	Brownish yellow	Hard&Farible	Mild necrosis
	0.3	$05^{c} \pm 1.39$	Dark brown	Friable	Strong necrosis
Ist Subculture	0.00	$87^{a} \pm 1.71$	Yellowish	Compact	No necrosis
	0.1	$67^{b} \pm 0.31$	Yellowish Brown	Compact	No necrosis
	0.2	$06^{c} \pm 2.00$	Brownish yellow	Hard&Friable	Mild necrosis
	0.3	$03^{c} \pm 0.33$	Dark brown	Friable	Strong necrosis
2^{nd} subculture	0.00	$85^{a} \pm 0.61$	Yellowish	Compact	No necrosis
	0.1	$65^{b} \pm 1.86$	Yellowish Brown	Compact	No necrosis
	0.2	$03^{c} \pm 0.10$	Dark Brown	Hard&Friable	Strong necrosis
	0.3	-	-	-	Necrotic death
3^{rd} subculture	0.00	$85^{a} \pm 1.43$	Yellowish	Compact	No necrosis
	0.1	$65^{b} \pm 2.09$	Yellowish Brown	Compact	No necrosis
	0.2	-	-	-	Necrotic death
	0.3	-	-	-	-

The means with different letters in each column, within a cell, are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Table 4. Effect of Salt Stress on Regeneration Potential and rooting, shooting & conversion of *Daucus carota.var sativus* callus cultures.

Conc. of NaCl added	%age Shooting /culture tube (Mean)	% age Rooting /culture tube (Mean)	%age Conversion / culture tube (Mean)
0.00mg/l	$48^{a} \pm 0.11$	$35^{a} \pm 1.72$	$30^{b} \pm 2.09$
0.1mg/l	$56^{b} \pm 1.63$	$25^{b} \pm 1.66$	28 ± 0.03
0.2mg/l	-	-	-
0.3mg/l	-	-	-
0.4mg/l	-	-	-

The means with different letters in each column are significantly different according to Duncan's Multiple Range Test (<0.05 p value) ± Standard error of the mean.

Fig:1: *In vitro* growth of *Daucus carota* var. *sativus* with1.0 mg/l BAP+ 0.2 mg/2,4 in total absence of NaCl after 50 days of inoculation.

Fig:2: *In vitro* growth of *Daucus carota* var. *sativus* with1.0 mg/l BAP+ 0.2 mg/2,4 in 0.1mg/l NaCl after 50 days of inoculation.

Appendix I

Composition of MS medium (Based on Murashige and Skoog, 1962)

Ingredients Macronutrients	MS medium (mg/l)	Stock concentration (mg/l)
NH_4NO_3	1,650	33,000
KNO_3	1,900	38,000
$CaC_{12}.8H_20$	440	8,800
$MgSO_4.8H_2O$	370	7,400
KH_2PO_4	170	3,400
Micronutrients	**MS medium (mg/l)**	**Stock concentration (mg/l) 100X**
$MnSO_4.4H_2O$	22.3	2230
$ZnSO_4.H_2O$	8.6	860
H_3BO_3	6.2	620
KI	0.83	83
$NaMoO_4.2H_2O$	50	5000
$CuSO_4.5H_2O$	0.025	2.5
$CoCL_2.6H_2O$	0.025	2.5
Iron stock	**MS medium (mg/l)**	**Stock concentration (mg/l) 200X**
$FeSO_4.7H_2O$	5.56	5,560
Na_2-EDTA	7.40	7,460
Vitamins	**MS medium (mg/l)**	**Stock concentration (mg/l) 100X**
Glycine	2.0	200
Nicotinic acid	0.5	50
Pyridoxine-HCl	0.5	50
Thiamine-HCl	0.1	10

Sucrose: 30g/l Agar : 10g/ pH : 5.5-5.7

We can anticipate that importance of salinity as one most important obstacle to the agricultural system will be increased in the future. Enhancing the salt resistance in the widely utilized crops is very much needed. Salinity resistance can both help provide yield stability in agriculture and help limit salinisation in irrigation systems with improper drainage practices.

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