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Assessment of Salinity Tolerance of *Vigna mungo* Var. Pu-19 Using *ex vitro* and *in vitro* Methods

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Abstract: The *ex vitro* and *in vitro* response of *Vigna mungo* PU-19 to salinity stress was assessed on the basis of germination percentage, seedling parameters and the pattern of response under *in vitro* conditions. *Ex vitro* assessment was done by treating healthy seeds with different concentrations of NaCl for 24 h and germinating them on moist blotting paper in Petri dishes in three replicates. With increase in the concentration of NaCl the seedling length was decreased and above 2.5% NaCl, negligible growth was obtained. Protein concentration and GST activity was measured in one week old seedlings by Bradford dye-binding assay and Habig's method, respectively. Both increased in the seedlings, almost according to the increase in NaCl concentration. In the *in vitro* selection system NaCl was added to the optimum growth medium and response of cotyledonary node and node of seedlings was observed. Callusing was the main response with plantlets from cotyledonary node at 0.4% NaCl and embryogenic callus from node explants in 0.4% NaCl.

Key words: Saline tolérance, tissue culture, NaCl, protein, callus, *Vigna mungo*, NAA, BAP

INTRODUCTION

The productivity of crops depends on its complex interaction with climate and soil. Soil salinity is one of the major problems in arid and semi arid regions of the world, which hampers the agriculture output by lowering the yield of various crops. Plants resort to many adaptive strategies in response to abiotic environmental stresses such as high salt, dehydration, cold, heat and osmotic stress which affect plant growth (Shu *et al*., 2005). Although, salinity resistance is a complex trait resulting from the interaction of several morphological and physiological properties, it should be possible to select salt resistant cell lines with the hope to regenerate valuable plant material which could be successfully integrated in a breeding scheme. Broadly, salinity can be dealt with using technological advances in water and soil management, irrigation methodology and/or through biological approaches such as breeding of resistant varieties or cultivation of naturally salt tolerant crops. Conventional plant breeding methods have met with limited success in developing varieties tolerant to salt stress. Tissue culture approach involving *in vitro* selection of salt tolerant cell lines has been reported in a large number of plants viz; rice, alfalfa, *Pennisetum purpureum*. Shaterian *et al*. (2008) evaluated phenotypic and physiological responses as

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indicators of salt tolerance in diploid potato clones (*Solanum tuberosum* L. x wild relatives) utilizing a hydroponic sand-based system. Carretero *et al.* (2007) studied the *in vitro ex vitro* salt (NaCl) tolerance of cassava (*Manihot esculenta* Crantz) plants and obtained a correlation between *in vitro* and *ex vitro* behavior of the cassava plant regarding salt tolerance, which would allow the *in vitro* culture method to be used for selection of salt-tolerant plants.

Various halophytes accumulated compatible solutes, such as glycinebetaine and proline, when they were subjected to salinity stress. The solutes are thought to work as osmoregulators in the cells and as antioxidants, which relieve oxidative stress. Furthermore, a positive correlation between the solute amounts in the cells and tolerance to NaCl has been reported (Marcum and Murdoch, 1994; Heuer and Nadler, 1998; Girija *et al*., 2002). Prajuabmon *et al.* (2009) investigated the effect of salt stress on some physiological and biochemical characteristics in three rice cultivars differing in salt-tolerance ability (*Oryza sativa* L. cvs. Pokkali, Leuang Anan and KDML105) using *in vitro* methods. The results showed that all three cultivars of rice seedlings grown under high salinity had shoot and root length, fresh and dry weight of shoot and relative growth rate of shoot decreased, whereas the Na+ /K+ ratio and proline content of leaf were increased.

Vigna mungo is an important crop of the Indian agriculture as an important source of income and nutrition. It contains high protein content (25-32%). Though *Vigna mungo* is a salt tolerant plant species, variation in salt tolerance among the cultivars due to the continuous testing of tolerance has become has became a necessity and can be done by both *ex vitro* and *in vitro* method. Selection agents as NaCl for salt tolerance are added to the petridish or basal nutrient medium at different levels. Seeds or explants sustaining such environment will only survive. These can be selected as mutant/variant and allowed grow or to regenerate into complete plantlets. The objective of our investigation was to compare the *ex vitro* and *in vitro* response of *Vigna mungo* Var. PU-19 and on the basis of the pattern of response obtained, establish a simple method for assessment of salt tolerance in this species.

MATERIALS AND METHODS

The investigation was carried out in the *in vitro* Culture and Plant Genetics Laboratory in the Department of Botany, Lucknow University, Lucknow in the summer of 2008 and it took one year to complete the experiment in replicates and also check the repeatability of results. The seeds were procured from the Indian Institute for Pulse research, Kanpur.

In order to screen the control seeds of *Vigna* for NaCl tolerance, the seeds were grown in petridishes, using blotting paper method. Various concentrations of NaCl were prepared and healthy seeds were treated with NaCl for 24 h. Then the seeds (untreated and NaCl treated) were inoculated on blotting paper in the petridishes. Readings were taken after 2 and 10 days. In order to avoid infection, the blotting paper was changed every 2 days. Various parameters used for ascertaining salt tolerance and its mechanism were percent germination after 2 and 10 days, seedling length and protein concentration (Bradford, 1976) and GST activity.

Protein Extraction

The seedlings were used after one week of germination for protein extraction. The extracted protein was used for protein estimation and GST enzyme assay. One gram of seedlings of the control and treated plants were ground in liquid nitrogen and homogenized in double volumes (w/v) of buffer solution (0.2 M Tris-Cl pH 7.8, 1 mM EDTA, 20% glycerol

and 2 mM PMSF). The homogenate was centrifuged at 14,000 rpm for 20 min. The supernatant was used for protein estimation and enzyme activity assays.

Protein Estimation

Protein was estimated according to the Bradford dye-binding assay (Bradford, 1976). Bovine Serum Albumin (BSA) was used as the standard. To perform the assay, 2.5-10 μL plant protein extract was diluted to 50 μL with water and mixed with 2.5 mL of Bradford dye. The mixture was allowed to stay at room temperature for 5 to 10 min. Then optical density was read at A_{595}. First, the standard graph was plotted between A_{595} and the known amounts of BSA. Thereafter, the A_{595} of unknown plant protein samples was observed. The A_{595} of plant protein samples was compared with A_{595} of BSA of known quantities. The protein concentration was expressed in μg protein g^{-1} of the extract.

GST Assay

The GST activity was determined spectrophotometrically according to Habig *et al.* (1974), with slight modifications. The assay utilizes CDNB and GSH as substrates. The GST enzyme reaction yields the DNP-GS complex which absorbs at 340 nm. This principle is used to perform the assay. The final *in vitro* assay mixture consisted of 50 mM phosphate buffer pH 6.5, 1 mM CDNB, 1 mM GSH, 0.5 mM EDTA and the green leaves extract containing 100 μg proteins. The protein extract was added after incubating the reaction mix at 37°C for 5 min. The final volume of the reaction mixture was made up to 2.5 mL with water. The reaction was monitored spectrophotometrically at 340 nm. The A_{340} at 0 and 5 min was recorded to calculate ΔOD_{340}. The GST activity was calculated in μM/mg/min as the measure of DNP-GS complex formed.

Effect of Salt on Seeds of *Vigna mungo*

Seeds collected from homogenous population of *Vigna* were grown on Murashige and Skoog's basal medium after thorough washing with autoclaved water, 70% alcohol and 0.1% mercuric chloride. The explants obtained after 10 days of inoculation, were inoculated on MS selection medium supplemented with different combination of cytokinin and auxin. The selection medium contained varying concentrations of NaCl (0.10, 0.20, 0.40, 0.50 and 1.0%). The readings were taken at regular intervals.

Collection of Data

Morphogenetic response has been represented in terms of: Percentage of explants that produced callus, roots or shoots, number of roots per culture, number of shoots per culture, length of roots per culture, length of shoots per culture.

RESULTS

In order to screen the control seeds for NaCl tolerance, they were grown in petridishes. Concentrations of NaCl in range 0.5-5.0%, were used for the treatment of seeds for 24 h. The percentage of germination decreased with increase in salt concentration and germination was observed till 2.5% of salt, above this no germination took place. However, 50% of the seeds germinated at 2.0% of salt and the length of seedling decreased with increase in the concentration of salt, the maximum seedling length was observed for untreated seed and the minimum for 2.5% concentration of salt (Table 1, Fig. 1A, B). In general, it was seen that percent germination, seedling length decreased with increase in salt concentration. The

Table 1: Effect of various concentrations of the salt on morphological and biochemical parameters of *Vigna mungo* var.PU-19

Concentration of salt (%)	Germination (%)	Seedling length (cm)	Total protein (mg g^{-1})	GST activity (μm/mg/mL/min)
0.00	100.0	12.64	2.20	0.305
0.05	100.0	12.26	2.40	0.814
0.10	100.0	10.56	2.93	0.713
0.15	100.0	9.46	2.74	0.629
0.20	91.6	7.66	3.00	0.509
0.25	90.9	7.60	3.32	0.389
0.30	90.9	7.44	2.60	0.342
0.35	90.9	7.40	2.90	0.293
0.40	81.8	5.68	3.20	0.452
0.45	81.8	4.84	2.80	0.444
0.50	100.0	4.34	2.40	0.352
1.00	81.8	3.38	2.40	0.257
1.50	91.1	1.86	1.33	0.250
2.00	63.6	0.52	-	-
2.50	25.0	0.30	-	-

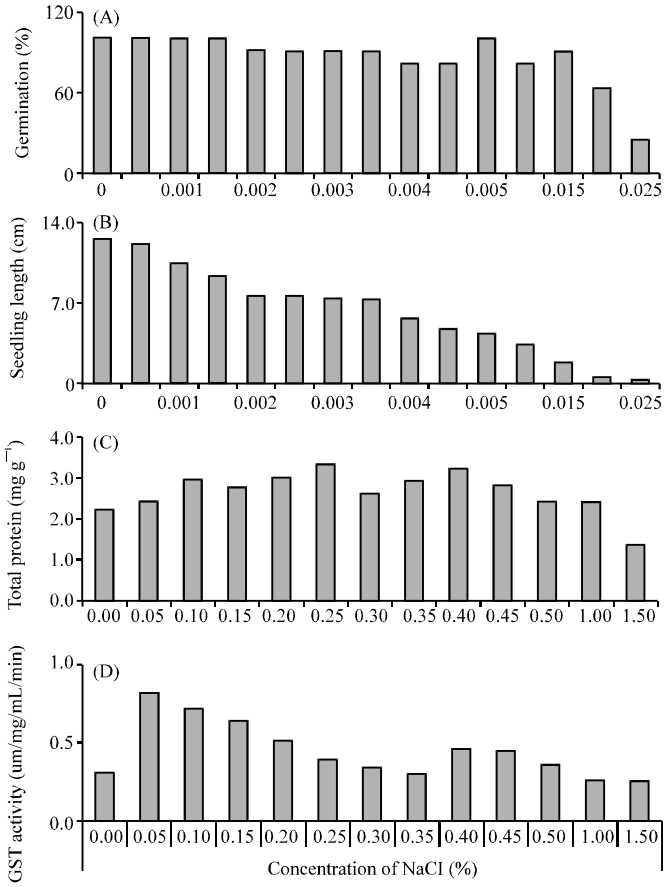

Fig. 1: (A-D) Effect of various concentrations of the salt on morphological and biochemical parameters of *Vigna mungo* var. PU-19

decrease was more prominent at the beginning, which progressively became less prominent during subsequent days of germination at all salinity levels. Furthermore, with increasing salt

Table 2: Effect of different concentration of salt on different explants obtained from control seedlings of *Vigna mungo* var. PU-19 on MS medium supplemented with NAA (1.5 mg L^{-1}) and BAP (1.5 mg L^{-1})

				No. of root		Length of root (cm)		No. of shoots		Length of shoots (cm)	
Explants	Percentage of NaCl in medium	Type of response	Callus wt. (g)	Range	AM±SE	Range	AM±SE	Range	AM±SE	Range	AM±SE
Cotyledonary	0	C	2.1	-	-	-	-	-	-	-	-
node	0.1	C+R	1.61	4-9	6.43±0.65	0.5-1.5	0.96±0.15	-	-	-	-
	0.2	C+R+PL	1.51	4-8	6.29±0.57	0.5-1.2	0.9±0.1	2-4	3.14±0.34	1.2-4.0	3.03±0.45
	0.4	C+R+PL	1.38	3-7	5.14±0.51	0.5-2.0	1.33±0.18	3-5	4.14±0.34	2-5	3.33±0.44
	0.5	C+R	0.89	3-6	4.29±0.42	0.4-1.8	0.96±0.19	-	-	-	-
	1	C	0.66	-	-	-	-	-	-	-	-
Node	0	C	1.17	-	-	-	-	-	-	-	-
	0.1	C+R	1.62	4-12	8±0.95	1.5-2.0	1.49±0.16	-	-	-	-
	0.2	C	1.45	-	-	-	-				
	0.4	EC	1.88	-	-	-	-	-	-	-	-
	0.5	C+R	1.73	2-8	4.71±0.71	0.2-1.6	1.11±0.21				
	1	C	0.75	-	-	-	-	-	-	-	-

C: Callusing, R: Roots, EC: Embryogenic Callus, PL: Plantlet

concentration the germination of seeds decreased progressively. The seedling vigour (length of root and shoot) increased gradually with 1-5 days of seed germination under the conditions of absence (control) and presence of various levels of salinity. However, salinity treatment resulted in decrease in the root and shoot length as compared to control values.

Protein Concentration (mg g^{-1})

Protein estimation was done in seedlings tissue. It increased with increased concentration of salt upto 1% and was highest at 0.25% and the least at 1.5% NaCl (Table 1, Fig. 1C).

GST Activity µm/mg/mL/min

GST activity at most salt treatments was higher than control. The maximum was observed at 0.05%, while, the minimum was at 1.5% concentration of the salt (Table 1, Fig. 1D).

Out of these concentrations of NaCl, on the basis of similar results the number of concentrations was reduced to 0.10, 0.20, 0.40, 0.50 and 1.0% for the study of *in vitro* morphogenetic response. Seeds were inoculated in Basal MS medium and after a week the seedling explants were cultured on MS medium supplemented with NAA and BAP (each 1.5 mg L^{-1}) and aforementioned concentrations of the salt. Two explants viz. cotyledonary node and nodal explants, showed good response with various combinations of growth regulators and were therefore used as explants (Table 2).

Cotyledonary Node as a Source of Explants

Cotyledonary nodes showed best response in Murashige and Skoog's medium supplemented with NAA and BAP. The response was 100% in the form of callusing. The callus was creamish and soft. The initiation of callus took 3 days.

Cotyledonary nodes in MS medium containing 0.2 to 0.4% sodium chloride showed similar response in the form of callus formation in 30% explants, only root formation in 20% explants and plantlet formation in 50% explants (Fig. 2A-J, 3A-J). The growth of callus was slow and it took 7 days for initiation of callus. The callus was light brown in colour.

At 0.1 and 0.5% NaCl the pattern of response was same i.e., callus and rhizogenesis. But the percentage of response at 0.1% NaCl containing medium was more than 0.5% NaCl containing medium (Fig. 2G, J).

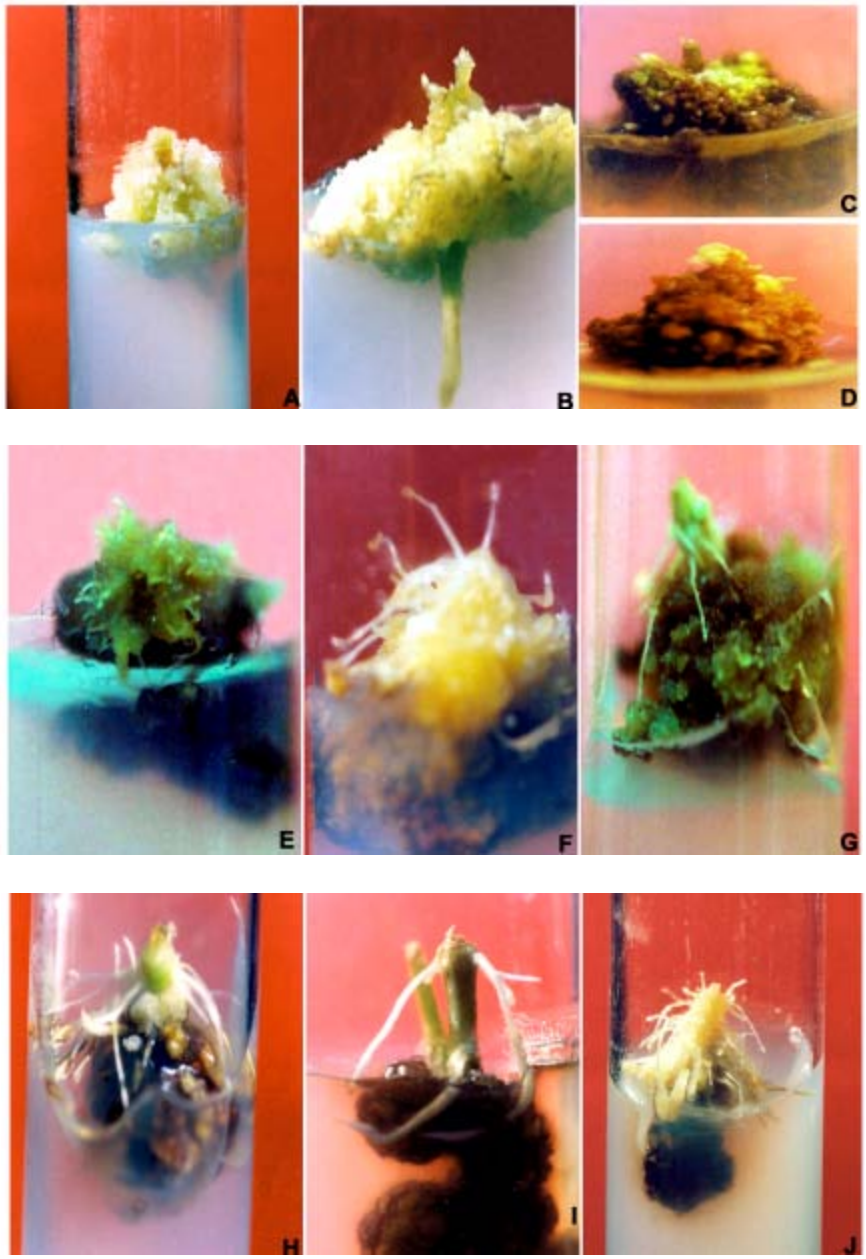

Fig. 2: A: Creamish callus at control from cotyledonary node explants, B: Creamish callus at control from nodal explants, C: Brown callus from cotyledonary node at 1% NaCl D: Brown callus from nodal explants at 1% NaCl, E and F: Embryogenic callus from nodal explants at 0.4% NaCl, G: Callus and rooting from cotyledonary node at 0.5% NaCl, H: Rooting from nodal explants at 0.1% NaCl, I: Rooting from nodal explants at 0.5% NaCl and J: Rooting from cotyledonary node explants at 0.1% NaCl

Fig. 3: A: Rooting from cotyledonary node explants at 0.2% NaCl, B-D: Shooting from cotyledonary node explants at 0.2% NaCl, E-F: Shooting from cotyledonary node explants at 0.4% NaCl and H-J: Plantlet from cotyledonary node explants at 0.2% NaCl

At 1.0% NaCl, callus initiation took place only after 20 days of inoculation with 30% response. The callus was formed only along the margins of the cotyledonary node explants. The callus was dark brown and very hard (Fig. 2C).

Callus formation was dominant response because it was observed in control as well as all the NaCl treatments. Callus of cotyledon was soft in 0.0, 0.1 and 0.2% NaCl and hard at 0.4, 0.5 and 1.0% NaCl concentration. The amount of callus formed was more in 0.0% NaCl in comparison to NaCl containing medium.

Node as a Source of Explants

The 100% response in the form of callus and roots was obtained in MS medium with 0.0, 0.1, 0.4 and 0.5% salt. Initiation was obtained within 6-15 days. The 4-12 roots were obtained at 0.1% NaCl with initiation starting on 4th day. Almost similar response (2-8 roots) was obtained at 0.5% NaCl although the growth was comparatively slow with initiation observed on 10th day of inoculation. In both the percentage response was 70 and 50%, respectively (Fig. 2H, I).

Callus formation was the dominant response. At 0.0% NaCl (control) creamish and soft callus was formed (Fig. 2B), however at 0.4% NaCl embryogenic callus formation was observed and proembryogenic cells were seen in histological preparation (Fig. 2E, F).

At 0.2 and 1% NaCl the response was slow with initiation starting on 12th day. Delayed callus formation was observed it was brown and hard, the percentage response was only 50 and 20%, respectively.

All these observations suggest that explants obtained from seedlings growing on Murashige and Skoog's basal medium showed varied response. Callusing was observed in cotyledonary as well as nodal explants while direct plantlet formation was observed in cotyledonary explants and embryogenic callus was observed only by nodal explants. Both seedling explants at higher concentration i.e., 0.4% NaCl gave the best response. *Ex vitro* experiments had revealed enhanced the protein content and GST activity at the same NaCl concentration. It is possible that a similar increase is helping the explants to overcome salt stress under *in vitro* conditions.

As is clear from the histogram (Fig. 4), in the cotyledonary explants, the percentage of response was higher at lower concentrations of salt. The concentrations 0.0 and 1.0% showed callus formation, while callus with roots was seen at 0.1, 0.2, 0.4 and 0.5%. The maximum root length as well as maximum callus weight was, 1.33±0.18 cm and 1.61 g, respectively, at 0.4 and 0.1% of the salt. The plantlets were formed at 0.2 and 0.4% of salt concentrations, its growth rate was better at 0.4%.

In the nodal explants, the percentage of response ranged from 20 to 100%. Callus and roots formed at 0.1 and 0.5% salt and the average root length was slightly more at 0.1% (1.49±0.16) in comparison to 0.5% (1.11±0.21) concentration of salt and the maximum callus weight, 1.88 g was obtained at 0.4% of salt.

DISCUSSION

In general, it was seen that percent germination, seedling length decreased with increase in salt concentration. Misra and Dwivedi (2004) studied the effect of salinity levels on green gram. It was observed that in the absence of salinity almost 100% germination was observed from day 1 onwards. However, in the presence of salinity the seed germination decreased. The decrease was more prominent at the beginning, which progressively became less prominent during subsequent days of germination at all salinity levels. Furthermore, with

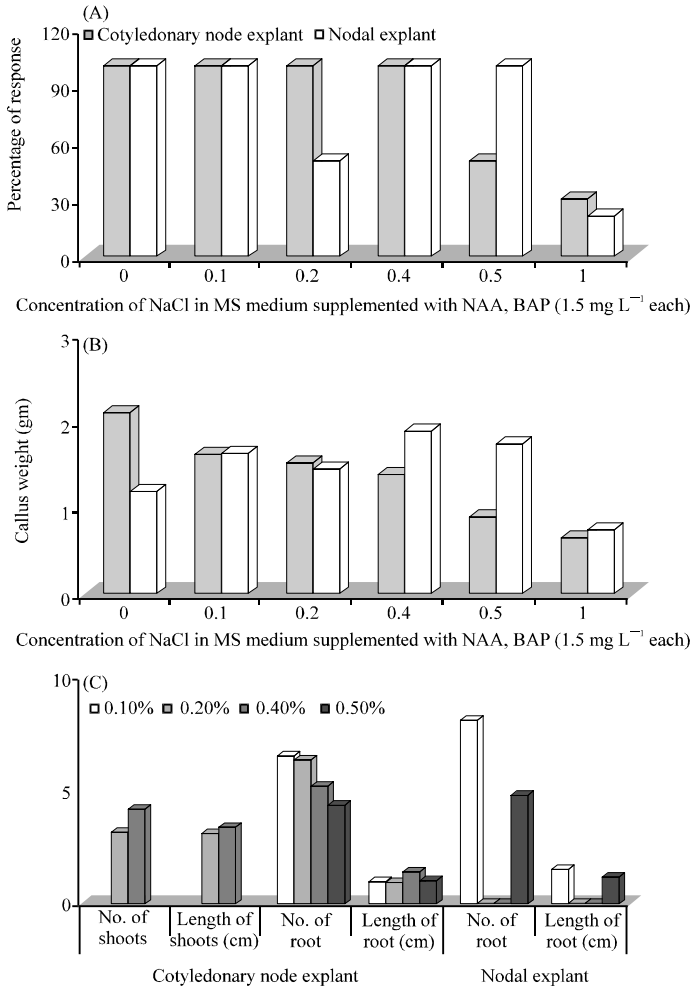

Fig. 4: Graphical representation of different growth parameters from seedling explants of *Vigna mungo* var. PU-19 in MS medium with 1.5 mg L^{-1} NAA, BAP and NaCl

increasing salt concentration the germination of seeds decreased progressively. The seedling vigour (length of root and shoot) increased gradually with 1-5 days of seed germination under the conditions of absence (control) and presence of various levels of salinity. However, salinity treatment resulted in decrease in the root and shoot length as compared to control values. Inhibition of germination due to salinity has been reported earlier (Ghoulam and Fares, 2001; Niazi *et al*., 1992). It is suggested that decrease in seed germination and depression in seedling vigour under saline stress is attributed to decrease in water uptake followed by limited hydrolysis of food reserves from storage tissues as well as due to impaired translocation of food reserves from storage tissue to developing embryo axis (Ghoulam *et al*., 2002; De-Lacerda *et al*., 2003).

The protein content increased with increased concentration of salt upto 1% and was highest at 0.25%. Similar variations in protein content has been reported by various workers in relation to salt stress. The crude protein content of both shoots and roots of the three

green bean cultivar was markedly lower under stress condition compared with control (Pessarakli *et al*., 1989). The impaired protein synthesis by other green cultivars such as red kidney beans (Frota and Tucker, 1978) and other types of plants such as barley (Helal and Mengel, 1979) cotton (Pessarakli and Tucker, 1985) alfalfa (*Medicago sativa* L., (Pessarakli and Huber, 1991), corn (Morilla *et al*., 1973) Pea (Kahane and Poljakoff, 1968) wheat (Abdul kadir and Paulen, 1982) tobacco (Ziono *et al*., 1967) have been reported previously by many investigators. In these studies, either decreased amino acid incorporation in to protein or the reduction in polyribosome levels due to salt stress was reported as the reason for the depressed protein synthesis by plants this may be reason for reduction in protein synthesis by plant. Ashraf (1989) worked on the effect of NaCl on water relations, chlorophyll and protein and proline contents of two cultivars of blackgram (*Vigna mungo* L.). The physiological basis of salt tolerance of two cultivars of blackgram, cv Candhari Mash (relatively salt tolerant) and cv Mash 654 (salt sensitive), was assessed in salinized sand culture at the flowering stage. Leaf protein and proline content was increased as a result of increasing salt concentration in both cultivars. High salt concentrations had no significant effect on the seed protein content of both cultivars.

Callus formation was the dominant response. At 0.0% NaCl (control) creamish and soft callus was formed (Fig. 2B), however at 0.4% NaCl embryogenic callus formation was observed and proembryogenic cells were seen in histological preparation (Fig. 2E, F). Similarly, Srivastava and Khare (2004) reported somatic embryogenesis under salt stress in *Justicea gendarussa*

Callusing was observed in cotyledonary as well as nodal explants while direct plantlet formation was observed in cotyledonary explants and embryogenic callus was observed only by nodal explants. Both seedling explants at higher concentration i.e., 0.4% NaCl gave the best response. *Ex vitro* experiments had revealed enhanced protein content and GST activity at the same NaCl concentration. It is possible that a similar increase is helping the explants to overcome salt stress under *in vitro* conditions. Gradual increase in the NaCl concentration in the growth medium lead to the development of salt-resistant lines capable of growing well even on 2.5% NaCl (Gosal and Bajaj, 1984). Using cell culture methods, salt tolerant cell lines have been developed and isolated in *Nicotiana* (Nabors *et al*., 1980), *Lycopersicon* (Meredith, 1978), *Medicago* (Croughan *et al*., 1978) and *Capsicum* (Dix and Pearce, 1981).

Selection of variants from salt sensitive callus elicit changes in gene regulation present already in the genomic makeup of the original plant (Winicov, 1991; Winicov and Button, 1991). Such changes are the result of an increase or decrease in gene expression as a result of selection for salt tolerance in tissue culture (Winicov *et al*., 1989). Cellular salt tolerance has been observed to be an inherited trait in tobacco although the plants themselves did not show improved salt tolerance (Bressan *et al*., 1987). In alfalfa, cellular salt tolerance often but not always correlates with the whole plant salt tolerance (McCoy, 1987 a, b). The regenerated plants from salt tolerant calli of *Brassica juncea* indicate acquisition of tolerance at the whole plant level (Jain *et al*., 1990).

Jaiwal *et al*. (1990) obtained salt tolerant lines from cotyledonary explants of *Vigna radiata*. NaCl not only effected the amount of callus produced at the proximal end of explants cultured on media supplemented with 25 to 200 mol m^{-3} NaCl, but also delayed the differentiation of shoots by 7-10 days. With increasing NaCl concentrations, number and mean length of shoots per explant decreased significantly. Cruz *et al*. (1999) work on tomato cultivars showed that the callus fresh weights were reduced by salinity, where as the free amino acids contents increased.

Pandey and Ganapathy (1985) have reported successful development of callus lines tolerant to NaCl stress in *Cicer arietinum*. A stable NaCl tolerant callus line was developed and fresh and dry weight was found to increase with increase in salinity. Thus, from above discussion it is observed that tolerant explants show increase in fresh and dry weight as compared to the salt-sensitive lines.

Recent reports have suggested that increase in leaf protein concentration with increasing concentration of salt is directly associated with stress tolerance. It has been also reported that salt stress favorably affects the vegetative growth and induced somatic embryo formation.

It can be concluded that the *in vitro* technique in connection with *ex vitro* technique is effective in assessment of salt tolerance. It is an efficient method to identify salt adapted genotypes within limited environmental conditions, that is, space and time period.

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