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SELECTION OF SALT TOLERANT PLANTS OF *NICOTIANA TABACUM* L. THROUGH *IN VITRO* AND ITS BIOCHEMICAL CHARACTERIZATION

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Sodium chloride tolerant organogenic callus lines of *Nicotiana tabacum* were developed *in vitro* on Murashige and Skoog [16] medium supplemented with BA, IAA and different concentration of NaCl. The maximum shoot bud regeneration was achieved from both tolerant and non-tolerant calluses on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA with or without NaCl within 4 weeks of culture. Standard growth parameters such as fresh weight and dry weight of organogenic callus, growth tolerant index and enzyme activity (peroxidase and catalase) were used as indicators of salt tolerance. The growth tolerance index in the 4-week after the beginning of treatments yielded significant differences among the non-tolerant and tolerant organogenic callus lines. The regenerated shoots were rooted on half-strength MS basal salts supplemented with 2% sucrose but devoid of growth regulator. The regenerated plants from tolerant callus lines were capable of growing *in vitro* in presence of 175 mM NaCl. SDS-PAGE profile showed that the progenies derived from tolerant sources were tolerant to salt. This investigation may help in the selection and characterization of salt tolerance in plant improvement programme.

Keywords: Enzyme activity – *In vitro* culture – *N. tabacum* – salt tolerance

INTRODUTION

Salinity is the major environmental factor that limits crop growth and productivity or destroys biomass is referred to as a stress or disturbance [4]. More amount of salt in soil or water adversely affects plant growth and development [19, 22]. About 25% of cultivated land throughout the world suffers from excess salinity, principally from

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NaCl. The main drawback is that there were no well-defined plant indicators for salinity tolerance that could practically be used by plant breeders for improvement of salinity tolerance in a number of important agricultural crops. Considerable improvements in salt tolerance have been made in crop species through conventional selection and breeding techniques [2, 17]. However, most of the selection procedures have been based on differences in agronomic characters [18]. Agronomic characters represent both the genetic and environmental effects on plant growth and include the integration of the physiological mechanism conferring salt tolerance. Plant regeneration is of crucial importance in the application of *in vitro* methods for plant improvement. A possible alternative to the conventional approach in crop improvement programmes is to utilize emerging biotechnologies such as somatic hybridization and/or recombinant DNA technology to accomplish transfer of these traits. However, application of either technique is dependent on the availability of a regenerative (i.e. embryogenic or organogenic) cell culture procedures [24]. So far, there are very few reports on *in vitro* selection of salt tolerant plants [5, 6, 21]. *N. tabacum* is widely used for commercial purpose and also in research. The leaves, which are used for, manufacture of cigarettes, bidi, hookah, tobaco and for purposes of chewing. In the present investigation, we report on *in vitro* selection of salt tolerant plants and its biochemical chacteristics.

MATERIALS AND METHODS

Plant material and culture condition

Leaf explants were collected from greenhouse grown plants of *N. tabacum*. The explants were surface sterilized with 0.1% mercuric chloride (w/v) solution for 2 min., followed by three rinses in sterile distilled water. The leaf explants (0.25 cm^2) were placed in the growth medium comprising of Murashige and Skoog [16] (MS) salts, vitamins, 3% (w/v) sucrose and supplemented with 6-benzylaminopurine (BA) alone (0, 0.25, 0.5, 1.0, 1.5 and 2.0) or in combination with IAA (indole-3-acetic acid) at various concentrations (0, 0.1, 0.25, 0.5). The pH of the media was adjusted to 5.8 using 0.1N HCl or 0.1N NaOH before autoclaving. The medium was gelled with 0.8% (w/v) agar (Qualigen, India), 15 ml of molten medium was dispensed into culture vessels. The medium was then autoclaved at 121 °C and 104 kPa for 15 min. The cultures were maintained by regular subculture at 4-week intervals to fresh medium. The cultures were incubated at 25 ± 2 °C in cool white fluorescent light (Phillips, India), with a photon flux density of 55 μmol m^{-2} s^{-1} under a 16 h photoperiod. Usually, 15 cultures were used per treatment and the experiment was conducted three times. The morphological changes were recorded on the basis of visual observations.

Determination of tolerant culture

Organogenic calli (200 ± 10 mg) derived from initial experiment were used for further experiment to determine tolerance ability. The organogenic calli were cultured on fresh MS medium with similar composition (already mentioned) along with various concentration of sodium chloride (0, 10 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM and 200 mM). The concentration of the test material was selected [i.e. LD_{50}, sub-lethal dosage: two concentrations below LD_{50}; another concentration above LD_{50}]. Morphological observations and shoot proliferation were recorded at 4-week intervals. Pre-weighed culture tubes containing 15 ml of culture medium were inoculated with similar quantities of calli, and the inoculated tubes were re-weighed to obtain the initial fresh weight of the leaf inoculum. The final weight minus initial weight of the proliferated leaf tissues in different treatments was used to calculate the percentage of growth against control. The cultures were incubated at 25 ± 2 °C in cool, white fluorescent light (55 μmol m^{-2} s^{-1}) under a 16 h photoperiod for 4 weeks. The experiment had 15 cultures per treatment and was repeated three times.

Determination of dry weight of organogenic callus

Every 4 weeks, the organogenic callus (both tolerant and non-tolerant sources) samples of known fresh weight (200 ± 20 mg) were dried to constant weight at 70 °C in an oven. The relative growth rate (RGR) in terms of fresh mass of the callus growth was calculated following the formula of Shah et al. [23].

$$\text{RGR} = \frac{\text{Ln (final mass)} - \text{Ln (initial mass)}}{\text{Weeks}}$$

Growth tolerance index (GTI) was calculated using the formula:

$$\text{GTI (\%)} = \frac{\text{Mean percent of organogenic callus growth in media with addition of NaCl}}{\text{Mean percent of organogenic callus growth in media without addition of NaCl}} \times 100$$

Chlorophyll and protein determination

Organogenic callus samples (500 ± 20 mg fresh weight basis) from each tolerant and non-tolerant sources were collected at 4-week intervals for estimation of chlorophyll. The organogenic callus was homogenised with 80% acetone in the dark. The amount of chlorophyll was estimated according to Vernon [26]. Pigment content was expressed as mg g^{-1} fresh weight of sample. The amount of total protein was estimated according to Lowry et al. [13]. Proteins in the unknown samples were estimated at 750 nm using bovine serum albumin (fraction V) as a standard; the results were expressed on the basis of grams per unit dry weight (g/dw).

Qualitative analysis of protein

Fifty microlitres of each sample (tolerant, non-tolerant and progenies from tolerant culture) was mixed with 50 µl of sample buffer containing 0.95% Tris, 2% SDS, 10% (v/v) glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue. The mixtures were hydrolysed for 10 min at 90 °C, then loaded into pre-formed wells in the stacking gel. A 12% polyacrylamide resolving gel, topped with 4% stacking gel was used as the separation medium. Electrophoresis was used as the separation medium. Electrophoresis was conducted at a constant current at 4 mA per well for 4 hours at 4 °C. After electrophoresis, the gels were stained with 1% Commassie Brilliant blue (G-250) dissolved in a mixture of methanol, acetic acid and distilled water (2 : 1 : 2) for 8–10 h. Destaining of the gel was made by using the same mixtures, but without the stain. After destaining, the gels were photographed and stored in 7% (v/v) acetic acid. Gel photographs were scanned through Gel Scanner (Bio Rad, USA) and the position of the protein band in the gel was expressed in comparison with standard protein markers.

Estimation of total amino acid content

Organogenic callus (500 ± 20 mg fresh weight basis) from each tolerant and non-tolerant sources were homogenized in 70% ethanol in a mortar and pestle. The homogenate was centrifuged at 4400 g for 10 min and the supernatant was taken. The extraction was repeated four or five times, and the supernatants were combined. An appropriate volume (5 to 10 ml) of this ethanolic extract was evaporated to dryness in a boiling water bath, and the residue was dissolved in 5 ml of 0.2 M citrate buffer (pH 5.0). Two ml sample was taken from the test tube and added 1 ml of a ninhydrin reagent (1 : 1, 4% ninhydrin in methyl cellosolve and 0.2 M acetate buffer). The samples were boiled for 20 min and cooled. Afterward, the volume was made up to 10 ml with distilled water, and absorbance was taken at 570 nm. Total free aminoacids were calculated from a standard curve prepared against glycine (0 to 100 µg).

Enzyme extraction and assay

Peroxidase

Organogenic callus samples (500 ± 20 mg fresh weight basis) from tolerant and non-tolerant sources were collected at 4-week intervals and homogenized with mortar and pestle in cold 0.1 M phosphate buffer (pH 6.1) containing 30 mg of insoluble polyvinylpyrrolidone and 15 mg sodium ascorbate. The homogenate was filtered through four layers of miracloth and centrifuged at 12,000×g for 10 min at 4 °C. The supernatant was used for the peroxidase assay. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H_2O_2 and 0.4 ml of crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbance at 420 nm was measured using a UV spectrophotometer (Jasco, UVIDEC-650, Japan). The levels of enzyme activity were expressed as μmol H_2O_2 destroyed/min/mg protein.

Catalase

Organogenic callus samples (500 ± 20 mg) from tolerant and non-tolerant sources were collected at 4-week intervals and homogenized with mortar and pestle in 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 1000 g for 10 min at 4 °C. One milliliter of supernatant was added to the reaction mixture containing 1 ml 0.1 M H_2O_2 and 3 ml 0.1 M sodium phosphate buffer (pH 7.0). The reaction was stopped by adding 10 ml 2% H_2SO_4 after 1 min incubation at 20 °C. The acidified reaction mixture with or without the supernatant was titrated against 0.01 M $KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. The catalase activity was expressed as μmol H_2O_2 destroyed/min/mg protein.

Differentiation of shoots buds from organogenic callus

The organogenic callus were transferred to various medium containing MS basal salts supplemented with BA (0.25 – 1.0 mg/l), IAA (0.1 – 0.25 mg/l) and different concentrations of sodium chloride (0, 100 mM, 125 mM, 150 mM, 175 mM and 200 mM) for shoot bud regeneration. All the cultures were grown in 100 mm Petri dishes and incubated at 25 ± 2 °C in a growth room under cool, white fluorescent lamps (55 μ E m^{-2} s^{-1}) for 4 weeks. The experiments had 15 cultures per treatment and repeated three times.

Induction of rooting and acclimatization

Regenerated shoots (both tolerant and nontolerant) were transferred to half-strength MS basal salts with 160 mM NaCl and supplemented 2% (w/v) sucrose and devoid of growth regulator. Rooted microshoots were planted in 2.5 cm earthern pots containing a pot compost comprised of cowdung : soil in the ratio of 1 : 1, respectively. The plantlets were maintained in a greenhouse for establishment.

Statistical analysis

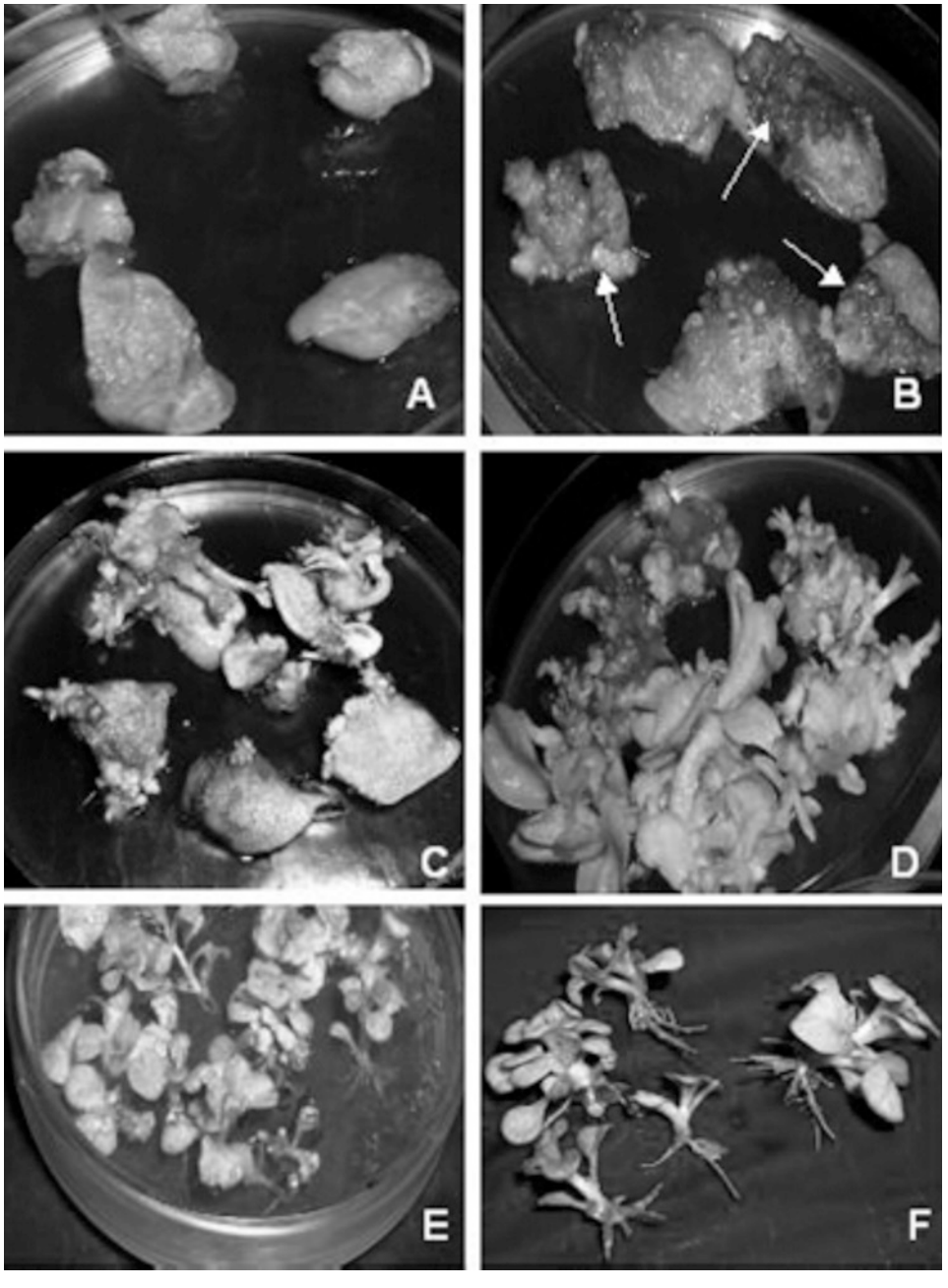
The data pertaining to mean percentage of explant responded per treatment, average number of shoots per culture, chlorophyll, protein, proline and total amino acid content, catalase and peroxidase activity in organogenic calli derived from tolerant and non-tolerant sources were statistically analysed by analysis of variance. Between the treatments, the average figures followed by the same letter within a column in the tables were not significantly different at the $P < 0.05$ level [15].

RESULTS AND DISCUSSION

Selection for growth medium for induction of organogenic callus

Leaf explants were enlarged within 6 – 7 days of culture on MS basal medium supplemented with different concentrations of IAA or NAA and BA or Kn. Initially, small greenish-white organogenic calluses were developed in the cut and upper surface of the explants (Fig. 1A, B). Subsequently, it covered the entire surface of the explant. The little callus growth was also obtained in MS media without growth regulators. The medium with either Kn or BA or BA supplemented with IAA promoted organogenic callus formation. The medium containing BA with IAA promoted rapid growth of organogenic calli as compared with BA alone. The rate of callus growth was decreased with increase of either BA or BA plus IAA. The maximum organogenic callus growth was observed in 1.0 mg/l BA and 0.1 mg/l IAA within 4 weeks of culture (data not shown).

Fig. 1. Regeneration of salt tolerant plants of *N. tabacum.* **A.** Leaf explants of *N. tabacum* cultured on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA after 7 days of culture. **B.** Organogenic callus formation (arrows) from leaf explants on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA after 2 weeks of culture. **C.** Regeneration of shoot buds from tolerant organogenic calli cultured on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA, 175 mM NaCl after 2 weeks of subculture. **D.** Elongated shoots derived from tolerant organogenic calli cultured on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA, 175 mM NaCl after 4 weeks of subculture. **E.** Salt-tolerant shoots were rooted on 1/2 strength MS medium supplemented with 175 mM NaCl after 7 days of culture. **F.** Rooted tolerant plantlets ready for transfer to greenhouse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Selection of NaCl tolerant organogenic callus

Proliferated organogenic calli grown in the MS basal salts supplemented with 1.0 mg/l BA, 0.1 mg/l IAA for two weeks were transferred to similar medium with different concentrations of sodium chloride (0, 10 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM and 200 mM). The calli were rapidly proliferated into compact green structures in 2 weeks on medium containing up to 150 mM NaCl. Few small shoots grew at higher concentration of NaCl (175 mM) and subsequently, it grews rapidly into greenish white calli. The culture having 200 mM NaCl did not show any sign of growth and turned brown within 1 week of transfer. The cultures that showed growth at 175 mM NaCl were separated and subcultured on respective medium at 4-week intervals. Both tolerant and non-tolerant organogenic calli were again transferred onto a similar medium with 175 mM NaCl or without NaCl. Significant growth was achieved on medium containing 175 mM NaCl as compared with the control medium (non-tolerant) (Table 1). Suppression of growth, tolerance

Table 1
Effect of different concentrations of NaCl on response of organogenic callus formation from leaf explants of *N. tabacum* on MS basal medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA and 3% (w/v) sucrose after 4 weeks of culture

NaCl concentration (mM)	Percent of explant response (mean ± S.E)*
0	80.4 ± 1.8^{g}
10	74.8 ± 1.2^{f} (–6.96)
50	68.6 ± 1.8^{e} (–14.7)
75	65.8 ± 1.0^{d} (–18.2)
100	62.2 ± 1.3^{c} (–22.6)
125	60.4 ± 1.8^{c} (–24.9)
150	52.6 ± 1.6^{b} (–34.6)
175	36.8 ± 0.8^{a} (–54.2)
200	0

*15 replicates/treatment; repeated three times. Parenthesis indicates the percentage of reduction (–)/increase (+) relative to control. Mean having the same letter in a column were not significantly different by Post-Hoc multiple comparison test $P < 0.05$.

levels and rates of growth reduction at lethal concentrations of salts vary widely among different plant species [9]. The growth tolerance index (GTI) of non-tolerant and tolerant organogenic culture showed significant variation on medium containing different concentration of NaCl (Fig. 2). Hasegawa et al. [8] reported the growth characteristics of NaCl-tolerant and non-tolerant cells of *N. tabacum*. Subsequently, Watad et al. [27] compared between the stable NaCl-selected *Nicotiana* cell line and

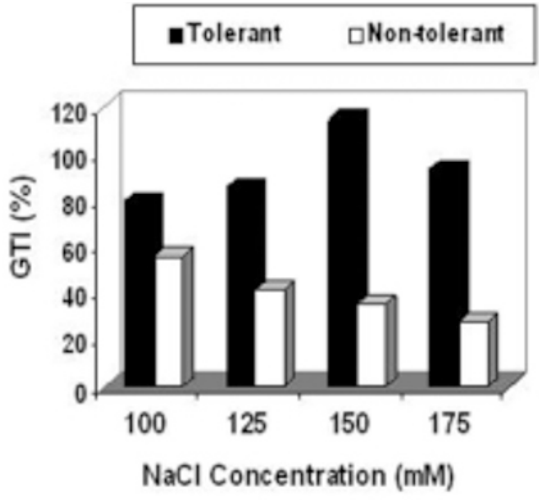

Fig. 2. Effect of NaCl concentration on Growth Tolerance Index (GTI) of tolerant and non-tolerant organogenic calli of *N. tabacum* cultured on MS basal salts supplemented with 1.0 mg/l BA, 0.1 mg/l IAA and 3% sucrose

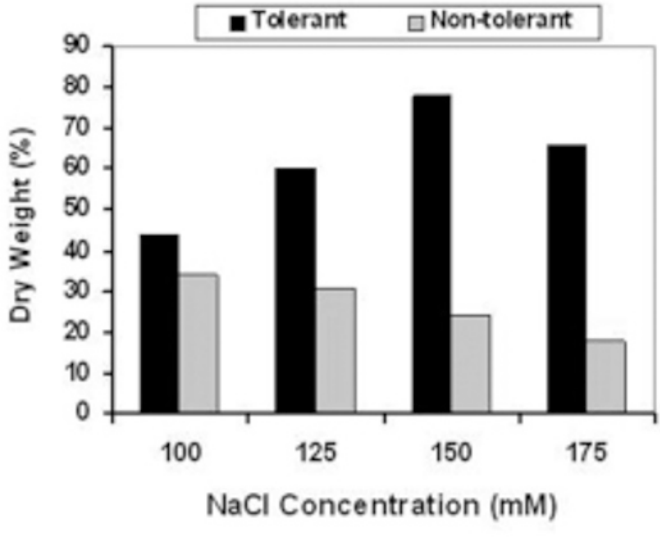

Fig. 3. Effect of MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA and 3% sucrose and different concentrations of NaCl on dry weight of tolerant and non-tolerant organogenic calli of *N. tabacum* after 4 weeks of culture. Mean of 10 replicates/treatment; repeated three times

wild type. The tolerant cultures produced significantly more fresh and dry biomass at 175 mM than the non-tolerant cultures. The growth of non-tolerant culture was, however, stimulated by low concentration of NaCl, which declined with further increase in NaCl and showed a reduction in their fresh weight. The retardation of growth may be due to the fact that certain amount of the total energy available for tissue metabolism is channeled to resist the stress [7]. Dry weight of organogenic callus was increased with increase in the NaCl concentration in the culture medium. Tolerant organogenic cultures showed a greater dry weight gain than non-tolerant ones (Fig. 3). Both tolerant and non-tolerant organogenic cultures were maintained for a prolonged period on similar fresh media.

Table 2
Chlorophyll content (mg/g fresh weight) of tolerant and non-tolerant calli of *N. tabacum* cultured on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA and different concentration of NaCl after 4 weeks of culture

NaCl concentration (mM)	Source of organogenic callus	Chlorophyll content (mg/g fresh weight) (mean ± S.E)*		
		Chlorophyll a	Chlorophyll b	Total chlorophyll (a + b)
	Tolerant			
0		5.36 ± 0.8^{c}	4.82 ± 0.6^{c}	10.2 ± 1.4^{c}
100		6.11 ± 0.5^{d} (+14.0)	5.10 ± 0.5^{d} (+5.80)	11.2 ± 1.0^{d} (+9.8)
125		6.35 ± 0.4^{d} (+18.5)	5.42 ± 0.7^{d} (+12.4)	11.8 ± 1.1^{d} (+15.7)
150		7.12 ± 0.7^{e} (+32.8)	5.82 ± 0.4^{d} (+20.7)	12.9 ± 1.1^{e} (+26.5)
175		8.28 ± 0.6^{f} (+54.5)	5.94 ± 0.8^{d} (+23.2)	14.2 ± 1.4^{f} (+39.2)
	Non-tolerant			
100		5.25 ± 0.6^{c} (–2.05)	4.38 ± 0.8^{c} (–9.12)	9.63 ± 1.4^{c} (–8.5)
125		5.06 ± 0.7^{c} (–3.61)	4.10 ± 0.4^{c} (–14.9)	9.16 ± 1.1^{c} (–10.2)
150		3.72 ± 0.6^{b} (–29.1)	3.11 ± 0.2^{b} (–35.5)	6.83 ± 0.8^{b} (–33.3)
175		1.13 ± 0.05^{a} (–78.5)	1.07 ± 0.06^{a} (–77.8)	2.2 ± 0.1^{a} (–78.4)

*10 replicates/treatment; repeated thrice. Mean having the same letter within a column were not significantly different by Post-Hoc multiple comparison test $P < 0.05$ level.

Biochemical analysis

The result showed that the chlorophyll content in the tolerant organogenic calli was higher as compared to non-tolerant ones. The organogenic calli derived from tolerant sources showed higher chlorophyll content (Chlorophyll a and b) which varied from 5.36 to 8.25 and 4.82 to 5.94 mg/g, respectively, as compared with non-tolerant ones. The chlorophyll a and b content in the tolerant sources increased 54.5% of chlorophyll a and 23.2% of chlorophyll b in the presence of 175 mM NaCl (Table 2). The decrease in chlorophyll content at high NaCl concentrations might be due to the disruption of some chloroplasts [20] or to changes in the lipid protein ratio of pigment-protein complexes, as well as because of increase of chlorophyllase activity [12]. Protein content increased in the organogenic culture derived from the tolerant sources as compared with non-tolerant cultures (Table 3). The higher content of soluble proteins has been observed in salt tolerant than non-tolerant cultivars of barley [11], finger millet [25], sunflower [3] and rice [14]. The SDS-PAGE protein pattern indicated that 10 polypeptides appeared in both salt tolerant as well as non-tolerant organogenenic callus lines (Fig. 4). The results also revealed that four new polypeptides (70.6, 75.2, 114.2 and 138.2 kDa) were detected in salt-tolerant shoot as well as progenies derived from tolerant sources. The 54 kDa protein was responsible for salt tolerance in case of finger millet has been reported by Uma et al. [25]. The total amino acid content was increased in the tolerant organogenic calli as compared with non-tolerant one (Table 4). Similar observations have been reported in higher plants

Table 3
Total protein content (µg/g fresh weight basis), total amino acid content (µmoles/g dry weight basis) and proline content (µmoles/g fresh weight basis) of tolerant and non-tolerant organogenic calli of *N. tabacum* on MS medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA and different concentrations of NaCl after 4 weeks of culture

NaCl concentration (mM)	Source of callus	Total protein content (mean ±S.E)*	Total free amino acid content (mean ±S.E)*	Proline content (mean ±S.E)*
	Tolerant			
0		1223 ± 16.8	1600 ± 11.2	2.1 ± 0.6
100		1442 ± 12.6	2142 ± 12.6	2.8 ± 0.4
125		1851 ± 14.7	2565 ± 10.5	3.6 ± 0.8
150		2428 ± 11.6	3782 ± 9.8	4.5 ± 0.6
175		2602 ± 20.2	4020 ± 12.8	5.9 ± 0.7
	Non-tolerant	932 ± 8.7	1352 ± 12.8	0.6 ± 0.04
100		1036 ± 14.4	1384 ± 9.7	1.2 ± 0.3
125		1128 ± 18.2	1456 ± 10.6	1.6 ± 0.4
150		1332 ± 12.6	1474 ± 11.7	2.2 ± 0.6
175		1214 ± 10.5	1228 ± 13.2	2.8 ± 0.5

*10 replicates/treatment; repeated thrice.

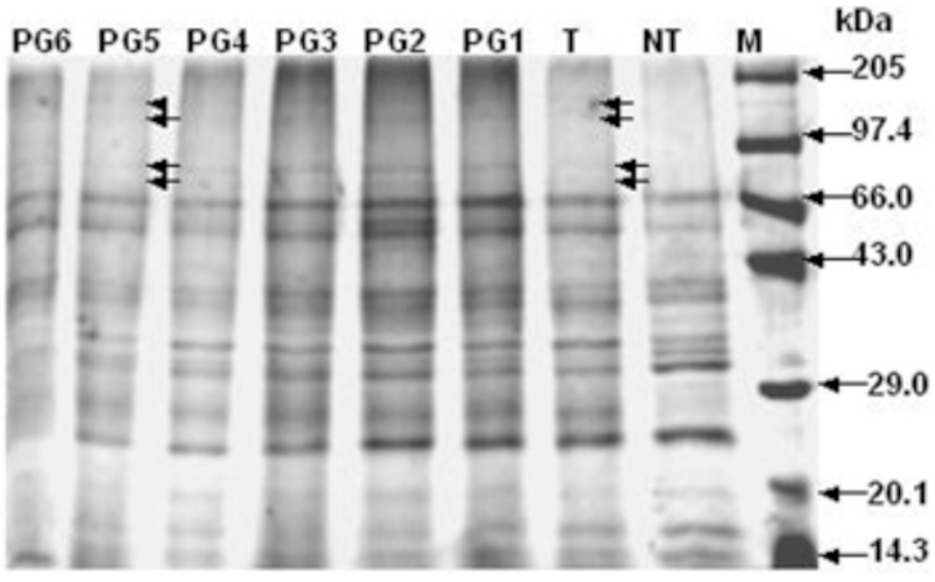

Fig. 4. SDS-PAGE protein profiles of the non-tolerant (NT), tolerant (T) plants and progeny from tolerant culture (PG1-PG6). Values in the margin are the kDa values of the major polypeptide fractions (M). The arrows identify bands discussed in the text

[1]. The amino acid content in tolerant organogenic calli was about twices higher than in the control. Proline is known to play a major role in osmoregulation in stressful environment in many plant species. The proline content increase with increase of NaCl concentration in the culture medium in case of tolerant calli (Table 3). The level of salt tolerance in callus lines has also been correlated with the level of accumulated proline as proline overproducers have been reported to be salt tolerant in different plant systems [5, 17].

Enzyme activity

The activities of specific enzymes are important for plant metabolism under conditions of salt stress, and therefore may play a subtle role in salt tolerance. During a period of 4 weeks of culture, the activities of both catalase and peroxidase were significantly higher in tolerant organogenic calli grown in the medium having NaCl in comparison with non-tolerant one (Table 4). The catalase and peroxidase activity were increase about 146% and 97.8%, respectively, in case of tolerant lines and reduction about 73.3% and 46.4% in case of non-tolerant lines grown on the medium containing 175 mM NaCl.

Table 4
Catalase and peroxidase (µmol H_2O_2 destroyed/min/mg.protein) of tolerant and non-tolerant organogenic calli of *N. tabacum* grown on MS basal medium supplemented with 1.0 mg/l BA, 0.1 mg/l IAA and different concentrations of NaCl after 4 weeks of culture

Source of organogenic callus	Enzyme activity (mean ± S.E)*				
	NaCl concentration (mM)				
	0	100	125	150	175
Catalase					
Tolerant	23.4 ± 1.3	32.4 ± 2.1 (+38.5)	41.2 ± 1.5 (+76.0)	53.2 ± 1.4 (+127.3)	57.6 ± 1.7 (+146.1)
Non-tolerant	16.5 ± 1.1	22.6 ± 1.2 (+37.0)	26.8 ± 1.0 (+62.4)	30.4 ± 2.1 (+84.2)	28.6 ± 1.8 (+73.3)
Peroxidase					
Tolerant	18.6 ± 1.0	22.6 ± 1.3 (+21.5)	26.4 ± 1.6 (+41.9)	30.2 ± 1.5 (+62.4)	36.8 ± 1.0 (+97.8)
Non-tolerant	12.8 ± 1.2	14.6 ± 1.4 (+14.0)	16.2 ± 1.8 (+26.6)	19.6 ± 1.2 (+53.1)	18.8 ± 1.6 (+46.9)

Parenthesis indicates the percentage of reduction (–) / increase (+) relative to control.
*10 replicates/treatment; repeated thrice.

Regeneration of shoots from organogenic culture

Both tolerant and non-tolerant organogenic calli of *N. Tabacum* were transferred to different regeneration media with or without NaCl for shoot development and multiplication. The organogenic calli derived from tolerant sources developed a large number of shoots in media having 1.0 mg/l, 0.1 mg/l IAA and 175 mM NaCl (Fig. 1C, D) With increase of either BA or IAA in the culture medium decline the percentage of regeneration and number of shoot buds/culture. The tolerant organogenic cultures grown more in the medium having NaCl as compare to non-tolerant one. The number of shoot buds per culture varied significantly between 2.1 to 45.4 on different culture medium (Table 5). The organogenic calli derived from non-tolerant sources were growing very slow in the medium having 175 mM NaCl, however, the calli derived from tolerant sources showed rapid growth of shoots in the medium having 175 mM NaCl. When increase the concentration of either BA or IAA in the medium showed decrease the regeneration process. Heszky et al. [10] reported that the presence of NaCl in the culture media does not affect regeneration potential. The effect of NaCl on plant regeneration in rice has been documented [6].

Table 5
Effect of various media on shoot bud regeneration from tolerant and non-tolerant organogenic calli of *N. tabacum* after 4 weeks of culture

MS medium +3% sucrose			Percent of shoot bud regeneration (mean ± SE)* (average number of shoot buds/culture)	
BA	IAA	NaCl (mM)	source of organogenic callus	
			tolerant	non-tolerant
0.0	0.00	0	4.8 ± 0.7^{a} (2.6)	6.2 ± 0.5^{a} (4.8)
0.5	0.00	0	54.6 ± 1.1^{b} (12.6)	58.8 ± 1.1^{e} (18.6)
0.5	0.10	0	65.8 ± 1.4^{d} (20.3)	72.2 ± 1.6^{f} (30.4)
1.0	0.10	0	74.5 ± 1.2^{e} (36.8)	78.6 ± 1.2^{g} (34.6)
1.0	0.10	175	82.6 ± 1.3^{f} (45.2)	34.8 ± 1.0^{d} (17.4)
1.0	0.25	175	66.6 ± 1.4^{d} (28.4) +	26.2 ± 0.9c (10.3) +
1.5	0.25	175	62.4 ± 1.1^{c} (26.2) +	8.5 ± 0.8^{b} (2.8) +

*15 replicates/treatment; repeated thrice. +: Browning of the culture. Means having the same letter in a column were not significantly different by Post-Hoc Multiple comparison test $P < 0.05$.

Induction of rooting and field acclimatization

The shoots regenerated from both tolerant and non-tolerant sources were excised and cultured on half-strength MS medium without growth regulators. A high percentage of rooting (95%) was obtained on half-strength MS medium devoid of growth regulators. The shoots derived from tolerant sources were rooted maximum in the medium with 175 mM NaCl and devoid of growth regulator (Fig. 1E, F). Non-tolerant shoots were not survived in the medium having 175 mM NaCl. The rooted plantlets were transferred to greenhouse condition for acclimatization. About 90% of tolerant plants were survived in the greenhouse condition.

In conclusion, the tolerant organogenic callus lines were grown in NaCl containing medium. Peroxidase and catalase activities were significantly higher in tolerant organogenic callus lines as compared to non-tolerant ones. Protein content was the maximum in tolerant calli than in non-tolerant ones. Therefore, it was also concluded that the proteins were involved in induction of tolerance to sodium chloride. Development of salt tolerant callus lines may be useful in crop improvement programmes. Further investigations are necessary to know the molecular mechanism of salt tolerance in *Nicotiana tobaccum*.

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