African Journal of Biotechnology Vol. 10(36), pp. 6947-6953, 18 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.777
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effect of NaCl on *in vitro* plant regeneration from embryogenic callus cultures of 'cv IR 64' *indica* rice (*Oryza sativa* L.)

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Accepted 2 June, 2011

In vitro **experiments were conducted to assess the effect of salt stress on callus induction, survival, fresh weight, regeneration, proline level and total protein content in salt sensitive indica rice cv. IR 64. For callus induction and regeneration, seeds and calli were subjected to 25, 50, 75 and 100 mM NaCl mediated salt stress which caused significant reduction in proliferation when compared to the control. Gradual reduction in regeneration was observed with increasing salt concentrations (25 to 100 mM). The relative regeneration was maximum at 50 mM. Similarly, a significant increase in proline content (6.5 fold in callus culture and 9.2 fold in the leaves) was observed at 50 mM NaCl after 30 days. However, the adverse effect of salt was more pronounced on total protein content, except at 50 mM. These results suggest that proline accumulation is an index of salinity tolerance and this important international variety can be genetically manipulated to develop salinity tolerant crop.**

Key words: Abiotic stress, callus proliferation, proline assay, salinity, total protein.

INTRODUCTION

Salinity is one of the major constraints to crop production throughout the world (Flowers, 2004), especially in the arid and semi-arid regions of the world, where it adversely affects photosynthesis (Munns and Tester, 2008), various growth phases (Epstein et al., 1980) and reduces productivity (Vicente et al., 2004). Salt stress is believed to reduce the optimum growth of crop plants by affecting water absorption as well as biochemical processes like nitrogen assimilation and protein biosynthesis (Dubey, 1994). Nearly 25% of the cultivable land around the world contains excessive amounts of salt, mainly NaCl (Shannon and Grieve, 1999). The semi-dwarf, elite indica rice cultivar IR 64 most widely grown in South Asia possesses many positive agronomic traits such as wide adaptability, tolerance to multiple diseases and pests, high yield potential and good eating quality (Wu et al., 2005). In spite of its superior traits, it is a salt sensitive cultivar (Sonali and Majumder, 2009) and is therefore regarded as an attractive system for *in vitro* selection and genetic transformation studies. Although, several reports on the development of transgenic IR 64 plants have already been published (Zhang et al., 1998; Khanna and Raina, 2002; Kumar et al., 2005), development of salinity tolerant IR 64 through *in vitro* selection strategy has not been achieved yet. Proline is reported to reduce the enzyme denaturation caused due to abiotic stresses and acts as a source of carbon, nitrogen and energy during, and recovery from, stresses (Kavi Kishor et al., 2005). Proline mediated salt-tolerance against stress damage by means of protein turnover machinery has been reported in *Pancratium maritimum* (Khedr et al., 2003). The aims of this study were therefore to analyze the effect of NaCl at embryogenic callus level to facilitate regeneration; also to provide a system for the analysis of proline and total protein content in callus cultures and leaf tissues in order to exploit proline over production for improving salt tolerance in rice.

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Abbreviations: **BAP**, 6-Benzylaminopurine; 2, 4-D, 2,4-dichlorophenoxyacetic acid; **FW**, fresh weight; Kin, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthalene acetic acid.

Table 1. Effect of NaCl on fresh weight of the callus of *O. sativa* (L.) IR 64 seeds.

NaCl concentration (mM)	Mean of callus Induction (15 d) (%)	Mean of callus Survival (30 d) (%)	Mean of calli FW (15 d) (g)	Mean of calli FW (30 d) (g)
0	92.9±0.63	92.76±0.68	0.18±0.015	0.31±0.01
25	86.03±0.44**	85.68±0.58**	0.14±0.015*	0.23±0.01**
50	60.7±0.49*	84.66±0.57**	0.06±0.025*	0.15±0.02*
75	53.3±0.24*	81.0±1.0**	0.05±0.025*	0.11±0.01**
100	46.6±0.20*	68.5±0.86*	0.04±0.02**	0.07±0.01**

Data represents the mean of three replicates ±SD. * and ** indicates significant difference at $P < 0.05$ and $P < 0.005$, respectively.

MATERIALS AND METHODS

Explant preparation

Seeds of *O. sativa* (IR 64) were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Healthy seeds were manually dehusked and surface sterilized with 0.1% (w/v) mercuric chloride for 4 min followed by 70% (v/v) ethanol for 30 s under aseptic conditions in a laminar air flow chamber. Seeds were thoroughly rinsed four times with sterile distilled water and blotted dry in 90 mm Petri dishes containing a layer of sterile filter sheet.

Growth media and culture conditions

The sterile seeds were inoculated on callus induction medium containing MS salts and vitamins (Murashige and Skoog, 1962) supplemented with13.5 µM 2,4 Dichlorophenoxyaceticacid (2,4-D), 1.3 µM Kinetin (Kin) and four different concentrations of NaCl, that is (25, 50, 75, 100 mM) to test their salt tolerance. The pH of the medium was adjusted to 5.75 and autoclaved at 121°C for 20 min and all the cultures were incubated at 24±2°C in the dark for 30 days. In order to determine the influence of NaCl on callus induction and growth, the callus induction frequency, callus survival rate and fresh weight (FW) of the callus cultures initiated at different concentrations of NaCl were observed after 15 and 30 days of inoculation. Friable and nodular embryogenic portions of the callus cultures were subcultured onto the same but fresh proliferation medium for another 24 days, before subjecting them to regeneration.

Regeneration of shoots

About 75 to 100 mg of callus tissue was inoculated on MS medium supplemented with 13.3 µM 6-Benzylaminopurine (BAP), 8 µM α-naphthalene acetic acid (NAA) and 25, 50, 75 and 100 mM NaCl. All the cultures were maintained in tissue culture room at 24±2°C under a light – dark cycle of 16:8 with a light intensity of 70 µmol m^{-2} s^{-1}. Frequency of regeneration was expressed as the number of seeds inducing callus at each salinity concentration and callus pieces that survived and developed at least one shoot of 1 cm in height (Lutts et al., 1999).

Estimation of proline

Proline estimation was carried out as described by (Bates et al., 1973). Proline was extracted from both untreated and treated (0, 25, 50, 75 and 100 mM NaCl) callus cultures and regenerated plants. The experiment was laid out in a completely randomized design. Three triplicates of randomly selected control and NaCl treated callus and leaf tissues with a fresh mass of 0.5 g were taken after 15 and 30 days of inoculation and then homogenized in 5 ml of 3% (w/v) sulphosalicylic acid. The residue was removed by centrifugation at 5000 g for 10 min and the supernatant was filtered through Whatman # 2 filter paper. The filtrate was reacted with an equal volume of ninhydrin and glacial acetic acid and incubated at 95°C for 1 h. The reaction was terminated by placing in an ice bath for about 30 min then extracted with 4 ml toluene and mixed vigorously for 15 sec. The toluene phase containing the chromophore was aspirated, warmed to room temperature for 10 min and the proline content was determined colorimetrically and expressed as µmol g^{-1} FW.

Extraction and analysis of total protein

The total protein was extracted by homogenizing 0.5 g each of control and NaCl treated callus cultures in 10 mL of 0.2 M perchloric acid. The homogenate was centrifuged at 5000 g for 10 min at 24°C. Ethanol-ether-chloroform (2:2:1; v/v/v) solvent mixture was used twice for the extraction of the pellet. To the residue, 0.2 M NaOH was added and left overnight. The supernatant was used for total protein estimation (Lowry et al., 1951).

Statistical analysis

For each NaCl treatment, the experiment was repeated thrice. Data was subjected to analysis of variance to evaluate the effect of salinity on callus induction and regeneration. Mean values were compared by one-way ANOVA and post hoc tests were performed using Dunnet's Test with the help of statistical software SPSS 10.00 for windows. $P < 0.05$ and $P < 0.005$ were used to define statistical significance.

RESULTS

Effect of NaCl on callus initiation and proliferation

Callus induction and proliferation were affected with increasing NaCl concentrations in the medium. Callus induction frequency as well as fresh weight decreased significantly at 25, 50, 75 and 100 mM NaCl (Table 1). There was no significant reduction in callus survival rate as evidenced by gradual reduction in the percentage of survival (68.5% at 100 mM NaCl) when compared to callus induction (46.6% at 100 mM NaCl), as the callus if initiated would mostly survived.

Effect of NaCl on regeneration

Callus cultures of IR 64 exhibited high percentage of regeneration (98%) in the non-saline control medium, whereas the rate decreased gradually with increasing concentrations of NaCl. Interestingly, the negative effect of NaCl was stronger in 25 mM NaCl as only 10% of the cultures developed plantlets and at relatively high salinity such as 50 and 75 mM, the final percentage regeneration significantly increased, 58 and 52% respectively (Figure 1A). Again there was a substantial reduction in regeneration at 100 mM, as only 12% of embryogenic callus cultures exhibited regeneration. The moderately high regeneration rate at 50 mM NaCl may be attributed to the increase in proline (Figure 1B) and total protein content (Figure 1C) and there exists a positive correlation between regeneration, proline and total protein content.

Effect of NaCl on proline content

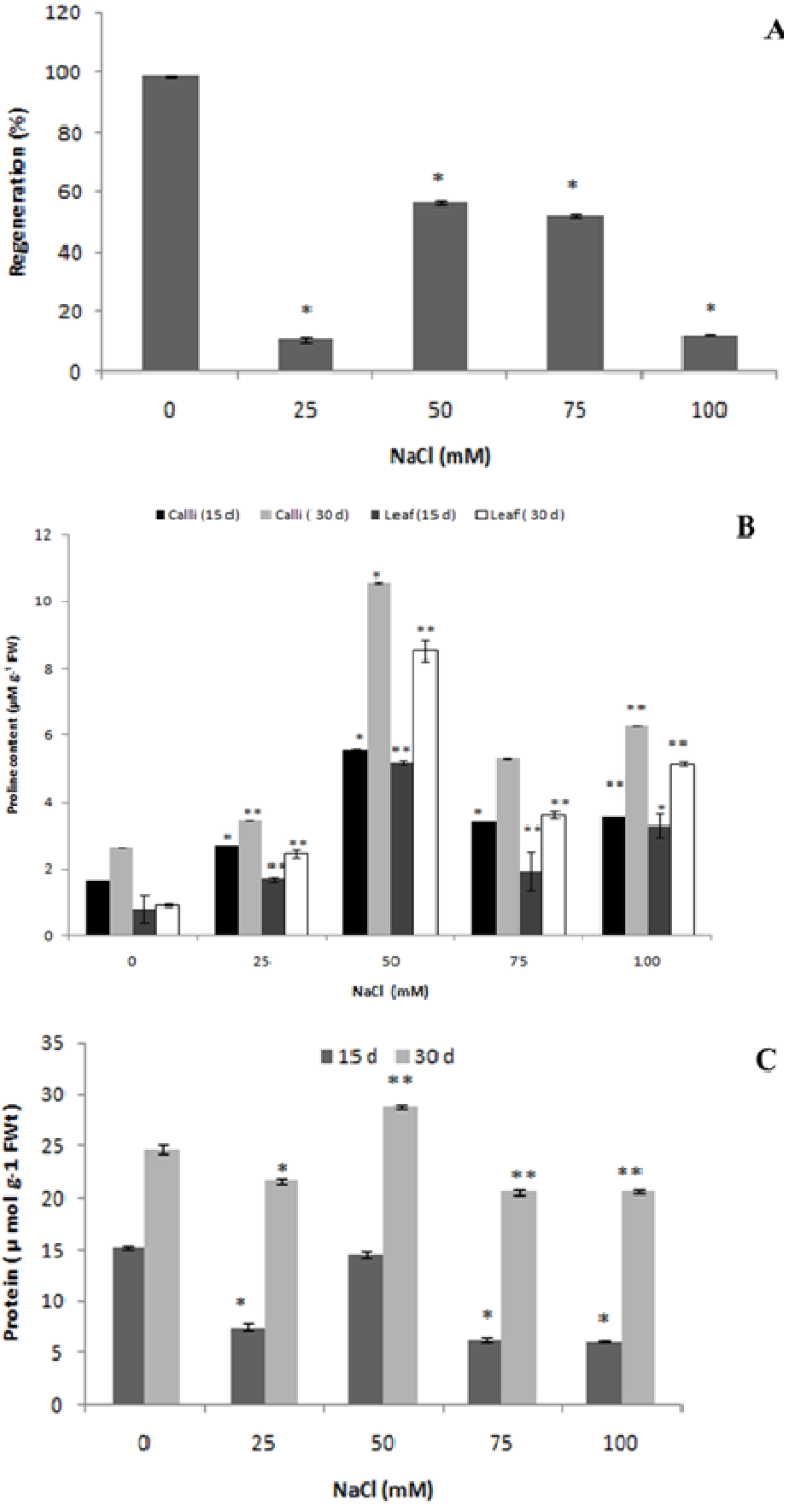
Compared to control, the increase in free proline level was found to be gradual in 25, 75 and 100 mM NaCl stressed callus cultures after 15 days (2.6, 3.3 and 3.5 µmol g^{-1} FW), whereas at 50 mM, there was a dramatic increase in the proline content, which is almost 3.4 fold (5.5 µmol g^{-1} FW). Similar results were observed in 25, 75 and 100 mM NaCl stressed callus cultures after 30 days (3.4, 5.3 and 6.2 µmol g^{-1} FW). At 50 mM, a significant increase in proline content (almost 4 fold, 10.5 µmol g^{-1} FW) was observed after 30 days. Leaves of plants regenerated from NaCl stressed callus cultures produced more proline than control, after 15 and 30 days. Similar to our earlier observation with callus cultures, only at 50 mM, leaves showed a remarkable increase in the proline content (8.54 µmol g^{-1} FW, almost 9.2 fold) and (5.18 µmol g^{-1} FW, 6.6 fold) after 30 and 15 days respectively. In all the remaining three treatment levels, the increase was found to be gradual (Figure 1B).

Effect of NaCl on total protein

Variation in the total protein content of the callus cultures was observed 15 and 30 days after inoculation with NaCl (Figure 1C). In all the 3 independent experiments, the total protein content in both control and stressed callus cultures were higher after 30 days in comparison to 15 days after inoculation. The protein content of NaCl treated callus cultures decreased considerably in all the concentrations tested except at 50 mM after 15 days. Protein content in the control callus was 15.24 µmol g^{-1} FW, whereas it was 7.5, 6.2 and 6.1 µmol g^{-1} FW at 25, 75 and 100 mM NaCl respectively after 15 days (Figure 1C). The positive correlation between regeneration and increase in proline suggested that this salt sensitive variety can be manipulated to have salt tolerance by *in vitro* selection and transformation with proline coding *p5cs* gene.

DISCUSSION

Incorporation of salinity stress in the medium in the form of NaCl during callus induction and regeneration will pave the way for studying the effects of salt stress on the different stages of development. The effect of *in vitro* selection of rice cells with salt-tolerant phenotype for producing fertile plants with improved salt tolerance has been reported (Winicov, 1996). Decrease in fresh weight of the calli is an indicator of the negative effect of NaCl on callus induction. The dramatic decrease in the fresh weight of the callus with increasing salt concentration was previously reported in indica rice cultivars such as Pusa Basmati 1 and Basmati 370 (Shankhdhar et al., 2000). The reduction might be as a result of reduced water availability in the culture medium due to increased NaCl concentration. As plant regeneration is a suitable parameter for analyzing the effects of salinity at the cellular level, callus cultures were exposed to regeneration medium containing various concentrations of NaCl. Except at 25mM the regeneration frequency decreased with increasing concentration of NaCl. High levels of NaCl in the regeneration medium caused a marked reduction in the regeneration of salt tolerant lines in other rice cultivars (Reddy and Vaidyanath, 1986; Binh et al., 1992). It has also been reported that 128 mM NaCl was found inhibitory to regeneration (Basu et al., 1997). On the contrary, many researchers have reported the improvement of regeneration from NaCl treated callus cultures and the positive effect of salt pretreated callus cultures on plant regeneration at high frequency in various indica rice cultivars (Lutts et al., 1999; Binh et al., 1992). Similarly, maximum callus growth at 50 mM and reduction in growth at 150 and 250 mM NaCl has been reported (Zhang et al., 2004). Proline may act by stimulating osmotic adjustment and consequently enhancing regeneration. Proline supported the growth of salt-adapted callus and the positive effect on the growth with low levels of NaCl could be due to an increase in free proline as reported earlier in indica rice callus cultures (Kavi Kishor, 1988). There was a significant variability in proline content of control and NaCl stressed callus cultures and leaves of regenerated plants, after 15 and 30 days of inoculation. As observed earlier, the lower levels of proline in control callus may be due to an increased rate of degradation (Pandey and Ganapathy, 1985). The results clearly indicated that the salt level triggered proline accumulation in the stressed cultures. Overall, the endogenous level of free proline in the callus tissues and leaves of plants regenerated from NaCl adapted culture was significantly higher than those from the control cultures. Proline accumulation in rice seedlings of various varieties was seen under all forms of


A
Regeneration (%)
120
100
80
60
40
20
0
0
25
50
75
100
NaCl (mM)
B
Calli (15 d)
Calli (30 d)
Leaf (15 d)
Leaf (30 d)
Proline content (µM g-1 FW)
12
10
8
6
4
2
0
0
25
50
75
100
NaCl (mM)
C
15 d
30 d
Protein (µ mol g-1 FWt)
35
30
25
20
15
10
5
0
0
25
50
75
100
NaCl (mM)

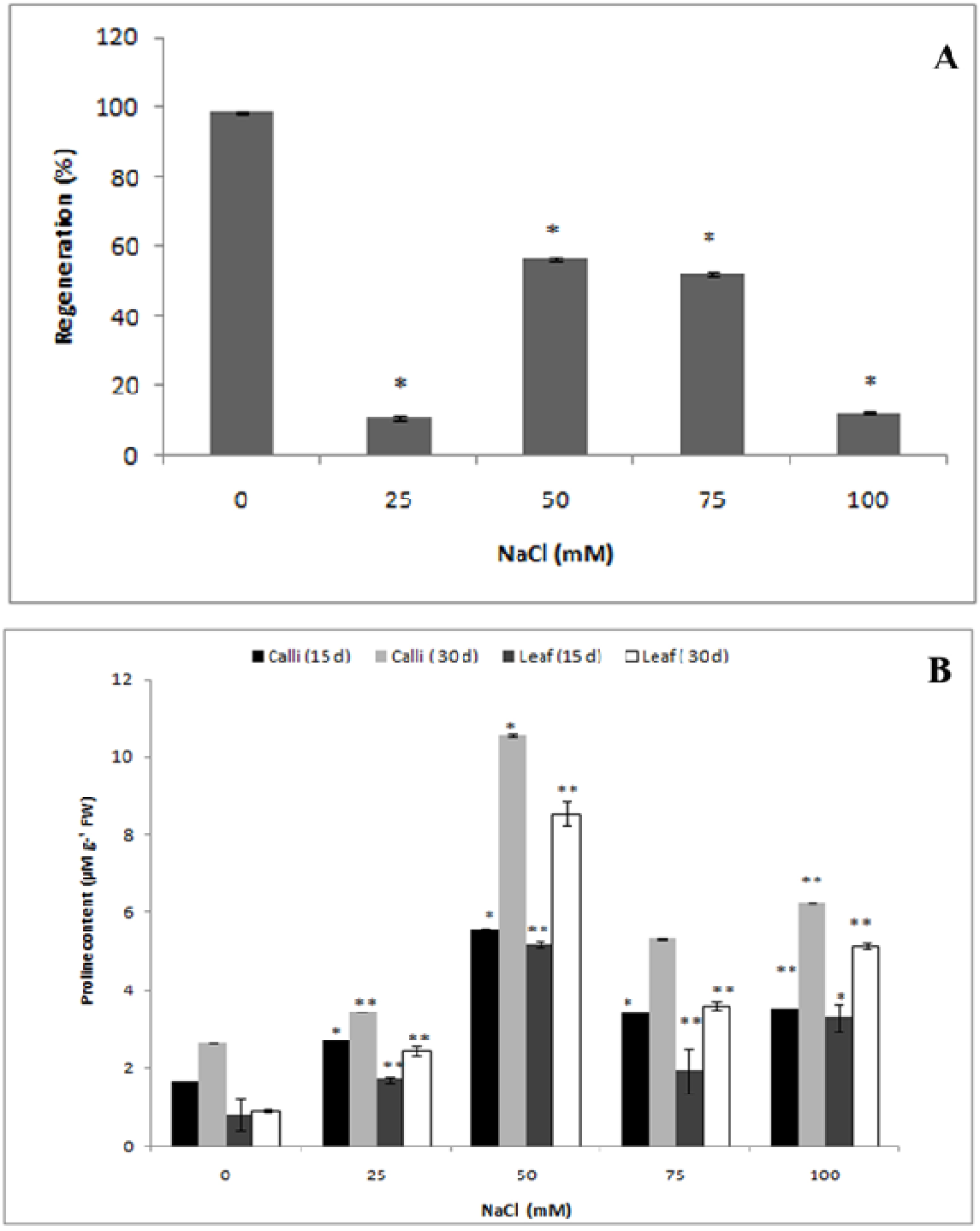


Figure I. continues.

stress and the proline content increased with increasing exposure to stress (Liu et al., 2000). Enhanced level of proline in the cultures may be due to an alteration in the amino acid pool (Yoshiba et al., 1997), and the fresh biosynthesis of proline may be due to osmotic stress or breakdown of proline rich proteins (Greenway and Munns, 1980) during stress conditions. Accumulation of proline in plants is widely believed to be one of the osmotic adjustment mechanisms to overcome water deficit caused by drought and high salt concentrations (Caballero et al., 2005). In several indica rice cultivars, a similar kind of increased proline accumulation in stressed

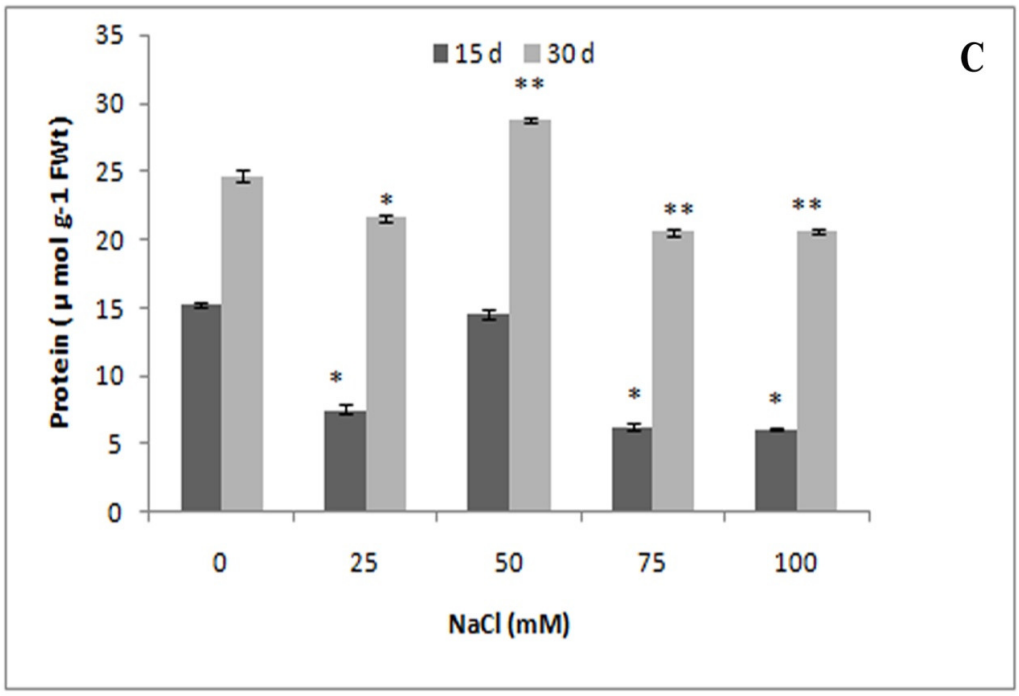


Figure 1. Regeneration percentage of *O.sativa* (L.) embryogenic callus cultures at various concentrations of NaCl (A) , Effect of NaCl on proline content of NaCl treated callus cultures and regenerated plants after 15 & 30 days (B) , Total protein content of callus cultures at various concentrations of NaCl after 15 & 30 days (C). Values are Mean ± SD * and ** indicates significant difference at *P < 0.05 and P < 0.005* respectively.

callus cultures compared to the control was reported (Reddy and Vaidyanath, 1986; Kavi Kishor, 1988; Summart et al., 2010). On the contrary, correlation between stress tolerance and accumulation of proline and protein in higher plants may not be applicable to all plants (Petrusa and Winicov, 1997). In addition to the various known roles of proline, it is also involved in the synthesis of key proteins that are necessary for stress responses (Nanjo et al., 1999). Only in 50 mM NaCl treated callus culture, the protein level is almost comparable to the control, which may be due to the presence of NaCl tolerant cells within the callus cultures and increase in protein synthesis. Interestingly, NaCl treatment has no significant effect on the total protein content of callus cultures after 30 days, as the reduction is only 0.3 to 0.4 fold less at 25, 75 and 100 mM NaCl. Similar to the results observed after 15 days, 50 mM NaCl enhanced the total protein content considerably after 30 days. Among the treatments, a maximum reduction of 6.1 µmol g^{-1} FW of protein was observed with 100 mM treatment after 15 days. A similar kind of decrease in protein contents in response to NaCl mediated stress were reported in other indica rice cultivars like 27814 ECD-1 and IR 58 (Viji et al., 1996). In our study, with the increasing concentrations of NaCl, the protein concentration decreases the reason might be plants under stress would have a powerful protein turnover machinery to degrade stress damaged and environmentally regulated proteins (Khedr et al., 2003). Proline content in both NaCl stressed callus culture and leaves of regenerated plants showed variation between 50 mM and 25, 75 and 100 mM and the synthesis of proline may be an adaptive strategy of the callus cultures to facilitate regeneration. Further investigations are in progress to develop transgenic abiotic stress tolerant IR 64 plants and to understand the effect of proline biosynthetic gene (*p5cs*) in genetically transformed IR 64 plants.

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