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# ***In Vitro* Selection of Salt Tolerant Calli Lines and Regeneration of Salt Tolerant Plantlets in Mung Bean (*Vigna radiata* L. Wilczek)**

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## **1. Introduction**

Salinity is one of the important abiotic factors in limiting plant productivity (Munns, 2002). 19.5% of the irrigated agricultural land is considered saline (Flowers & Yeo, 1995). Although minerals are essential for plants, their excess quantity in the soil is injurious to plants. Plants exposed to saline environment suffer from ion excess or water deficit and oxidative stress linked to the production of reactive oxygen species (ROS), which cause damage to lipids, proteins and nucleic acids (Hernandez, *et al.*, 2000). Oxidative stress is considered to be one of the major damaging factors in plant cells exposed to salinity (Gossette, *et al.*, 1994; Hernandez, *et al.*, 1995; Khan & Panda, 2002; Queiros *et al.*, 2007). The process of salt response and tolerance has been studied at the whole plant level (Hasegawa *et al.*, 2000; Jogeshwar, *et al.*, 2006). However the structural complexity of the whole plant makes it difficult to separate systemic from cellular salinity tolerance mechanism (Hawkins & Lips, 1997). The importance of plant tissue culture in the improvement of salt tolerance in plants has been pointed long back (Dix, 1993; Hasegawa, *et al.*, 1994; Nabors *et al.*, 1980; Tal 1994). In recent years tissue culture techniques are being used as a useful tool to elucidate the mechanism involved in salt tolerance by using *in vitro* selected salt tolerant cell lines (Davenport, *et al.*, 2003; Gu, *et al.* 2004; Lutts *et al.*, 2004 ; Naik & Harinath, 1998; Rao & Patil. 1999; Venkataiah. *et al.*, 2004). Besides, these lines have been used to regenerate salt tolerant plants (Chen *et al.*, 200; Jaiwal & Singh, 2001; Miki *et al.* 2001; Ochatt *et al.*, 1999; Rao & Krupanidhi, 1996; Shankhdhar, *et al.*, 2000). The selection of crop varieties for greater tolerance to saline environment will allow greater productivity from large saline lands.

## **2. Materials and methods**

### **2.1 Callus induction and culture**

Seeds of mung bean cultivar (cv) PS-16 were obtained from Agriculture Research Station Gulbarga, India. Seeds were washed thoroughly and imbibed in sterile water for six hours and then soaked in 70% (v/v) ethanol for 4 min and then surface sterilized for 1 min by 0.1% HgCl<sub>2</sub> (m/v). The seeds were germinated on sterile filter paper soaked in sterile water in

culture tubes and allowed to germinate for 5 days at 16/8h (light/dark) photoperiod at  $42 \mu \text{mol m}^{-2} \text{s}^{-1}$  irradiance provided by cool fluorescent tubes and incubated at  $26 \pm 1 \text{ }^\circ\text{C}$ . Cotyledons from 5 days old seedlings were used as source of explants for callus induction. The cotyledons were cut along the four side ( i.e. leaf apex, petiole region and leaf margin of both the sides) with a sterile scalpel and cultured on Murashige and Skkog's (MS) medium supplemented with  $9.09 \mu\text{M}$  2, 4-Dichlorophenoxy acetic acid (2,4-D) and  $5.76 \mu\text{M}$  kinetin(Kn) solidified with 8% (m/v) agar, before solidifying the pH of the media was adjusted to 5.6, the cultures were incubated under 16/8h (light/dark) photoperiod and allowed to grow for four weeks.

## 2.2 Selection of salt tolerant lines

Callus tissue grown on MS medium as described above was directly exposed to different concentrations of NaCl (50, 100, 150 and 200 mM) and the concentration at which growth was completely inhibited was determined. Cell survival was nonexistent at 200 mM. Consequently a concentration of 150 mM was used for selection and a portion of calli developed at this concentration was thereafter sub- cultivated on fresh MS medium containing same concentration (150mM) of NaCl and allowed to grow for 8 weeks. After this, calli were then transferred on MS medium without NaCl for 4 weeks. To test the stability of salt tolerant trait. Callus line growing healthily, on medium without NaCl were again transferred to MS medium containing 150 mM NaCl for 4 weeks, surviving callus lines showing good growth at this stage was considered to be NaCl tolerant. The salt tolerant calli was picked up and were further grown for a period of 4 weeks and used for enzyme, protein and proline estimation.

## 2.3 Assessment of growth

Callus tissue (~250 mg) of both selected and non-selected were grown MS medium supplemented with  $9.09 \mu\text{M}$  2, 4-D and  $5.76 \mu\text{M}$  Kin containing 0-150 mM NaCl. The increase in fresh weight of the callus was determined after four weeks. Three replicates each consisting of 10 culture tubes were maintained per treatment, the relative growth rate of callus was calculated as the  $(\text{FM}_f - \text{FM}_i) / \text{FM}_i$ , where  $\text{FM}_f$  and  $\text{FM}_i$  were the final and initial fresh weight respectively. Samples of callus tissues were stored in  $-80 \text{ }^\circ\text{C}$  for further analysis.

## 2.4 Lipid peroxidation

Lipid peroxidation was determined in terms of Malondialdehyde (MDA) content using the Thiobarbitric acid reaction (TBARS) according to Heath and Packer (1968). Frozen callus tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle. Subsequently about 100 mg of powder was homogenized in  $5 \text{ cm}^3$  of TCA (0.1% w/v) and centrifuged at 10,000g for 10min at  $5 \text{ }^\circ\text{C}$ .  $1 \text{ cm}^3$  of supernatant was mixed with  $4 \text{ cm}^3$  of 0.5% TBA reagent in 20% TCA. The mixture was then heated at  $95 \text{ }^\circ\text{C}$  for 30 min, cooled over ice and centrifuged at 10,000g for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected for non specific absorbance at 600 nm. MDA content was calculated using an extinction coefficient ( $\epsilon$ ) of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$  and expressed as  $\text{nmol g}^{-1} \text{ FW}$ .

## 2.5 Determination of ascorbate

Ascorbic acid (As A) was extracted in 5% (m/v) methophosphoric acid with sand at 4 °C. The homogenate was then centrifuged at 3000 g for 20 min at 4 °C. Ascorbate content was quantified in the supernatant as described by Shukla, *et al.*, (1979). An aliquot of 1 cm<sup>3</sup> of the supernatant was mixed with 2.5 cm<sup>3</sup> of 1% (v/v) freshly diluted Folin - Ciocalteu reagent. The reaction mixture was allowed to stand for 40 min at room temperature, then the absorbance was recorded at 730 nm, using ascorbic acid as standard.

## 2.6 Superoxide dismutase activity (SOD) (EC 1.15.11)

The activity of SOD was determined based on its ability to inhibit the auto oxidation of pyrogallol. The measurement was based on the modified method of Markland and Markland, (1947). The reaction mixture consisted of 0.25 M pyrogallol in 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 cm<sup>3</sup> of enzyme extract. The reaction was initiated by light illumination and the rate of oxidation was measured spectrophotometrically at 429 nm as per the procedure described by Nakono & Asada, (1981). SOD activity is expressed in units/mg protein. One unit is defined as the amount of the enzyme which caused 50% inhibition of pyrogallol oxidation.

## 2.7 Ascorbate peroxidase activity (APX)

The activity of APX was determined by measuring the decrease in absorbance at 290 nm and the amount of Ascorbate oxidized to dehydroascorbate was calculated from the extinction coefficient 2.8 (Nakano and Asada, 1981). The reaction mixture (2 cm<sup>3</sup>) consisted of 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbic acid, 1.0 mM H<sub>2</sub>O<sub>2</sub> and 0.2 cm<sup>3</sup> enzyme extract.

## 2.8 Catalase activity (CAT; EC 1.11.1.6)

Catalase activity was measured according to method of Chandlee and Scandalios (1984). One gram of frozen callus was homogenized in a pre chilled pestle & mortar with 5 ml of ice cold 50 mM phosphate buffer. The extract was centrifuged at 4°C for 20 Min. at 12,500 X g. The supernatant was used for enzyme assay. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0) 0.4 cm<sup>2</sup> 15 mM H<sub>2</sub>O<sub>2</sub> and 0.1 cm<sup>2</sup> of enzyme extract. The decomposition of H<sub>2</sub>O<sub>2</sub> was followed by decline in absorbance at 240 nm.

## 2.9 Peroxidase activity (POX; EC 1.11.1.7)

Peroxidase activity was assayed by the method of Kumar and Khanna (1982). Assay mixture contained 2 ml of 0.1 M Phosphate buffer (pH 6.8) 1ml of 0.01M pyrogallol, 1 ml of 0.005 M H<sub>2</sub>O<sub>2</sub> and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5N H<sub>2</sub>SO<sub>4</sub>. The purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5N H<sub>2</sub>SO<sub>4</sub> at zero times. The activity was expressed in unit mg<sup>-1</sup> protein. One unit (U) is defined as the change in the observance by 0.1 min<sup>-1</sup> mg<sup>-1</sup> protein.

### 2.10 Protein estimation

Samples of frozen callus tissue were ground in Tris-HCl (60 mM, pH 6.8) at 4°C, using prechilled mortar and pestle. The extract was centrifuged (4000g for 15 min at 4°C) and the supernatant was used for protein estimation according to Bradford (1976).

### 2.11 Proline estimation

Proline was extracted and determined calorimetrically by the method of Bates, *et al.*, (1973). Callus tissue (~200 mg) was homogenized with 4 cm<sup>3</sup> of 50 mM phosphate buffer (pH 7.8) containing 1% (w/v) polyvinyl pyrrolidone (PVP) 0.01% (w/v) Triton X-100, and centrifuged at 800 rpm for 15 min. Proline was determined in the supernatant by measuring the absorbance of proline-ninhydrin product formed, at 520 nm in a spectrophotometer using toluene as a solvent.

### 2.12 Regeneration of plantlets from NaCl tolerant calli

Plantlets were regenerated from salt tolerant and non-tolerant (control callus) callus. For regeneration of plantlets, callus was transferred in fragments of about 300 mg directly to MS medium supplemented with 0.5-2 mg/l BAP, alone or in combination with 0.25 and 0.5 mg/l NAA. After the shoots reached a height of 3-4 cm individual shoots were excised and transferred to rooting media which consisted of MS medium supplemented with 0.5 and 1 mg/l IBA.

### 2.13 NaCl tolerance test of regenerated plantlets

After the regenerated shoots produced roots, they were transferred to Erlenmeyer flasks containing the same rooting medium supplemented with 150 mM NaCl (Chen, *et al.*, 2001). The survival rate of plantlets regenerated from non selected callus and NaCl selected callus was determined by using the formula

$$\frac{\text{The number of surviving plantlets} \times 100}{\text{Total number of tested plantlets}}$$

### 2.14 Statistical analysis

The results presented are an average over three independent experiments. Data were processed with analysis of variance (ANOVA) and the means were compared using students test at a significance level of = 0.05.

## 3. Results and discussion

### 3.1 Establishment of salt tolerant lines

The NaCl tolerant mungbean cell lines obtained in this study were selected from callus cultures initiated from cotyledon explants. The direct recurrent selection procedures were successful in selection of mungbean cell lines exhibiting tolerance to 150 mM NaCl.

Callus tissue cultured on medium containing 50 mM NaCl showed good cell proliferation and appeared morphologically similar to control, where as callus tissue cultured on medium



containing 100 mM NaCl showed moderate cell proliferation and small portions showing brownish color indicating early stages of necrosis. When callus tissue was cultured on medium supplemented with 150 mM NaCl, a great portion of the cells did not show any sign of growth and turned brown within 2 weeks of culture. However, small clusters of cells survived on 150 mM NaCl containing medium. Subsequently the cell clumps that proliferated in the presence of the salt were picked up and sub cultivated on NaCl free medium for 4 weeks. The salt tolerant cell lines were again transferred on to a fresh medium supplemented with 150 mM NaCl. On this medium cell clumps proliferated in to a mass of callus exhibiting friable nature. The selection processes resulted in establishment of NaCl-tolerant callus lines showing good proliferation of callus.

Cell lines exhibiting tolerance to NaCl have been previously selected from a range of legumes Gosal & Bajaj,1984) pasture legumes, (Smith & MeComb, 1981) chick pea, (Pandey & Ganapathy,1984,1985) Alfalfa (Wincicov, 1991) red gram (Rao & Krupanidhi,1996) pea (Ochatt, *et al.*, 1999) soybean (Liu & Van Staden,2000) ground nut (Jain, *et al.*, 2001). And in several other plant species such as, canola (Tyagi & Rangaswamy, 1997, Jain, *et al.*, 1991a, b) finger millet, (Pius, *et al.* 1993) Cotton (Gosset, *et al.*, 1994) Potato (Olmos & Hellin, 1996; Queiros, *et al.*, 2007) rice (Dang & Nguyen, 2003; Shankdhar *et al.*, 2000) Sunflower (Davenport, *et al.*, 2003) chilli (Venkataiah, *et al.*, 2004) sugarcane (Gandonou, *et al.*, 2006).

### 3.2 Growth of callus

Growth rate of adapted callus line in NaCl supplemented media was steady and sustainable but it was lower than the growth of the control callus grown on stress-free medium.

Reduction in the growth is a common phenomenon in cultured cells grown on medium supplemented with NaCl (Cushman *et al.*, 1990; Greenway & Munns, 1980; Jain *et al.*, 1991 a, b; Luts *et al.*, 2004; Rus *et al.* 1999; Shankdhar *et al.*, 2000; Thomas *et al.*, 1992; Venkataiah *et al.*, 2004) and it has been interpreted that a certain amount of the total energy available for tissue metabolism is channeled to resist the stress (Cushman, *et al.*, 1990). However 200 mM NaCl tolerant calli obtained either by direct or indirect selection process did not sustained a regular growth on salt supplemented media, these calli suffered with time a decrease in growth and all callus tissues died when the culture period was prolonged beyond one month. A similar response was reported in salt tolerant potato calli lines (Queiros, *et al.*, 2007) and in *Chrysanthemum* sp. (Hossain, *et al.*, 2007).

However, at a given concentration of NaCl, selected calli maintained higher growth rate than non-selected calli when grown on NaCl supplemented medium (Table-1). Similar to our findings, Kumar and Sharma (1989), Patil & Rao (1999) & Gulati & Jaiwal (2010) reported that NaCl tolerant lines maintained higher growth rate when compared to non selected calli in this species. Most of the previously published reports in legumes indicated that selected cell lines maintained higher rate of growth on saline media when compared to their wild type for example, Pea (Hassan & Wilkins, 1988; Olms, *et al.*, 1995) red gram (Krupanidhi & Rao,1996) and in other plants like tobacco (Hascgawa *et al.*, 1980) colt cherry (Ochatt & Power, 1989) rice (Basu, *et al.*, 2002; Kishor & Reddy, 1986) finger millet (Pius, *et al.*, 1993) sugar cane (Gandonou, *et al.*, 2006).

Serial No	NaCl ( $\mu\text{m}$ )	non- selected callus (mg)	selected callus (mg)	MDA (nmol g <sup>-1</sup> freshweight)	
				Non-selected callus (mg)	Selected callus (mg)
1	00 (Control)	778.0 $\pm$ 0.67 <sup>b</sup>	780.0 $\pm$ 0.45 <sup>b</sup>		
2	50	806.6 $\pm$ 0.34 <sup>a</sup>	865.3 $\pm$ 0.83 <sup>a</sup>	84.0 $\pm$ 0.75 <sup>c</sup>	82.6 $\pm$ 0.13 <sup>c</sup>
3	100	647.6 $\pm$ 0.97 <sup>c</sup>	740.0 $\pm$ 0.56 <sup>c</sup>	90.0 $\pm$ 0.86 <sup>c</sup>	82.3 $\pm$ 0.21 <sup>c</sup>
4	150	449.3 $\pm$ 0.53 <sup>d</sup>	614.6 $\pm$ 0.36 <sup>d</sup>	140.6 $\pm$ 0.54 <sup>b</sup>	88.0 $\pm$ 0.43 <sup>b</sup>

Data represents average of three replicates; each replicate consists of 10 cultures.

Mean  $\pm$  Standard error.

Mean followed by the same superscript in a column is not significantly different at P = 0.05 levels.

Table 1. Effect of NaCl on fresh weight and MDA content of selected and non-selected callus of *Vigna radiata* L.

### 3.3 Analysis of proline and protein contents

Proline and protein content in the selected and non-selected callus line is presented in table-2. Both protein and proline contents were significantly ( $p < 0.05$ ) affected with an increase in the concentration of NaCl. NaCl tolerant callus line maintained increased protein levels than control callus when exposed to 150 mM NaCl. Increased protein content in NaCl tolerant callus has been reported earlier (Chen, *et al.*, 2001; Queiros, *et al.*, 2007). Extra protein bands in *In Vitro* raised NaCl tolerant *V. radiata* plants have been reported by (Hassan, *et al.*, 2004). Similarly a gradual increase in the Proline content of tolerant callus line was noticed with an increase in the concentration of NaCl from 100 to 150 mM, maximum being at 150 mM NaCl level. (Kumar & Sharma, 1989; Patil & Rao, 1999) reported higher proline in selected calli when compared to non selected calli in this species. Accumulation of proline has been widely advocated for use as parameter of selection of cell lines for salt tolerance and it has been shown that proline over producing lines are more tolerant than their parent cultivars (Kavi Kishor *et al.*, 1995). Increased proline levels in NaCl tolerant callus has been earlier reported in many legumes like, soybean (Liu, & Van Staden, 2000) ground nut (Jain, *et al.*, 2001) and in other plants like tobacco (Wattad, *et al.*, 1983) canola (Jain, *et al.*, 1991 a,b; SashiMadan, *et al.*, 1995) tobacco (Gangopadhyay, *et al.*, 1997b) barley (Chaudhuri, *et al.*, 1997) *Citrus* (Piqueras, *et al.*, 1996) alfalfa (Petrusa, *et al.*, 1997) basmati rice (Basu, *et al.*, 2002) bamboo (Singh, *et al.*, 2003) chili (Venkataiah, *et al.*, 2004) sugarcane (Gandonou, *et al.*, 2006). On the contrary some workers did not observe any appreciable increase in free proline content (Dix & Pearce, 1981, Errabii, *et al.*, 2006; Jain, *et al.*, 1987). Higher proline levels in salt tolerant callus may be due to an increased rate of synthesis or a decreased rate of oxidation of this compound as suggested by Wyn-Jones & Gorham (1984). Patnaik & Dabata (1997) suggested that salinity induced proline accumulation could be due to putrescence oxidation. There are divergent reports concerning the role of proline in salt tolerance. High levels of proline in NaCl selected cells have been suggested as a factor conferring salt tolerance (Kumar & Sharma, 1989; Pandey & Ganapathy, 1985; Wattad *et al.*, 1983). In contrast proline accumulation has also been excluded as the mechanism of tolerance in some cases (Dix & Pearce, 1981; Errabii *et al.*, 2007; Tal *et al.* 1979). The significant levels of proline observed in our study supports the former. It is well known fact that proline stabilizes the structure and function of various macromolecules (Kavi Kishor, *et al.*, 2005; Rhodes, 1987; Smirnoff & Cumbes, 1989).

Serial No.	NaCl (µm)	Protein mg g <sup>-1</sup> FW		Proline mg g <sup>-1</sup> FW		Ascorbic acid mg g <sup>-1</sup> FW	
		Non-selected callus	Selected callus (mg)	Non-selected callus	Selected callus (mg)	Non-selected callus	Selected callus (mg)
1	00 (Control)	22.6 ± 0.43 <sup>c</sup>	27.2 ± 0.76 <sup>c</sup>	7.6 ± 0.53 <sup>c</sup>	8.8 ± 0.54 <sup>d</sup>	8.4 ± 0.44 <sup>a</sup>	22.9 ± 0.45 <sup>c</sup>
2	50	28.2 ± 0.87 <sup>b</sup>	42.0 ± 0.56 <sup>b</sup>	10.5 ± 0.19 <sup>b</sup>	18.2 ± 0.76 <sup>b</sup>	8.6 ± 0.35 <sup>a</sup>	26.5 ± 0.45 <sup>b</sup>
3	100	32.2 ± 0.56 <sup>a</sup>	47.0 ± 0.23 <sup>a</sup>	16.2 ± 0.65 <sup>a</sup>	28.2 ± 0.55 <sup>a</sup>	6.4 ± 0.87 <sup>b</sup>	42.7 ± 0.50 <sup>a</sup>
4	150	16.4 ± 0.32 <sup>d</sup>	19.2 ± 0.65 <sup>d</sup>	5.9 ± 0.25 <sup>d</sup>	12.2 ± 0.22 <sup>c</sup>	5.5 ± 0.43 <sup>c</sup>	27.1 ± 0.25 <sup>b</sup>

Data represents average of three experiments; Mean ± standard error Mean followed by the same super script in a column is not significantly different at P= 0.05 level.

Table 2. Effect of NaCl on protein, proline, and Ascorbic acid content in non-selected and selected callus on *Vigna radiata* L.

### 3.4 Lipid peroxidation

Oxidative damage to lipids was determined as lipid peroxidation in terms of amount of malondialdehyde (MDA). MDA content of tolerant callus was less when compared to that of sensitive callus line (Table-1). Sumithra, *et al.*, 2006) reported low levels of lipid peroxidation in salt tolerant *V. radiata* cv Pusa bold than in salt sensitive cv CO 4. Jain, *et al.*, (2001) reported 4 fold increases in MDA content of sensitive callus of ground nut when grown on saline medium where as in the tolerant callus line the increase was only 1.1 fold. The results indicate that NaCl tolerant callus maintained membrane integrity when growing in saline environment however sensitive calls line were unable to maintain membrane integrity under salinity stress resulting in decreased growth and metabolic imbalance. Changes in the cell wall have been shown to be important for salt adaptation (Binzel, *et al.*, 1985; Curz, *et al.*, 1992). Salt stress is known to result in extensive lipid peroxidation (Davenport, *et al.*, 2003; Hernandez, *et al.*, 2000; Khan & Panda, 2000; Kholova *et al.*, 2009; Queiros *et al.*, 2007). In the present investigations it was noticed that the extent of lipid peroxidation was higher in NaCl sensitive cell lines than NaCl adopted cell lines, such observations were also made in ground nut (Jain *et al.*, 2001) wheat (Sarin, *et al.*, 2005) eggplant (Yaser, *et al.*, 2006). It seems that due to low peroxidation in NaCl tolerant callus line, membrane integrity might have been maintained, thus preventing protein denaturation which is supported by the fact that NaCl tolerant callus lines had higher protein content. Lipid membranes are vulnerable targets for stress induced cellular damage and the extent of damage is commonly used as a measure of stress (Gadallah, 1999; Zhou, *et al.*, 1992).

### 3.5 Ascorbic acid content

Ascorbic acid content increased gradually in NaCl tolerant calli line than in the control line. An increase of about 18% was found in callus tissue grown at 50 mM NaCl, while in callus grown on medium supplemented with 100 and 150 mM NaCl ascorbic acid content



significantly increased to 30% and 59% respectively (Table-2). It was previously reported that the response of many plants cells to salinity is the increased synthesis of ascorbic acid. A positive correlation between ascorbic acid content and salinity was also reported in NaCl tolerant cell lines in several species (Gosset, *et al.*, 1996; Sarin, *et al.*, 2005; Qlmos & Hellin, 1996; Queiros, *et al.*, 2007). This antioxidant compound is one of the most effective free radical scavengers implicated in the adaptations of plants to stress (Shigeoka, *et al.*, 2002; Smirnoff, *et al.*, 2000; Queiros, *et al.*, 2007). It is reported that, high levels of endogenous ascorbate is essential to effectively maintain the anti-oxidant system that protects plants from oxidative damage due to abiotic stress (Shigeoka, *et al.*, 2002).

### 3.6 Antioxidant enzymes

NaCl stress affect plant processes that lead to the formation of reactive oxygen species (ROS) super oxide radical ( $O_2^-$ ) Hydrogen peroxide ( $H_2O_2$ ) and hydroxide radicals (OH). The ROS cause oxidative damage to membrane lipids, proteins and nucleic acid. Accelerated detoxification is fundamental in development of tolerance to NaCl. To control the levels of ROS and protect the cells from injury under stress conditions, it is important that ROS should be scavenged.

Plants protect cells and sub cellular systems from the effects of reactive oxygen species (ROS) by enzymes such as Super oxide dismutase (SOD) Ascorbate peroxidase (APX) and Catalase (CAT). In the present investigation it was noticed that NaCl significantly altered the activities of SOD, APX & CAT with an increase in the levels of NaCl from 50 to 150 mM NaCl level. Maximum activity of these enzymes (Table-3) was noticed in callus tissue tolerant to 150 mM NaCl, where as in sensitive callus the activity of these enzymes was highly inhibited.

Serial No.	NaCl ( $\mu$ m)	SOD (EU mg <sup>-1</sup> protein)		APX (EU mg <sup>-1</sup> protein)		CAT (EU mg <sup>-1</sup> protein)	
		Non-selected callus (mg)	Selected callus (mg)	Non-selected callus (mg)	Selected callus (mg)	Non-selected callus (mg)	Selected callus (mg)
1	00 (Control)	40.0 $\pm$ 0.76 <sup>b</sup>	40.6 $\pm$ 0.34 <sup>c</sup>	0.95 $\pm$ 0.23 <sup>b</sup>	0.88 $\pm$ 0.67 <sup>d</sup>	22.3 $\pm$ 0.56 <sup>a</sup>	22.9 $\pm$ 0.41.6 <sup>c</sup>
2	50	42.8 $\pm$ 0.45 <sup>a</sup>	50.5 $\pm$ 0.56 <sup>b</sup>	1.40 $\pm$ 0.12 <sup>a</sup>	2.10 $\pm$ 0.56 <sup>b</sup>	20.9 $\pm$ 0.75 <sup>b</sup>	26.5 $\pm$ 0.45 <sup>b</sup>
3	100	38.2 $\pm$ 0.36 <sup>c</sup>	75.8 $\pm$ 0.65 <sup>a</sup>	0.71 $\pm$ 0.34 <sup>c</sup>	2.94 $\pm$ 0.67 <sup>a</sup>	16.4 $\pm$ 0.67 <sup>c</sup>	42.7 $\pm$ 0.50 <sup>a</sup>
4	150	32.6 $\pm$ 0.65 <sup>d</sup>	50. 6 $\pm$ 0.48 <sup>b</sup>	0.58 $\pm$ 0.56 <sup>b</sup>	1.80 $\pm$ 0.56	12.6 $\pm$ 0.45 <sup>d</sup>	27.1 $\pm$ 0.25 <sup>b</sup>

Data represents average of three experiments. Mean  $\pm$  standard error.

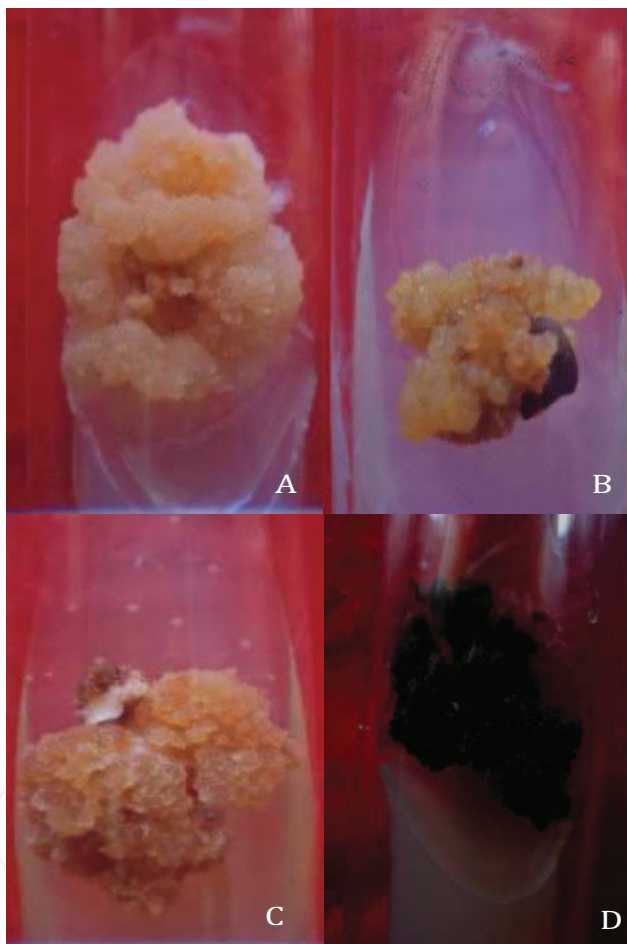
Mean followed by the same superscript in a column is not significantly different at P= 0.05 levels.

Table 3. Effect of NaCl on antioxidant enzymes in non-selected and selected callus in *Vigna radiata* L.

Serial No.	Growth regulators (mg/l)	Frequency of shoot induction (%)	Number of shoot buds per explants.	Average length of plant let (cm)
1	BAP 0.5	0.0	00	00
2	BAP 1.0	21.66 ± 1.6 a	2.90 ± 0.16 a	3.8 ± 0.25 a
3	BAP 2.0	26.00 ± 1.4 b	8.20 ± 0.16 b	5.8 ± 0.34 b
4	BAP 2+NAA0.25	30.10 ± 1.8 c	12.80 ± 0.34 c	7.2 ± 0.55 c
5	BAP 2 +NAA 0.5	40.13 ± 1.8 d	14.80± 0.64 d	8.6 ± 0.68 c

Data represents average of three experiments. Mean ± standard error. Mean followed by the same superscript in a column is not significantly different at P= 0.05 levels.

Table 4. Effect of growth regulators on Frequency, Number of shoots, and mean shoot length from cotyledon derived callus in *Vigna radiata*.

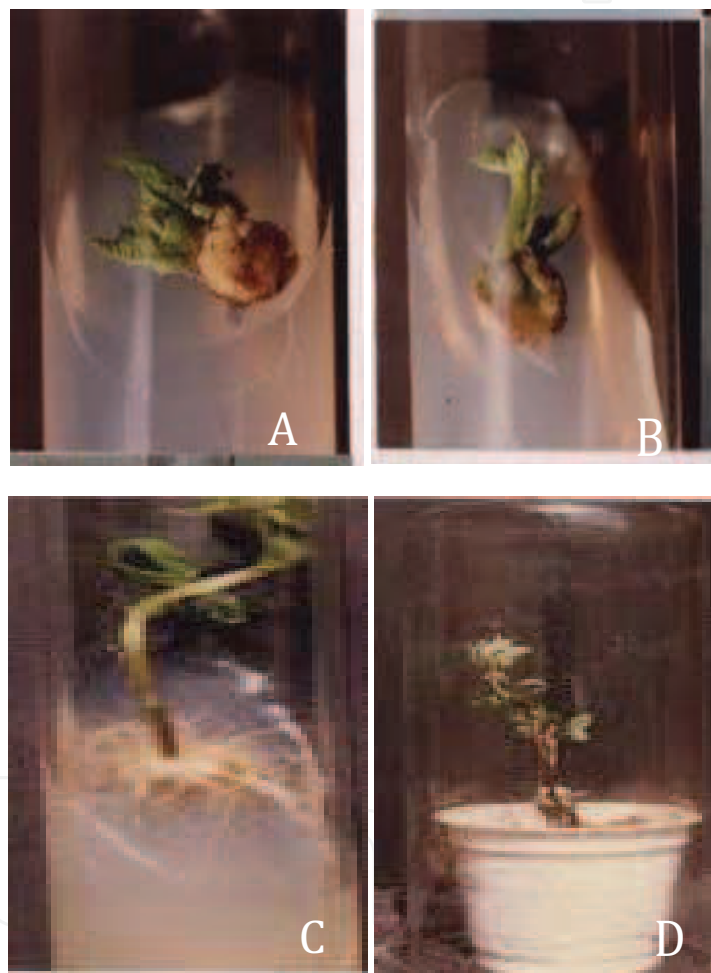


- A) Callus on NaCl-free medium.
- B) Surviving callus on 150mM NaCl.
- C) NaCl-tolerant callus growing on 150mM NaCl.
- D) NaCl sensitive callus on 150mM NaCl supplemented medium (Note complete darkening and death of Callus).

Bar  =1 cm

Fig. 1. Photographs showing effect of NaCl on callus cultures of *Vigna radiata*

SOD, APX and CAT are considered as useful enzymes to help plants defend salt stress (Koca, *et al.* 2006, Shen, *et al.*, 2002). Sumithra *et al.*, (2006) have reported increased SOD and CAT activity in NaCl tolerant cultivars in this species. There are previous reports indicating increased activity of SOD in pea (Hernandez, *et al.*, 1993) wheat (Sairam, *et al.*, 2003) cotton (Gossett, *et al.*, 2004) potato (Queiros, *et al.*, 2007) sweet potato (He, *et al.*, 2008). Apart from SOD activity considerable increase in the activity of the enzymes APX and CAT was noticed in tolerant callus line than in sensitive callus when grown on different concentrations of NaCl. increase in the activities of SOD, CAT, APX in callus tissue raised from NaCl tolerant callus in cotton than in plants obtained from sensitive callus was reported by Gossett, *et al.*, (2004). Elkahoui, *et al.*, (2004) reported increased APX activities in NaCl adopted cell lines in periwinkle and Mittova, *et al.*, (2002) in salt tolerant wild tomatoes.



- A) Initiation of multiple leafy shoot buds on MS +2 mg/l BAP + 0.5 mg/ NAA
- B) Further elongation of shoot after 25 days of culture
- C) Rooting of the shoot on MS + 1 mg/l IBA.
- D) A potted plant in a polycup containing 3 : 1 garden soil and sand.

Fig. 2. Photographs showing organogenesis from NaCl tolerant callus

Catalase is a common enzyme found in all living organisms which are exposed to oxygen, where it functions to catalyze the decomposition of  $H_2O_2$  to water and oxygen (Chelikani, *et*

al., 2004).  $H_2O_2$  is a harmful by product formed as a result of stress conditions to prevent damage it must be quickly converted into less dangerous substance like gaseous oxygen and water molecules. Hence the role of catalase is important in reducing stress related damages. Willekens, et al., (1997) suggested that the function of catalase in the cell is to remove the bulk of  $H_2O_2$  where as peroxidase would be involved mainly in scavenging  $H_2O_2$  that is not taken by catalase. In the present investigations it was noticed that both catalase and peroxidase activity increased in NaCl tolerant callus. From this observation it can be inferred that catalase/peroxidase might have acted co-operatively to remove  $H_2O_2$  at a minimum expense of reducing power.

### 3.7 Regeneration of plantlets from NaCl selected and non selected callus

For regeneration of plantlets calli pieces approximately  $250 \pm 10$  mg were transferred to MS medium supplemented with 0.5-2 mg/l BAP alone or in combination with 0.25 and 0.5 mg/l NAA. On MS medium without growth regulators and 0.5 mg/l shoot bud initiation was not observed; however on MS medium supplemented with 1 & 2 mg/l BAP shoots were initiated and the frequency of shoot formation was 21.66 and 26.00% respectively. Supplementing NAA at 0.25-0.5 mg/l further enhanced the frequency of shoot formation increased to 30.10 & 40.13% respectively.

Regeneration from various seedling explants viz shoot tip, cotyledons, cotyledonary node of mung on BAP supplemented medium has been reported earlier (Mathews, 1987; Gulati & Jaiwal, 1992; Kaviraj & Rao, 2009; Mendoza, et al., 1999). There is only one report of regeneration from callus cultures in mungbean (Rao, et al., 2005), similarly plantlets have been regenerated from NaCl tolerant callus in many species like *Citrus sinensis* (Ben-Hyyim & Goffer, 1989) *Brassica juncea* (Jain, et al., 1991; Kerthi, et al., 1989; Sashimadan, et al., 1995) *Triticum durum* (Zair, et al., 2003) *Ipomea batatas* (He, et al., 2009) In the present study however, it was noticed that the formation of shoots from NaCl tolerant callus was lower (14.00% than that from NaCl-non selected calli (40.13%) and the number of multiple shoots per callus piece was reduced to 3.33 from 14.80, a phenomenon reported earlier by few workers (Basu, et al., 1997; Chen et al., 2001; Ochatt, et al., 1999; Queiros, et al., 2007; Zair et al., 2003).

## 4. Conclusions

In conclusion NaCl stress induces severe oxidative stress in mungbean callus where the antioxidant defense system seemingly fails to combat with the stress induced oxidative damage. However it is possible to select callus line tolerant to elevated levels of NaCl stress by sudden exposure to high concentration of NaCl, accordingly a NaCl tolerant cell line was selected from cotyledon derived callus of mung bean which proved to be a true cell line variant. It can also be concluded that, the salt tolerant cell line could overcome the adverse effect of NaCl and maintained better growth on NaCl supplemented medium when compared to salt sensitive cell lines (controls). This conclusion is based on the following observations (a) cells which have been removed from the selection pressure for at least four passages retained tolerance to NaCl after transferring to NaCl (150 mM) medium (b) High accumulation of proline was noticed in salt tolerant cell lines compared to salt sensitive cell lines. (c) The activity of anti-oxidant enzymes was high. The results presented in this paper suggest that the mechanism of enhanced tolerance in mungbean callus is via improved

synergistic and protective effects of antioxidant enzyme like SOD, APX and CAT. It is also concluded that the callus lines were able to grow in elevated concentration (150mM NaCl) by maintaining membrane integrity, high levels of protein and proline, which supports the hypothesis that proline plays a significant role in protecting the cells from oxidative stress as reported in other grain legumes like chickpea and pigeonpea.

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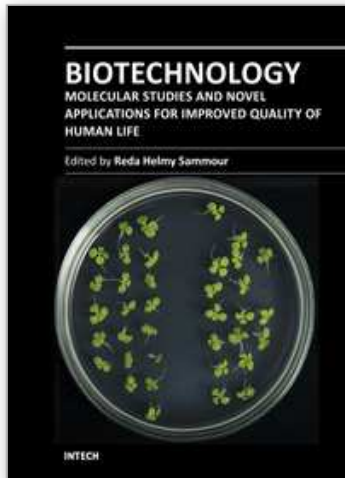
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