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Development of NaCl-tolerant Line in *Tanacetum cinerariaefolium* (Trevir.) Schultz-Bip Through Shoot Organogenesis of Selected Callus Line

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ABSTRACT

Plants were regenerated successfully through shoot organogenesis of a NaCl-selected callus line of *Tanacetum cinerariaefolium* (Trevir.) Schultz-Bip developed through stepwise increase in NaCl concentration in MS medium. Increasing NaCl level concentration (0, 5, 10, 15, 20, 25, 30, 35, 40, 45mM) from low level to high level was found to be a better way to isolate NaCl-tolerant callus line, since direct transfer of callus to high saline medium was detrimental to callus survival and growth. Among different media and growth regulator treatments, MS media containing 1 mgl^{-1} BA and 1 mg l^{-1} NAA or 1 mgl^{-1} BA, 2 mgl^{-1} NAA and 0.5 mgl^{-1} GA_3 for shoot organogenesis in selected callus line and B5 medium supplemented with 2 mgl^{-1} NAA showed best response for root regeneration. As increasing NaCl concentrations (From 0 to 45 mM) the ability of shoot and root regeneration were decreased. The selected callus line showed significance increase in proline content and decrease in pyretrine content. Based on growth performance and proline content (20 mM in callus line and 35 mM in shoot culture) could be considered as NaCl-tolerant line showing all positive adaptive features towards the salinity stress. Further studies about agronomic performance of obtained plants under saline soil condition are necessary for understanding to check the genetic stability of the induced salt-tolerance plants.

Keywords: Acclimatization, callus induction, *in vitro*, pyrethrins, salinity stress

INTRODUCTION

*Tanacetum cinerariaefolium i*s an important medicinal perennial herb belonging to the family *Asteraceae*. The genus *Tanacetum* encompasses many species found mainly in the temperate regions (Anderson, 1987). *Tanacetum cinerariaefolium* has been widely used in traditional medicine in Iran as well as in the insectiside industry because of their Pyrethrins production. Pyrethrins are currently the most economically important natural insecticide of plant origin (Casida, 1973). They are a mixture of six compounds produced by acidification of two acids (chrysanthemic acid and pyretric acid) with three ketons-alcohols (pyrethrolone, jasmolone and cinerolone). Pyrethrins have ideal pest control agents that very effective against a broad range of insects with little development of resistance strains and have knock -down and killer effects on insects (Crombie, 1980). One of the major advantages of pyrethrins over all other insecticide is their low toxicity to mammals and other warm blooded animals (Jovetic and DeGooijer 1995). One of the major stresses affecting plant productivity is salinity. Salinity is a limiting factor in arid and semi arid areas (Bajji *et al*., 1998; Matsumoto *et al*., 2003; Orcutt and Nilsen, 2000; Turiian and Ayaz, 2004). Plants exposed to salt stress undergo exchange in their metabolism in order to cope with the change taking place in their environment. Tissue culture can be use as a new method for increasing genetic exchange ability of plants and obtaining salt resistant lines (Larkin and Scoweroft 1981; Liu and Zhu, 1997). Millions of hectares of land in Iran is suffering with problems of salinity and lying either un-utilized or semi-utilized. Massive efforts are going on for developing techniques for the identification of successful plant genotypes for proper utilization of such degraded soil. Different strategies are in progress for the development of NaCl-tolerant plants. *In vitro* selection procedure and *Agrobacterium*-mediated transformation offer a meaningful tool for development of such tolerant lines. Enhanced tolerance to salt stress, by over expressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts, has been reported by Badawi et al. (2004). Number of papers have been published on development and isolation of NaCl-tolerant cell/callus lines using *in vitro* technique (Watad et al., 1983; Olmos et al., 1994; Tal, 1994; Barakat and Abdel-Latif, 1996; Patnaik and Debata, 1997a), but only a few reports are available on successful regeneration of plants from such tolerant lines showing stability in salt tolerance character (Winicov, 1996; Patnaik and Debata, 1997b; Zhang et al., 2001). The main constrain behind the development of NaCl-tolerant plants from such callus/cell lines is the loss of regeneration potentiality or genetic instability of salt tolerance at whole plant level. In the present experiment an attempt has been made to develop stable NaCl-tolerant *Tanacetum cinerariaefolium* plants by selection of NaCl-tolerant callus line and their subsequent differentiation under NaCl stress condition.

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MATERIALS AND METHODS

Callus induction and growth

Petiole and stem segments measuring about 1.5-2 cm length and 30–40-day-old leaves (≈10 mm^2) were used as explants. They were washed first under running tap water at least 30 min, surface sterilized in 70% alcohol for 1 min, rinsed twice with sterile distilled water, immersed in 1.5% sodium hypochloride for 10 min and rinsed four to five times in sterile distilled water for 5 min duration each. Then petiole and leaf segments were cut and transferred to 150 ml glass jars with 25 ml MS (Murashigue and Skoog, 1962), SH (Sckenk and Hildebrant,) and B5 (Gamborg, 1988) medium containing various concentration of 2,4-D and NAA alone or in combination with kinetin (Kin) plus 3% sucrose and 0.8% agar for callus induction. To obtaining best medium with optimum BA (6-benzyladenine) and NAA, calli from best treatment were transferred to MS, B5 and SH medium supplemented with various concentrations of BA (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, and 4 mg l^{-1}), NAA (1, 2, 4, 6 and 8 mg l^{-1}). The calli induced from explants in best treatments were subcultured on the same medium surface in every 4 weeks. Each callus was divided into 2–4 mm diameter pieces during transfer and subcultured up to 11 times. The cultures were kept at 25 ± 3 °C under a 16-h photoperiod with a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1} provided by white fluorescent lamps.

Shoot regeneration

For plant regeneration, calli were transferred to MS, B5 and SH medium supplemented with various concentrations of cytokinins (BA; 0, 1, 2, 3, and 4 mg l^{-1}), (Kin; 0, 1, 2, 3, and 4 mg l^{-1}), (2ip 0, 1, 2 and 3 mg l^{-1}) and adenine (0, 1, 2 and 3mg l^{-1}) and different concentrations of auxins (IAA: 0, 1, 2 and 3 mg l^{-1}, 2,4-D: 0, 0.2, 0.4, 0.6, 0.8 and 1 mg l^{-1}) and GA3 (0, 0.1, 0.2 and 0.4 mg/l-1) plus 3% sucrose and 0.8% agar. The cultures were kept at 25 ± 3 °C under a 16-h photoperiod with a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1} provided by white fluorescent lamps.

Rooting

Regenerated shoots measuring about 3-4 cm in length were transferred to rooting medium, which consisted of different medium (MS, B5 and SH) supplemented with different concentrations of naphthalene acetic acid (NAA) at 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5mg l^{-1} plus 3% sucrose and 0.8% agar. The cultures were kept at 25 ± 3 °C under a 16-h photoperiod with a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1} provided by white fluorescent lamps.

Estimation of free proline content

Proline content was measured spetrophotometrically using the method of Bates et al. (1973). Two hundred and fifty milligrams of callus/leaf tissue was homogenized with 5ml 3% sulpho salicylic acid and centrifuged at 5000 rpm for 10 min. Supernatant was treated with acid-ninhydrin and acetic acid, boiled for 1 h at 100 ◦C. The reaction was then terminated in an ice bath. Reaction mixture was extracted with 2ml toluene. Absorbance of chromophore containing toluene was determined at 520 nm.

In vitro *NaCl treatment and selection procedure*

After three subcultures, the fast growing from MS media containing 2 mg l^{-1} 2, 4-D in combination with 0.2 mg l^{-1} BA were selected for NaCl treatment. At first, the lethal concentration of NaCl in the medium for callus survival was determined. For this propose friable calli (high growth rate with yellow or yellow green color, no dense) were transferred to MS medium containing methylene blue and different concentration of NaCl (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60 mM). Salt tolerance rate of callus detected by evaluation plasmolysis response of cells in different concentration. Highest salt concentration that cells did not show any plasmolysis response or plasmolysis can back to normal condition was found 10 mM. Cells at this concentration did not show any plasmolysis response and distance between cell wall and cell membrane. Up to 30 mM increasing plasmolysis response were observed. At high salt concentration 45 mM cells completely plasmolysed (Fig 1. a, b, c) After determination tolerance rate of cells direct selection procedures was applied to develop NaCl-tolerant callus line. In this procedure, the callus pieces (approximate 500 mg fresh mass) were transferred to the MS, SH and B5 medium but supplemented with different concentration of NaCl (0, 5, 10, 15, 20, 25, 30 , 35, 40 and 45 mM) and maintained for 3 consecutive months with regular subculture at 30 days interval. Calli cultured on MS, SH and B5 medium devoid of NaCl were considered as control. In stepwise method, approximately 500mg of callus (fresh mass) was first transferred to MS, SH and

B5 medium supplemented with 5mM NaCl and maintained for 1months. Only surviving and fresh calli were Transferred to 10mM NaCl medium and maintained for another 1 month. Thereafter surviving calli was transferred to 15mM NaCl and maintained for another 1 month. Increasing the salt concentrations was increased till 45 mM NaCl. After determining response of different medium in various concentration of salinity calli were transferred to the best regeneration medium. Thereafter regenerated shoot (3–4 cm in length) of selected calli were transferred to the best proliferation medium but supplemented with different concentration of NaCl (0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 mM) and response of shoot proliferation in salinity condition was determined. Separate control line (shoots developed from Control calli) was maintained in MS medium having the same compositions except for the NaCl and treated as control. After all the propagation steps and salinity experiments had been optimized, the content of pyrethrins in different salinity levels (0, 20 and 40 mM) in callus and shoot culture experiments were measured and compared with pyrethrins content of mother plant flowers for obtaining salt effect on pyrethrins.

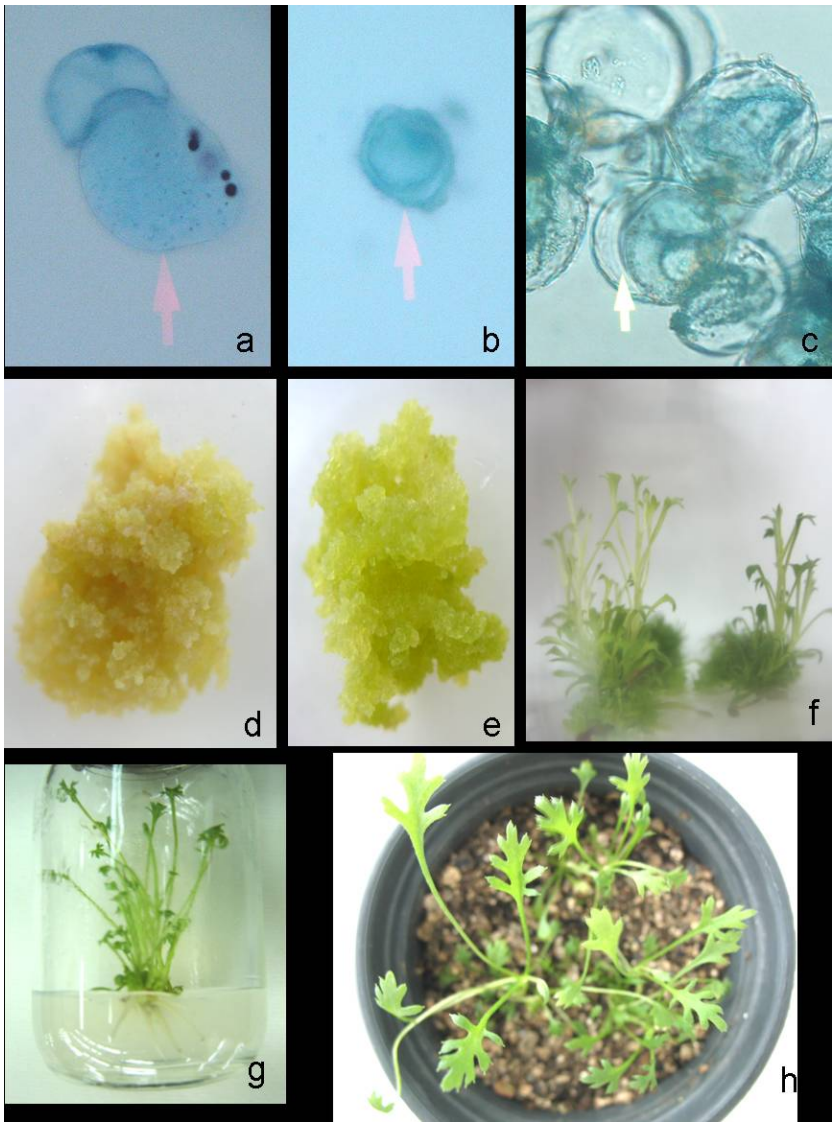

Figure 1. Response of pyrethrum cell in different concentration of NaCl in MS medium. (A) No wrinkle in MS Medium without salinity (B) plasmolysis response of cell in medium contain 20 mM NaCl. (C) plasmolysis response of cell in medium contain 40 mM NaCl. Arrow indicates the situation of cellwall. (D, E) The soft, mostly friable, yellow and nonorganogenic calli in MS medium containing 0.6 mg/l-1 BA and 4 mg/l-1 NAA, SH containing 0.4 mg/l-1 BA and 2 mg/l-1 NAA with light green color. (F) The emergence of large number of shoots from the calli on MS medium supplemented with 1 mg/l-1 NAA and 1 mg/l-1 BA 25 days after culture. (G) Rooting of a regenerated shoot in B5 medium supplemented with 2 mg l-1 NAA 21 days after culture. (H) two-week-old acclimatized plants growing in greenhouse.

Culture condition, acclimatization and data analysis

After 3 weeks, the rooted plantlets were thoroughly washed in distilled water to remove the agar and transferred to small pots containing 1/3 vermiculite, 1/3 perlite, 1/3 sand (V.V). The pots placed under transparency box and maintained under 25±3 ^{0}C temperature and 70% relative humidity. After an adaptation period (4 weeks), acclimatized plantlets were transferred to greenhouse (Fig 1. h). All the cultures were grown at 25 ± 2 °C under 16 h photoperiod supplied by two with a photosynthetic photon flux density of 40 µmol m^{-2} s^{-1} provided by white fluorescent lamps. This experiment was conducted as a complete randomized design in a factorial arrangement with four replicates and each replicate contain 12 explants. Duncan's multiple range test (DNMRT) was used for comparison among treatment means.

Assay of Pyrethrins

Pyrethrins were extracted as previously described (Berthomeeuf *et al*., 1996). After 28 days of culture, wet tissue were frozen in liquid nitrogen and ground in glass mortar with ¼ anhydrous sodium sulphate [v/fresh weight(fw)] to obtain a homogenous powder. Pyrethrins were extracted (twice) after maceration of the powder (15 min and 72 h, respectively with petroleum ether (b.p 35-60C; 5 ml g -1 FW). Solid matter was spun down (10000 g for 30min at 4 C) and the two extracts were combined and evaporated to dryness under a nitrogen stream. The residue was then dissolved in n-hexane (3ml), filtered through a 0.45- µm filter (Millipore) and analyzed by HPLC. HPLC analyses were performed at 230 nm using a Beckman HPLC system (166 gold pumping system, 168 diode array detector spectrophotometer and new gold software), a nova pack C18 column (2.0*150mm ID, 4 µm, waters) fitted a centry guard coulomn (3.9*4.0mm ID, 4 µm, waters), and a solvent mixture of CH3CN:H2o in gradient mode. The gradiant was the following: from 20 to 80 % CH3CN in 15 min (Linear gradient mode), 80 % CH3CN in 10 min (isogretic segment); re-equilibration at initial condition for 15 min. the flow rate was 1ml min-1. peal areas were compared with peak areas of authentic samples (Pyretrin technical mixture, OSI, France), and pyrethrins were quantified as mg/100g dry weight (DW). The calibration curves were plotted at 230 nm, using a commercial pyretrin mixture. Curves with linear over the range of concentration studied (0.01-0.9 µg/µl 1). This allowed detection of less than 1 mg total pyrethrins per 100gg DW. Pyrethrin content in callus and tissue culture were recorded after 4 weeks of culture. At least three replicate were used for each measurement.

RESULTS

Callus induction

The response of the different *Tanacetum cinerariaefolium* explants on different media containing different levels and combination of auxins and BA were tested. No callus was observed from leaf and petiole explants on any of the media that tested without growth regulators or media containing BA alone and auxins alone. This examination demonstrated that a combination with auxin and cytokinin is needed for optimum callus induction. Highest rate of callus induction (100%) were observed in SH medium containing NAA (2 mg l^{-1}) and BA (0.2 mg l^{-1}) in petiole culture. This treatment show significant differences with other induction treatments. Of the various concentrations of 2, 4-D tested in all media and different concentration of BA, the maximum response was observed on MS medium in the presence of 2 mg l^{-1} 2, 4-D in combination with 0.2 mg l^{-1} BA where 89% of leaf explants produced (data not shown) callus. Between axuins, NAA was effective than 2, 4-D in callus induction. The calli were produced from the explants after 2 weeks in incubation media. The calli were soft, friable and nonorganogenic in all concentrations of NAA in combination with BA. Calli in various concentrations of 2, 4-D in combination with BA were compact, light yellow to greenish and non morphogenic.

Callus growth

Evolution the growth index of calli that were cultured in different medium (MS, B5 and SH medium) supplemented with various concentrations of BA and NAA indicate that MS medium containing 0.6 mg/l-1 BA and 4 mg/l-1 NAA, SH containing 0.4 mg/l-1 BA and 2 mg/l-1 NAA and B5 containing 1 mg/l-1 BA and 4 mg/l-1 NAA were best treatments and showed significant difference with other treatments (Data not shown). The calli were soft, mostly friable, light green to yellow and nonorganogenic (Fig. c,d).

Effect of salinity on callus growth in direct selection procedure

Calli in different salt concentrations and different media exhibited different growth rates. The lowest change in fresh mass (FM) was observed in low NaCl concentration (5mM). Although 5mM NaCl treatment in all tried medium showed highest growth rate among the different treatments, but it was always lower than control callus. Calli of 5 and 10 mM NaCl treatments showed almost uniform growth rate with mostly yellow or yellow-green color. Between different media, MS medium showed best callus growth compared to other medium in first, second and third subculture. At 15 mM NaCl treatments callus in SH and B5 medium did not show any growth (at first subculture) but, at second subculture calli in SH medium started to growth that was much lower than control and MS medium treatment. At 20 mM NaCl treatment, callus in all media did not show any growth (at first subculture). But, in MS medium at second subculture calli started to growth. High salinity levels (25, 30, 35, 40 and 45 mM NaCl treatments) showed severs inhibitory effect in all subcultures. Inhibition of growth at 25, 30 35, 40 and 45 mM NaCl treatments resulted in 100% death of callus tissue (turned brown and dark brown) after successive subculture. In high salinity levels callus on MS medium showed gradually growth in third subculture (Table 1).

Table 1. Growth performance of *Tanacetum cinerariaefolium* callus grown under in vitro NaCl stressed condition in different media.

		First subculture		Second subculture		Third subculture	
Salt concentration (mM)	medium	Color	Callus growth rate	Color	Callus growth rate	Color	Callus growth rate
0	MS	YG	+++[z]	YG	+++	YG	+++
	B_5	Y	++	Y	++	Y	++
	SH	YG	++	YG	+++	YG	++
5	MS	Y	+++	YG	+++	YG	+++
	B_5	Y	++	Y	++	Y	++
	SH	YG	++	YG	++	YG	++
10	MS	Y	++	Y	++	YG	++
	B_5	Y	+	Y	+	Y	+
	SH	Y	++	Y	++	Y	++
15	MS	YW	*++	Y	++	Y	++
	B_5	W	=	YW	=	YW	=
	SH	YW	=*	Y	*+	Y	+
20	MS	C	=	Y	+	Y	++
	B_5	W	=*	YW	=*	W	=*
	SH	YW	=*	YW	=*	Y	=
25	MS	D	=**	Y	+	Y	++
	B_5	D	0	D	0	Y	0
	SH	D	0	D	0	D	0
30	MS	D	0	D	0	Y	++
	B_5	D	0	D	0	D	0
	SH	D	0	D	0	D	0
35	MS	D	0	D	0	Y	*+
	B_5	D	0	D	0	D	0
	SH	D	0	D	0	D	0

[z]Desirable Growth (+++), Medium Growth (++), Little Growth (+), Without Growth (=), Dead Callus (0), little burn (*), High burn (**), YG=Yellow-Green, YW= Yellow-Whit Y= Yellow, D= Dark, C= Creamy

Effect of salinity on callus growth in stepwise selection procedure

In stepwise method, a slight decline in growth was observed after calli were transferred from 5 to 10 mM NaCl medium (observed at 3th months) in all media. Similar kind of decline in growth rate was observed during the transfer of calli from low concentration of salt to high concentration salt medium (observed at ninth month). Lowest growth rate of callus was observed in high salinity level (40 mM). Control calli exhibited higher callus growth rate. In high salinity levels (45 mM NaCl) sever reduction of callus growth and lethality was observed in all media. Between different media that used in this experiment, MS medium showed highest growth rate and fresh mass than other media (Data not shown).

Effect of NaCl on Shoot regeneration, shoot growth and Status of proline pool

MS media containing 1 mgl^{-1} BA and 1 mg l^{-1} NAA or 1 mgl^{-1} BA, 2 mgl^{-1} NAA and 0.5 mgl^{-1} GA_3 for shoot organogenesis in selected callus line and B5 medium supplemented with 2 mgl^{-1} NAA showed best response for root regeneration (data not shown). Salinity decreased the proliferation rate, average shoot length and fresh mass of shoot. The highest rate proliferation rate, average shoot length and fresh mass of shoot were observed in NaCl free (Control) medium. Minimum average shoot length, fresh mass and proliferation rate were observed in high salinity treatment (Table 2) (Fig 1. f). Salinity decreased significantly rooting ability (both Mean root length and Number of roots/shoot). Highest reduction was observed in highest salinity levels. Highest number of roots was observed in medium contenting 10 mM NaCl. Also, The same result obtained in mean root length (Table 2) (Fig 1. g). The effects of NaCl, on proline contents of treated and control calli and shoot, are presented in Table 2. Control callus maintained as control proline level throughout the experimental period. In direct selection procedure as compared to control, NaCl treated calli accumulated higher proline. The proline content was proportional to the stress level. Highest proline content was observed in 20 mM NaCl treated callus on first month. In 15 mM treatment, proline content increased significantly in the first month, which then slightly decreased leading to stable level. In 5 and 10 mM NaCl treatment, proline level gradually increased and finally reached a plateau (data not shown). In high salinity levels callus turned to brown and dark brown color and died. In stepwise selection procedure, stepwise increase in NaCl concentration resulted in a steady increase in both callus and shoot proline content. This increase was more evident at 20 mM NaCl in callus and 35 mM NaCl in shoot culture. Comparing proline content between shoot and callus culture, indicate proline content of callus were much lower than shoot (Table 2).

Table 2. Affect of different concentrations of NaCl on proliferation, average shoot length, shoot fresh mass, number of roots/shoot, mean root length and callus and shoot proline content of *Tanacetum cinerariaefolium* in selected time.

Salt concentration (mM)	Number of shoot/explant	Mean shoot length (mm)	Fresh mass of shoots	No. of roots/shoot	Mean root length(mm)	proline content (µM)	
						callus	shoot
0	18 a[z]	13.8 a	92.8 a	9b	5 ab	15.8g	198.7i
5	15.8 b	11.8b	69b	9.5b	5ab	16.5g	189.6j
10	13.8 c	10.5 b	55.7 c	10.6a	6a	28.9 f	364.1h
15	12.8 cd	1.2b	54 c	7c	4bcd	53.2 b	463.1f
20	11.7 d	11b	53.7 c	5.4d	4.2bc	74.7a	798.7e
25	11.6 d	8 c	45.2 d	4.4e	3cde	43.6 c	823.6d
30	11.3 d	6.5d	41.8 d	1.5f	3cde	37.9 d	1722.6 b
35	9 e	5.4 de	44.2	1.2f	3cde	34.5e	1820a
40	6.3 f	5.2 e	24.3d	1.2f	2.5de	35.2e	1389c
45	4.5 g	6 de	24.2 e	1.2f	2e	28.8f	448g

[z]Means within a column followed by the same letters are not significantly different by new Duncan's multiple range test ($P > 0.05$).

Effect of salinity on pyrethrins content

After optimizing all steps and salinity experiments, the content of pyrethrins in different salinity levels (0, 20 and 40 mM) in callus and shoot culture (stepwise selection procedure) experiment were measured and compared with pyrethrins content of mother plant flowers for obtaining salt effect on pyrethrins. The analysis demonstrated a direct correlation between the levels of salinity and pyrethrins contents in cells and tissues (Table 3).

Table 3. Content of pyrethrin components (mg 100g DW) in the original plant flower, callus and shoot under various concentration of NaCl (mM).

Tissue or cells	Salt Concentaration (mM)	Total pyrethrins	CinerinII	CinerinI	JasmolinII	JasmolinI	PyrethrinII	PyrethrinI
Flower	-	3.395	0.545	0.678	0.193	0.256	0.38	1.343
Shoot	0	0.024	-	0.0004	-	0.0025	0.0184	0.0027
	20	0.0286	-	0.0027	-	0.0022	0.0198	0.0029
	40	0.0083	-	-	-	0.0024	0.0023	0.0037
Callus	0	0.0004	0.0003	-	-	-	-	0.0001
	20	0.0004	0.0003	-	-	-	-	0.0001
	40	0.0001	-	-	-	-	-	0.0001

Pyrethrin I was the main product present in young flower. Also, pyrethrin II was main product synthesised in differentiatied shoot at non salinity condition (control). Also, comapring pyreththrin accumulation between different callus (fraible, semicompact and compact callus) showed that pyrethrin accumaulation in fast growing and fraible callus were lowest than other type of callus (data not shown). Although jasmolin II and cinerin II were not detected in the extract of shoot and most pyretrins in callus include pyrethrin II, jasmolin II, I and cinerin I were not detected. The amount of all pyrethrins in flower were higher than shoot and callus. Salinity incraesed some pyrethrins but total pyrethrins showed reduction in shoot and callus culture in high salinity level (Table 3).

DISCUSSION

Callus line selection procedure is likely to play an important role in the recovery of stable variant plants with improved salt tolerance (Winicov, 1996). Results obtained from this experiment strongly indicate that stepwise increase in NaCl concentration from a relatively low level to cytotoxic level is a better way to isolate NaCl-tolerant callus line since direct transfer of callus to highest selection procedure was found to be lethal. Similar results have been reported earlier in other crops (Binh et al., 1992; Patnaik and Debata, 1997a). The process of adaptation of tissues in stressful media is gradual process. Under stress condition, one of the strategies that plants have adopted is to slow down their growth. This reduction in growth not only helps the plant to save the energy for defense purpose but also limits the risk of heritable damage (May et al., 1998). The procedure of stepwise adaptation by sequential treatment of callus from low to high concentrations of NaCl was of added advantage because this procedure helped in better selection of adapted line (Collin and and Dix 1990). Proline, as ubiquitous messenger of stress response, probably plays a signaling role in these adaptive processes (Vranova et al., 2002). Most of the works so far done in relation to *in vitro* selection is based on ion-homeostasis and proline pool (Watad et al., 1983; Olmos et al., 1994; Patnaik and Debata, 1997a). Among the different parameters responding to NaCl stress, rapid accumulation of free proline within the cell is the most significant one. In the present experiment with increase in NaCl stress there was an increase in endogenous free proline content, which was more prominent in case of stepwise route. Whenever, calli were transferred to a higher saline medium from a lower one, a sharp increase in endogenous free proline content was recorded. Interestingly, this accumulation is reversible, i.e., the withdrawal of NaCl from the medium (for 3 months) results in a sharp fall in proline content. Disappearance of high level of proline under unstressed condition was also observed in other crops (Goas et al., 1982; Watad et al., 1983; Chandler and Thorpe, 1987). According to Fukutaku and Yamada (1984) proline acts as a reservoir of nitrogen and carbon sources for post stress growth. This may be the reason that when NaCl was withdrawn from the medium for 3 months, proline level fell down to control level. On retransfer to NaCl medium, high proline accumulates just to balance the osmoregulation. These results indicate that NaCl induced proline synthesis and accumulation are significant adaptive features of plant cells for their survival and growth in saline environment. Also, the shoot proline content was greater than callus proline content. Shoots accumulated significantly high proline under salinity stress. The selected callus line exhibited several positive characters (better growth and proline content) towards salinity stress. This might be the result of some small changes in genetic makeup (somaclonal variation), of which several are beneficial for salt resistance, or due to a single mutation with a pleiotropic effect. However, further studies need to be undertaken to resolve this issue. Most of the work done so far on plant regeneration from selected callus/cell line was through somatic embryogenesis (Binh et al., 1992; Patnaik and Debata, 1997b; Zhang et al., 2001). In the present study, plants were regenerated from callus through shoot organogenesis. Among cytokinins, BA (in combination with NAA and GA3) was found to be an effective inducer of organogenesis in selected callus line. However, previous studies with *Tanacetum cinerariaefolium* demonstrated that combination cytokinin and auxin is necessary for inducing callus regeneration from petiole and leaf (Hitmi et al., 1998). This experiment indicated that shoot regeneration from callus is not easy and step by step method is necessary for shoot regeneration. Using 2, 4-D at first step produced semi compact calli with green color. At second step using BA produced compact callus with green point without regeneration response. The results clearly showed that calli of *Tanacetum cinerariaefolium* could regenerate on MS media contains 1 mg l^{-1} BA and 1 mg l^{-1} NAA or 1 mg l^{-1} BA, 2 mg l^{-1} NAA and 0.5 mg l^{-1} GA_3. Pal and Dhar (1985) reported shoot regenerate from callus in two steps. But, Hitmi *et al*., (1998) obtained shoot regeneration from callus in one step. This difference can be due to different cultivar, culture condition, explant type and medium composition. In the present study, regeneration frequency, mean shoot lenght and fresh mass of regenerated

shoot were decreased under salinity. Decrease in regeneration frequency in NaCl-treated callus line may be due to the presence of NaCl in the regeneration medium or may be due to the partial loss of regeneration potentiality of the selected line during 9 months long NaCl treatment, imposed at the time of callus line selection. Comparing salinity levels showed a significant decrease in plant average shoot length under salinity stress, but the magnitude of decrease was more severe in high salinity level. Rooting (root initiation and number of roots/ plant) of plants was strongly affected by NaCl treatment. This could be the reason of their stunted growth as poor root system may result in inefficient absorption of nutrients from the medium. Evaluation the pyrethrins content in different type of callus indicated that fast growing and friable callus accumulate the lowest levels of pyrethrins where as callus in which cells are more compactly associated grow relatively slowly and accumulate higher levels of pyrethrins (data not shown). Also, the pyrethrins content in regenerated shoot were higher than in callus. The breakdown of organization and the disruption of cell-cell communication during rapid growth is the factor determining the loss of decrease of expression of the biosynthetic potential. Alternatively, it is possible that the growth rate itself is the determining factor in secondary product formation by allowing the diversion of precursors from primary pathway (associated with rapid growth) to secondary pathway (Mak and dovan 1994). Differentiation occur in two stages (Lindsay and Yeoman 1983): cytodifferentiation (changes in subcellular organism and in the pattern of metabolism) and organogenesis (changes in cell organization). Charlwood et al., 1989 reported that cytodifferentiation or even morphological differentiation is a prerequisite for synthesis and accumulation of monoterpenes. We postulate that in compact callus cytodifferentiation leads to the formation of structures necessary for enzyme activity in the pyrethrin pathway. The positive effect of organogenetic on the production and accumulation of secondary metabolite has been explained by the formation of the specialized structure such as surface oil gland and internal secretory canals (Zito 1994). The involvement of this structure in pyrhetrin production has been suggested by both analytical and anatomical evidence (Zito et al., 1983; Zito and Tio 1990). Salinity decreased pyretrins content in both callus and shoot culture. Must reduction was in shoot culture. Proline status of plant organs (shoots in this research) and callus culture has been described in this research. Osmotic stress induced accumulation of proline content. The highest free proline content were 74.7 mM at 20 mM NaCl concentration and 1820 mM at 35 mM NaCl concentration in callus and shoot culture respectively. Sharply increase in free proline content in both culture indicated that tissues required accumulation more free proline for osmiprotection to adapt to an environment with an ionic stress. This phenomena, however was not true in cases of high concentration of NaCl. Accumulation of free proline content as a response to stress was higher in shoot culture. This result may be due to high ability of complete and mature structure in producing precursors of proline and may be due to photosynthesis activity of shoot than callus. Jain et al, 1993 reported changes in polypeptide pattern in *Brassica juncea* in different part of plant at different stage of plant growth in stressful medium. Also, using mature and complete tissue can help better to synthesis and accumulation of proline. Free proline content in high salinity concentration was decreased in both cultures. This result can be due to decreasing protein synthesis because of ionic poisons. Accumulation of Proline is a response to drought and salinity stress. Increasing proline content in most cases indicates increasing resistance to salinity (Sumaryati et al., 1992). Based on growth performance and proline content (20 mM in callus line and 35 mM in shoot culture) could be considered as NaCl-tolerant line showing all positive adaptive features towards the salinity stress. Further study on agronomic performance of these plants under saline soil condition need to be undertaken to check the genetic stability of the induced salt-tolerance.

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