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Research Article

In vitro mutagenesis to improve salt tolerance in rice (*Oryza sativa L.*)

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Abstract
Rice is the most important food crop in the world which has been severely affected with salinity. Aim of this study was to improve the salt tolerant ability of two moderately susceptible rice cultivars (White ponni and BPT-5204) using Ethyl Methane Sulfonate under *in vitro* condition. In this experiment salt tolerant callus were recovered in seeds treated with 0.5% EMS cultured on MS media supplemented with different concentrations of NaCl and sea water (starts with 0%, 0.5%. 1.0% and 1.5% NaCl to 10% and 25% seawater). The extant of salt tolerance was determined by callus growth parameters like callus induction frequency, relative growth rate and regeneration frequency. The reduction in growth parameters were more at 25% (v/v) sea water and 1.5% (w/v) NaCl treated media. Significant number of plants (17.23% and 22.49% plantlets respectively) was obtained in callus recovered in 3hr and 5hr EMS treated seeds cultured on 25% sea water and 1.5% NaCl treated media. Proline is an important osmaticm accumulated more under salt stress to maintain the turgor pressure and protect the plants from extreme desiccation. Under higher salt concentration BPT-5204 accumulated the higher amount of proline than white ponni. The plantlets obtained from higher concentration of salt can be transferred to field for the selection of elite lines.

Keywords
salt stress, proline, growth parameters, rice and mutagenesis

INTRODUCTION

Rice (*Oryza sativa* L.) is the stable food for about 90 % population of Asian countries. In Asia, more than two billion people getting 60-70% of their energy requirement from rice and its derived products. In India, it has been cultivated in around 44 million hectare with an average production of 90 million tonnes and the productivity of 2.0 tonnes/ha. The estimated rice requirement for 2025 is about 140 million tonnes (Deepa Sankar, 2015). It necessitates world needs to produce 40% more rice to feed the increasing population (Swapna and Shylaraj, 2017). Among the various yield limiting factors that adversely affecting crop production, salinity stands one of the most destructive environmental stress in the world (Nikam *et al*., 2015). Salinity alone persists as serious threats to agriculture in arid and semi-arid regions of the world. Rice is classified as moderately sensitive to salt in the field condition (Khan *et al*., 2016). About 6.5% (831 million ha) of the world's total area (12.78 billion ha) is affected by salt with salinity levels ranging from 2 to 4 ds m^{-1} (Rudra, 2013). In India, 4 million ha of rice cultivating land is affected with salt. In Tamil Naidu, the coastal area is around 0.68 million ha, out of which problem soils constitutes 30 % and saline soils constitute 21.7%. Problem soil which includes both alkaline and saline soils (Jauhar Ali *et al*., 1999). Accumulation of high concentration of salt affects rice growth, development and yield. Salt interferes with plant adaptation, and stress responses. Plant exposed to salt causes varied range damage to plant such as reduced number of leaf, tiller, stunted growth, sterility in rice if imposed during pollination and fertilization. Hence salt causes major destruction to rice production. To increase the production to meet the food requirement of growing population it is necessary to identify salt tolerant genotypes. Variability for salt tolerance is very limited in the available rice germplasm. Field screening also select for variability already available in the population. Field screening does not create any new variability. Hence employing mutagenesis under *in*

vitro condition creates a new variability by somaclonal variation. *In vitro* salt screening proved to be a potent alternative to the conventional breeding methods for the identification of promising salt tolerant genotype (Suprasanna *et al*., 2012). Mutagens may cause genetic changes in an organism, breaks the undesirable linkages which releases the many new promising traits (abiotic, lodging resistance and dwarfness) for the improvement of crop plants. Among the chemical mutagens, EMS is reported to be the most effective and frequently used one in the crop improvement (Shah *et al*., 2008). Crop plants have been evolved various mechanisms such as osmo regulation, ion exclusion, ROS (Reactive oxygen species) scavenging and membrane impermeability which reduces the plant damage. The osmoregulation was realized mostly by accumulation of compatible osmolytes like proline, sucrose, polyols, trehalose and quaternary ammonium compounds (QACs) such as glycine betaine, alinine betaine and proline betaine (Qiao *et al*, 2010). Proline is one of the most common compatible osmolytes accumulated more under stress condition. Aim of this study was to evaluate the effective *in vitro* regeneration system for the development of saline tolerant mutant in rice cultivars. The effect of NaCl on embryogenic callus was facilitated by the analysis of proline content in callus cultures.

MATERIALS AND METHODS

Seeds of two rice cultivars namely white ponni and BPT-5204 (high yielding fine grain quality rice, moderately susceptible to salt) were obtained from Department of Rice, Tamil Nadu Agricultural University, Coimbatore. Manually dehusked healthy seeds were surface disinfected with 0.1% (w/v) $HgCl_2$ for 5 min followed by 70% (v/v) ethanol for 30 sec under aseptic condition in a laminar air flow chamber. Surface disinfected seeds thoroughly rinsed with sterile distilled water for four to five times and blot dried in 90 mm petri dishes containing a layer of sterile filter sheet.

The surface disinfected seeds were inoculated on callus induction medium containing MS salts and vitamins (Murashige and Skoog, 1962) was supplemented with 2 mg L^{-1} 2, 4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} kinetin (Kin). Sterilized seeds were treated with 0.5 percent EMS solution at different time interval (control, 1, 2, 3 and 5 hour). Treated seeds were washed with sterile water and inoculated on callus induction medium supplemented with different concentrations of NaCl (0, 0.5, 1, 1.5 % NaCl) and sea water (10% and25% sea water). pH of the media was adjusted to 5.78 with 0.1 N HCl or NaOH and autoclaved at 121°C for 20 min. Cultures were incubated at 27 ± 1°C in the dark for 30 days. Friable and nodular embryogenic calli were sub-cultured on the same fresh medium for about 12-24 days for better proliferation of the calli before subjecting them to regeneration. Two weeks old well proliferated embryogenic callus was transferred to regeneration medium.

About 75 to 100 mg of callus tissue was transferred to regeneration media supplemented with 5 mg L^{-1} 6-Benzyl aminopurine (BAP) and 0.05 mg L^{-1} α-naphthalene acetic acid (NAA) treated with different concentration of NaCl (0.5, 1, 1.5 %) and sea water (10 and 25%). Cultures maintained at 27±1°C with day and night cycle of 16:8 h at 70 µmol m^{-2} s^{-1} light intensity. Regeneration frequency was expressed as the number of NaCl surviving callus inoculated to number of calli regenerated into 1 cm shoot (Lutts *et al.,* 1999).

Proline content of tissues was estimated by colorimetric method as described by Bates *et al*. 1973. Proline was extracted from control as well as salt treated (0, 0.5%, 1%, and 1.5% NaCl) callus. The experiment was laid out in a completely randomized design with three replications. 0.5 g fresh mass of 15 days old calli selected randomly grown on different medium was homogenized in 5 ml of 3% (w/v) sulphosalicylic acid. The residues were removed by centrifugation at 5000 g for 10 min and the supernatant was filtered with whatman No. 2 filter paper. The filtrate was reacted with an equal volume of ninhydrin and glacial acetic acid with the incubation of reaction mixture at 95°C for 1 h. The reaction was terminated by placing in ice bath for about 30 min. Reaction mixture was extracted with 4 ml toluene by vortex vigorously for 15 sec. The upper phase containing the chromophore was measured calorimetrically. Obserbance (OD) observed at 520 nm and the concentration of proline was expressed as µg/g on fresh weight (FW) of the callus.

Effect of NaCl on growth of the callus was determined by observing growth parameters like relative growth rate (RGR), callus induction frequency (CIF) and frequency of embryogenic calli (FEC) at 15 days after inoculation on NaCl treated media. Fresh weight of the calli was recorded at the beginning and end of the culture period. The different parameters were calculated according to Smith and McComb (1983) as follows

1. **Relative growth rate (RGR)**

It is calculated on the basis of initial and final weight of the callus as follows

$$\text{Relative growth rate} = \frac{final\ weight - initial\ weight}{initial\ weight}$$

2. Callus induction frequency (CIF)

Callus induction frequency was calculated as follows

$$\text{Callus induction frequency} = \frac{number\ of\ calli}{number\ of\ incubated\ seeds} \times 100$$

3. Frequency of embryogenic calli (FEC)

Frequency of embryogenic calli was calculated as follows;

Frequency of embryogenic calli =

$$\frac{\text{number of embryogenic calli}}{\text{number of incubated seeds}} \times 100$$

4. Number of days to be taken for callus induction

From the date of inoculation to the first day of visual appearance of calli on the surface of the germinating seeds were taken as the number of days for callus induction.

5. Callus regeneration frequency

Callus regeneration frequency was calculated as follows

Callus regeneration frequency (%) =

$$\frac{\text{No.of callli produced plants}}{\text{No.of calli inoculated}} \times 100$$

Experiment was laid out in completely randomized block design with three replications under *in vitro* condition. Mean value of different NaCl treatments were subjected to analysis of variance in AGRES software to evaluate the effect of salt on the callus induction and regeneration.

RESULTS AND DISCUSSION

In vitro culture technique was widely adopted in cereals to get the desirable variants for salt and other stress. In this study mutation is combined with salinity screening under *in vitro* condition to select salt tolerant callus lines (somaclones) in desirable direction. This technique allows us to select for promising genotypes either by direct or indirect regeneration *via* callus (Raveendar *et al.,* 2008: Hassanein, 2004 and Feng-Ling *et al.,* 2011). In the case of direct regeneration, stress agent is embedded in the culture medium, while under indirect regeneration, stressing agent is supplemented during callus initiation (Khaleda *et al*., 2007; New *et al.,* 2011) and regeneration stage (El-Sayed *et al.,* 2004). The present study was relied on indirect regeneration of plants through callus survived on salt treated media.

Mutation rate depends on the mutagenic dose and treatment duration. Mutagenic efficiency refers to the mutation rate at which mutation causes various biological effects is usually a measure of degree of damage to the cell (Girija and Dhanavel, 2009). Upon EMS treatment all the biological criteria was decreased with increasing the duration of mutagenic treatments (**Fig 2**). Maximum embryogenic callus induction (59%) was observed in BPT-5204 at 1 h EMS treated seeds on media containing 0.5 % NaCl, while white ponni recorded 57.66 % callus induction. Callus induction frequency was decreased with increasing EMS treatment duration from 2 hr to 3 hr and salt concentration from 1 to 1.5 % NaCl. Lowest callus induction frequency (29.66 %) was recorded at 5 hr EMS treated seeds inoculated on media containing no salt (**Table 1**). Compared to BPT-5204 callus induction frequency of mutagen treated seed was low in white ponni.

Callus induction frequency of two rice cultivars was decreased with increasing the concentration of the NaCl in the media represented in **Table 1**. Callus induction frequency recorded in BPT-5204 was higher than white ponni. Higher embryogenic callus induction frequency of 63.66 % at 10 % sea water treatment was recorded in

Table 1. Effect of salt tress and 0.5 % EMS on Biological criteria of the two cultivars

Varieties	Salt treatment	CIF (%)	RGR	Number of days and hours taken for callus induction	RF (%)	Proline (µg/g)	0.5%EMS treatment (hour)	CIF (%)
	NaCl (%)							
	Control	59.39	2.66	7 days and 2 hrs	61.55	418.66	Control	54.40
	0.5	64.22	2.62	7 days and 3 hrs	38.25	473.33	1	57.66
	1	50.33	2.28	7 days and 4 hrs	28.73	740.66	2	50.33
White ponni	1.5	40.83	2.97	8 days and 1.6 hrs	17.23	696.66	3	33.33
	Sea water						5	23.66
	10 %	46.33	1.37	6 days and 5 hrs	30.02	468.44		
	25 %	56.66	1.43	8 days and 1 hr	22.49	673.36		
	NaCl(%)							
	Control	63.33	1.46	5 days and 4 hrs	67.89	608.66	Control	68.66
	0.5	61.00	2.79	5 days and 8 hrs	47.33	660.00	1	59.00
	1	57.33	2.31	6 days and 2 hrs	38.00	685.33	2	54.00
BPT-5204	1.5	44.33	3.30	7 days and 2 hrs	22.33	803.33	3	43.66
	Sea water						5	29.66
	10 %	63.66	1.46	6 days and 2hrs	42.66	689.33		
	25 %	62.33	1.49	8 days and 0 hrs	33.23	798.25		

Notes: CIF= Callus induction frequency, **RF** = Regeneration frequency, **NDC=** Number of days for callus induction, **RGR=** Relative growth rate.

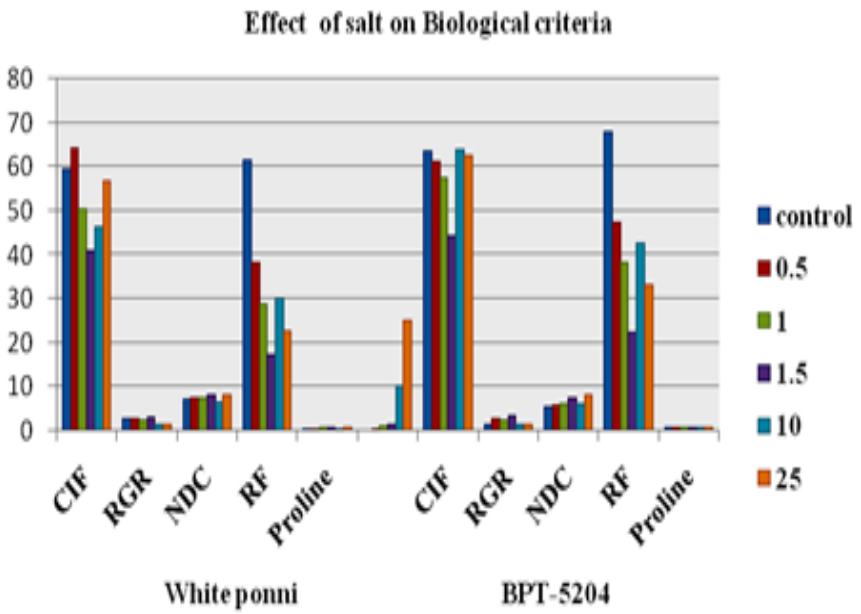

Fig. 1. Effect of salt stress on Biological criteria

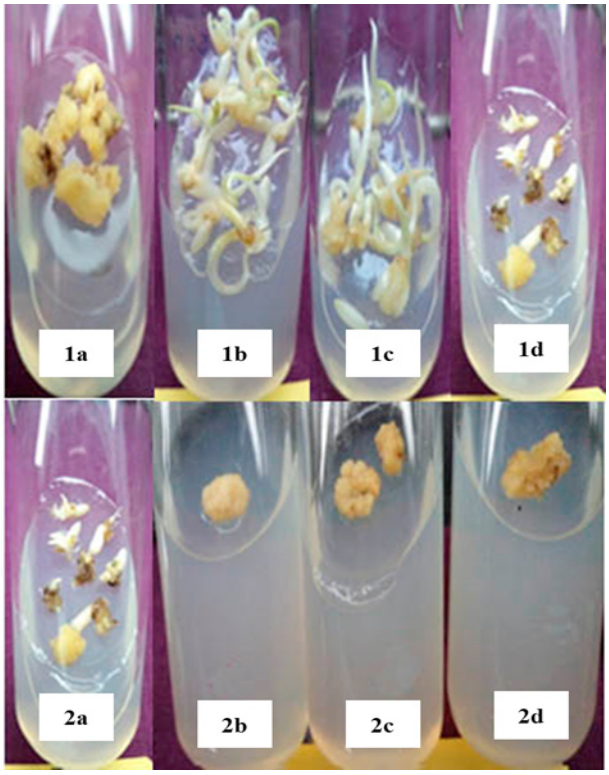

Fig. 2. 1 (a, b, c, d) Effect of 0.5 % EMS on callus induction in white ponni on treatment with different duration (a: 1 hr, b: 2 hr, c: 3 hr, d: 5 hr), 2 (a, b, c, d) Embryogenic callus induction in BPT-5204 on treatment with 0.5 % EMS for different duration (a: 1 hr, b: 2 hr, c: 3 hr, d: 5 hr).

BPT-5204. Salt treatment in the form of NaCl had more effect on callus induction frequency as compared to sea water treatment **(Fig 3**). Reduction in callus induction frequency was higher at 1.5 % NaCl followed by 1 % and 0.5. Callus induction frequency was reduced with increasing the concentration of the salt in the media from 0.5, 1.0 and 1.5%. This is because as the salt inhibit all the biological activity of cell. Reduction in biological criteria (callus induction and survival) is mainly due to drop in auxin level, inhibition of auxin synthesis and chromosomal aberrations or due to decline of assimilation mechanism. The present result was in accordance with the report of Ayolie *et al.* (2011); Rao and Patil, (2012). The number of days taken for callus induction was presented in **Table 1 and Fig. 1**. Under control the number of days taken for callus induction in BPT- 5204 was 5 days 4 hrs whereas in white ponni 7 days 2 hrs. Salt treatment increased the number of days taken for callus induction as observed at 1.5 % NaCl treatment (8 days and 1.6 hr) was higher in white ponni. The number of days taken for callus induction was increased with increasing the concentration of salt. The callus induction process is delayed with salt treatment of media is mainly due to inhibition of cell by salt which causes reduction in fresh weight, callus induction percentage and prolonged the rate at which callus induced. The decrease in fresh weight under the salt stress has already been reported in a number of studies (Ahmad *et al.,* 2007). Callus growth characteristic like relative growth rate (RGR) was also affected with salt stress. Reduction in relative growth rate was more in BPT-5204 than white ponni. Highest relative growth rate of 2.97 at 1.5% NaCl was observed in white ponni. A significant reduction in regeneration frequency

observed in both the cultivars. White ponni recorded a low regeneration frequency of 17.23% at 1.5% NaCl treatment. A significant per cent of plantlets (42.66%) were recorded in BPT-5204 at 10% sea water treatment (**Table 1** and **Fig 4**). The regeneration rate gradually decreased with increasing NaCl concentration in both the genotypes. Similar results on regeneration frequency of salt treated callus was reported in wheat (Alvarez *et al.,* 2003), tomato (Saleem *et al.,* 2005) and rice (Khaleda *et al.,* 2007; New *et al.,* 2011). In the same way, Raveendar *et al.* (2008) and Ayolie *et al.* (2011) showed that the reduction in regeneration capacity was higher at 25 % of sea water treatment.

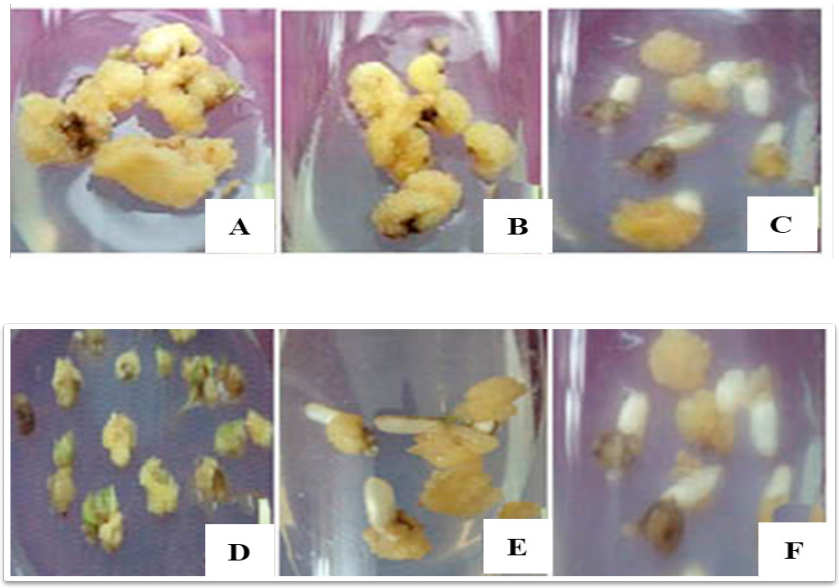

Fig. 3. Effect of salinity on callus induction in BPT-5204, A: control (creamy translucent calli), B: 0.5 % NaCl (calli with reduced size and slight change in colour), C: 1 % NaCl (calli blackened and necrosis of tissue), D: 1.5 % NaCl (non embrogenic and more no of necrotic colli), E : 10 % sea water (normal calli but reduced size), F : 25 % sea water

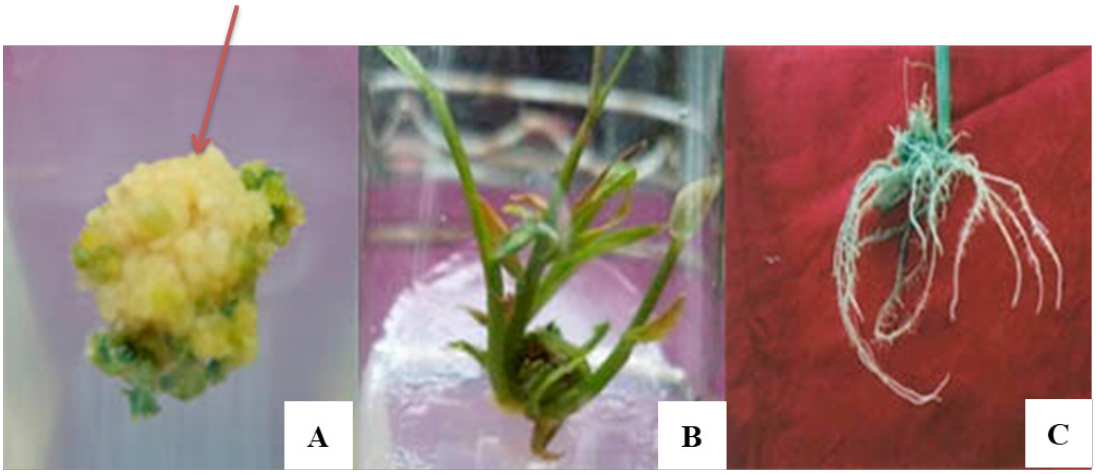

Fig. 4. Regeneration in white ponni on salinized medium, A: Green islet formation, B: Plantlets regeneration, C: Root regeneration.

Proline is an important stress protein was accumulated more under stress condition. BPT-5204 had a higher proline at higher salt concentration. Accumulation proline helps to maintain the osmotic adjustment and tissue water potential there by which reduces the damage caused by salt. It is well established that proline status of the plant organ and cell culture is an active research area in biotic and abiotic stress physiology (Aghaei *et al.,* 2009; Amirjani *et al.,* 2010; Sananda Mondal, 2011). Increase in the level of proline acts as a compatible osmosolute and hydrophobic protectant for a number of enzymes and cellular structure. Proline content of white ponni and BPT-5204 at control was 418.66 and 608.66 µg/g/ respectively in fresh weight (FW) basis. Free proline level of callus has increased in salt treated callus was gradual from 0.5 % to 1.5 % NaCl (**Table 1**). The higher total proline content (803.33 µg/g on fresh weight (FW) basis) was observed in BPT-5204 with 1.5 % NaCl treatment. Whereas the total proline content of NaCl adapted callus of white ponni, was decreased at 1% and 1.5 % NaCl treatment. This study adequately demonstrated that in vitro salt screening could be effective in the selection of salt tolerant genotypes in desirable direction. Some plants were recovered from callus grown on high salt concentration will be having salt tolerant potential can be used to improve cultivars. Stress protein, proline could be the potential marker in the selection of salt tolerant genotypes in callus stage which increase the buffering capacity of genotypes to salt stress.

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