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Increased Regeneration Efficiency in Seed Derived Callus of Rice (*Oryza sativa* L.)

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Abstract

In five cultivated rice varieties *e.g.,* Nonabokra, PNR 381, Taipei 309, Koshika and TKM 11 the response in the initiation of callus and its size varied greatly among varieties, salt concentrations (NaCl and Na_2SO_4) and their interaction. Better regeneration ability of Nonabokra, Koshika and TKM 11 was found to be correlated to small size callus with a high concentration of Na_2SO_4. The regeneration ability found here was dependent on variety/genotype, callus type and size, source of salt and their concentration. Regeneration ability enhanced three - tenfolds from Na_2SO_4 stressed callus compared to the control i.e. without Na_2SO_4. Vigorous rooting was also observed in the regenerated plants obtained from the calli induced in the medium containing Na_2SO_4. In NaCl supplemented medium, on the other hand, regeneration and rooting ability were poor.

Introduction

Rice (*Oryza sativa* L.) is the most important and staple food crop of Bangladesh as well as 60% of the global population (Uchimiya et al. 2002). As the population in the world is increasing continued increase of rice production poses a major issue for world food security. Global rice production has increased, almost threefold over the past three decades (Christopher 2002), but further increase in yield and nutrition may only be possible using the biotechnological tools to meet the rising demand of the population.

Recent advances in *in vitro* plant cell, tissue and organ culture as an additional method for crop improvement has been well augmented. Successful application of *in vitro* techniques of somatic tissue offers a wide scope of achieving somaclonal variants in regenerated plants from the rice calli. Seed derived calli of rice have been shown to be superior in efficient regeneration (Hiei et al. 1994). The regenerants could be obtained from calli under initial salt stress as a supplement to the callus induction medium (Kishor et al. 1999).

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Soil salinity is one of the important factors limiting the growth and productivity of rice in saline prone areas of the world (Lee et al. 2003). Initial salt (NaCl, Na_2SO_4) stress in the medium has been reported to improve the frequency of somatic embryogenesis, androgenesis and plantlet regeneration (Kishor et al. 1999; Lutts et al. 1999; Jain 1997; Karim and Zapata 1996). It is possible that such plantlets can be adapted to a saline environment resulting in enhanced crop production (Anitha and Reddy 1997).

In this paper, the result of NaCl and Na_2SO_4 stress on the seeds of five cultivated indica rice varieties are reported in so far as their effects on enhancement of embryogenic calli production as well as increased regeneration rates.

Materials and Methods

Mature seeds of five indica rice varieties *e.g.,* Taipei 309, Nonabokra, TKM 11, PNR 381 and Koshika were used in this study as test materials. Seeds were dehusked manually and sterilized in 0.1% $HgCl_2$ for 20 minutes followed by extensive washing under laminar airflow cabinet. The healthy and unwrinkled seeds of each variety were sorted out and cultured in callus induction medium (CIM) containing MS basal medium consisting of macro, micro nutrients, iron, vitamins, *Myo*-inositol, 3% sucrose with 2.0 mg/l 2, 4-D. Four different concentrations of NaCl and Na_2SO_4 (0.0, 2.5, 5.0 and 7.5 g/l) were added to the medium.

Four to five weeks old calli, initiated from seeds, were cultured onto shoot regeneration medium (SRM) containing MS basal salts supplemented with 1.0 mg/l of both Kn and NAA. Agar at a concentration of 8.0 g/l was added to solidify the media. All the media combinations were adjusted to pH 5.8 with 1N/0.1 N NaOH before autoclaving at 121°C for 15 min at 1.05 kg/cm^{-2} pressure (15 - 20 psi). Fifteen ml of both CIM and SRM were dispensed into sterile glass Petri dishes (60 × 15 mm) and test tubes (8" height), respectively. The seed and callus cultures were incubated at 24 ± 1°C in a dark chamber and under continuous lighting (provided by cool-white fluorescent and incandescent lights), respectively. For seed culture, six replications were used for each treatment and placed in a completely randomised experimental design. The experiment was repeated three times. Callus initiated from seeds were divided into two to three parts and cultured on to SRM in test tubes for obtaining regenerated plants.

Well developed, phenotypically normal and extensively rooted plantlets were chosen for acclimatization. The acclimatized plants were then transfered to soil in pot with Yoshida's (Yoshida et al. 1962) nutrient solution for further growth and development. Data on callus initiation, callus size, its structure, and regeneration efficiency under control as well as salt treatments were recorded.

The number of green and albino plants produced and the number of green plants survived during acclimatization were also recorded. A descriptive analysis was conducted using the recorded data.

Results and Discussion

Effect of salt on callus initiation: Seeds of all varieties enlarged in CIM at all concentrations of NaCl and Na_2SO_4 (0.0, 2.5, 5.0 and 7.5 mg/l) within one to two weeks. One month of culture was found suitable for obtaining sufficient amount of calli initiated from seeds of each variety. Most of the calli induced from seeds in CIM with salts was found to be light yellowish in colour. Occasionally a mixture of light yellowish and white calli initiated together. Light yellowish calli were compact, but white calli were less compact.

Addition of NaCl and Na_2SO_4 to the CIM showed affect in colour and type of callus. It was observed that higher rate of light yellowish and compact calli were initiated with Na_2SO_4 compared to control and NaCl treatment. Among the varieties, Taipei 309 produced better callus (light yellowish and compact) with

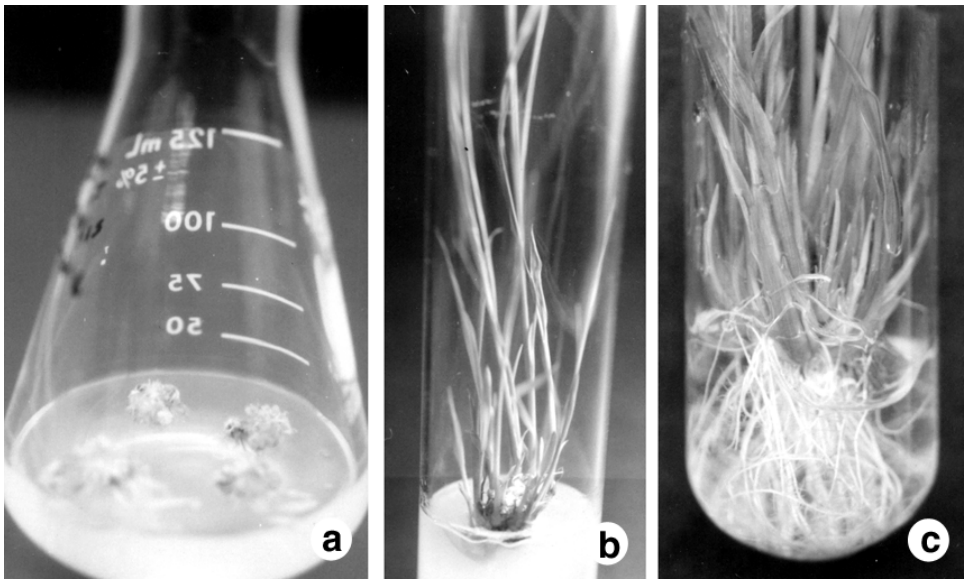

Fig. 1. Effect of Na_2SO_4 on regeneration of Nonabokra: (a) Callus initiation in Taipei 309 at 5.0 g/l Na_2SO_4. (b) Green plants regenerated from Nonabokra at 7.5 g/l Na_2SO_4. (c) Vigorous rooting from the base of regenerated plants of TKM 11 obtained from calli at 5.0 g/l of Na_2SO_4.

Na_2SO_4 compared to others (Fig. 1a, visual observation). Light yellowish and compact calli initiated from seeds with Na_2SO_4 showing better regeneration indicates that this type of treatment could be used for future studies.

Without salts in CIM, calli initiated from all seeds (100%) of all varieties cultured (Table 1). Per cent callus initiation from seeds varied among the varieties in different concentrations of salts added to CIM. High concentrations of both NaCl and Na_2SO_4 induced lower per cent of callus in Nonabokra, PNR 381 and Taipei 309 (Table 1). Several studies on chemical factors (salt) confirm our present findings (Chauhan et al. 2000; Kishor et al. 1999; Chauhan and Prathapasenan 1998).

Table 1. Effect of salt treatments on callus induction from seeds of five exotic rice varieties.

Variety	% callus initiation ± S.E.						
	Control	MS with NaCl (g/l)			MS with Na_2SO_4 (g/l)		
	0.0 g/l	2.5	5.0	7.5	2.5	5.0	7.5
Nonabokra	100 ± 0.0	100 ± 0.0	97 ± 3.3	96 ± 4.2	100 ± 0.0	88 ± 8.3	90 ± 10
PNR 381	100 ± 0.0	97 ± 3.3	100 ± 0.0	96 ± 4.2	100 ± 0.0	100 ± 0.0	100 ± 0.0
Taipei 309	100 ± 0.0	100 ± 0.0	96 ± 4.2	87 ± 6.7	90 ± 6.8	100 ± 0.0	82 ± 7.5
Koshika	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
TKM-11	100 ± 0.0	100 ± 0.0	97 ± 3.3	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

Effect of salt on callus size: Average callus size was found higher in control CIM, without NaCl and Na_2SO_4. With increasing NaCl concentrations average callus size reduced in all varieties tested (Table 2). Similar result was also found with Na_2SO_4 in all varieties. The reduced range of average callus size was found to be 2.2 to 3.3-folds with NaCl compared to control. On the other hand, the range was observed to be 1.7 to 4.1-folds lower with Na_2SO_4 compared to control

Table 2. Effect of salt treatments on callus size induced from seeds of five exotic rice varieties.

Variety	Average callus size in mm ± S.E.						
	Control	MS with NaCl (g/l)			MS with Na_2SO_4 (g/l)		
	0.0 g/l	2.5	5.0	7.5	2.5	5.0	7.5
Nonabokra	6.5 ± 0.25	3.9 ± 0.17	2.0 ± 0.10	2.3 ± 0.10	6.3 ± 0.23	2.2 ± 0.00	1.6 ± 0.05
PNR 381	4.6 ± 0.23	2.2 ± 0.10	1.8 ± 0.05	1.5 ± 0.05	4.6 ± 0.22	4.4 ± 0.15	2.7 ± 0.17
Taipei 309	7.3 ± 0.43	5.0 ± 0.27	4.3 ± 0.18	3.0 ± 0.13	5.3 ± 0.25	5.1 ± 0.24	2.9 ± 0.09
Koshika	4.4 ± 0.19	2.8 ± 0.10	2.1 ± 0.06	2.1 ± 0.11	3.7 ± 0.17	2.6 ± 0.09	2.3 ± 0.07
TKM-11	5.6 ± 0.35	2.3 ± 0.11	2.0 ± 0.08	2.2 ± 0.10	4.7 ± 0.29	2.0 ± 0.08	1.9 ± 0.09

(Table 2). This obviously suggests that salt concentration inhibits callus initiation and reduced callus size. Pushpalatha and Padmanabhan (1998) and Vaziri et al. (2004) found the same effect of NaCl in rice and soybean callus culture, respectively.

In Nonabokra, Na_2SO_4 reduced average callus size to a maximum of 4.1-folds. In PNR 381, NaCl reduced average callus size to a maximum of 3.1-folds. On the other hand, in Taipei 309, Koshika and TKM 11 varieties the average callus size reduced less than 3.1-folds when both Na_2SO_4 and NaCl were added as supplements. The callus size obtained varied among the varieties which may be due to genotypic effect.

Effect of salt on shoot regeneration: A beneficial effect was observed on shoot regeneration and height of regenerated plants of all varieties when Na_2SO_4 was added to the CIM. A correlation was found between increasing shoot regeneration and increasing concentration of Na_2SO_4 in Nonabokra, Koshika and TKM 11 varieties. Higher regeneration frequency of 43.8, 62.5 and 16.7% was observed in Nonabokra (Fig. 1b), Koshika and TKM 11, respectively at 7.5 g/l of Na_2SO_4 (Table 3). In Nonabokra, the regeneration rate was over threefolds higher at 7.5g/l Na_2SO_4 compared to the control. Similarly, in Koshika and TKM 11 the regeneration frequency increased over 6 and 2.5-folds, respectively at 7.5 g/l of Na_2SO_4 (Table 3).

Table 3. Shoot regeneration obtained from calli of five exotic rice varieties at different concentrations of NaCl and Na_2SO_4.

Variety	% regeneration in regeneration medium						
	Control	MS with NaCl (g/l)			MS with Na_2SO_4 (g/l)		
	0.0 g/l	2.5	5.0	7.5	2.5	5.0	7.5
Nonabokra	14.3 (20 ± 0.5)	5.9 (21 ± 0.3)	9.2 (19 ± 0.4)	11.8 (20 ± 0.3)	19.2 (20 ± 0.7)	28.6 (21 ± 0.4)	43.8 (24 ± 0.7)
PNR 381	4.4 (17 ± 0.0)	0.0	0.0	0.0	20.9 (19 ± 0.3)	33.3 (22±0.8)	25.0 (20 ± 0.2)
Taipei 309	30.9 (18 ± 0.4)	21.0 (18±0.3)	18.0 (19±0.6)	0.0	50.0 (19 ± 0.6)	81.3 (22±0.6)	37.5 (18 ± 0.3)
Koshika	10.0 (17 ± 0.7)	0.0	0.0	0.0	50.0 (17 ± 0.4)	57.0 (18±0.4)	62.5 (21 ± 0.3)
TKM-11	6.52 (16 ± 0.0)	0.0	0.0	12.5 (17 ± 0.5)	11.11 (16 ± 0.0)	13.0 (20 ± 0.7)	16.7 (21 ± 0.7)

Number in parenthesis is average height in cm of regenerated plants ± standard error.

Our results revealed that higher concentrations of Na_2SO_4 induced small size of callus from seeds, but higher numbers as well as higher rates of shoot regeneration were obtained from it. It indicates that high concentration of Na_2SO_4 initiated a higher number of embryogenic callus, which finally formed shoot primordia and subsequently a higher number of regenerated plants. Regenera-tion ability enhanced three to tenfolds with Na_2SO_4 at 7.5 g/l,

compared to the control suggesting that this salt concentration is an essential ingredient for higher rate of rice shoot regeneration.

A lower concentration of Na_2SO_4, *e.g.*, 5.0 g/l, was found suitable in induction of higher rate of shoot regeneration in PNR 381 and Taipei 309. In PNR 381 and Taipei 309 shoot regeneration was observed over 8 and 2.5-folds higher, respectively compared to the control (Table 3). At a higher concentration of Na_2SO_4 (7.5 g/l), the regeneration rate declined in both PNR 381 and Taipei 309. Our results indicate that higher regeneration rate varied among different varieties at different concentrations of Na_2SO_4. This indicates that regeneration of rice is not dependent on Na_2SO_4 concentrations alone; however, genotypic effect is also an important factor in higher rate of shoot regeneration. This confirms that rice shoot regeneration is dependent on genotype, salt type and their particular concentration along with callus type and their size.

A correlation was also found in our study between callus size and regeneration ability. Higher regeneration rate was obtained when small sized calli was used as sample materials, obtained from seeds of Nonabokra, Koshika and TKM 11 at higher concentration of Na_2SO_4 (7.5 g/l). Poor regeneration rate was obtained from the above mentioned varieties when bigger sized calli were used from seeds grown at a lower concentration of Na_2SO_4 (2.5 and 5.0 g/l) (Tables 2 and 3). On the other hand, NaCl caused reduction in callus size and also declined regeneration rate in Nonabokra and Taipei 309. No regeneration was found in PNR 381 and Koshika (Tables 2 and 3) with NaCl. Poor regeneration was observed in control.

Average regeneration rates of all varieties were also observed to be higher at all concentrations of Na_2SO_4 compared to control and NaCl (Fig. 2). Similarly, average height of regenerated plants was found to be greater when specific concentration of Na_2SO_4 (5.0 and 7.5 g/l) was added (Table 3, in parenthesis). This indicates that Na_2SO_4 is suitable for higher rate of regeneration and better elongation of regenerated plants than NaCl. For future study NaCl could be excluded for these varieties tested. Different studies showed that NaCl supplemented to the medium enhanced formation of somatic embryogenesis and shoot regeneration (Kishor et al. 1999; Jain 1997). However, our study revealed that Na_2SO_4 instead of NaCl enhanced shoot regeneration in particular varieties. This may due to a genotypic effect. Karim and Zapata (1996) also observed the same effect of Na_2SO_4 in rice androgenesis.

Effect of salt on root initiation: Rooting capability varied in the regenerated plants depending upon genotype as well as salt concentrations. Vigorous rooting was observed in plants regenerated from callus of Taipei 309 and TKM 11 induced in CIM containing 5.0 g/l Na_2SO_4 (Fig. 1c). However, plants of Nonabokra and TKM 11 showed better rooting at 2.5 g/l of Na_2SO_4 compared to

any of the concentrations of NaCl used. Better rooting plays an important role in acclimatization of regenerated plants to the ambient environment resulting in higher survival rate of plants.

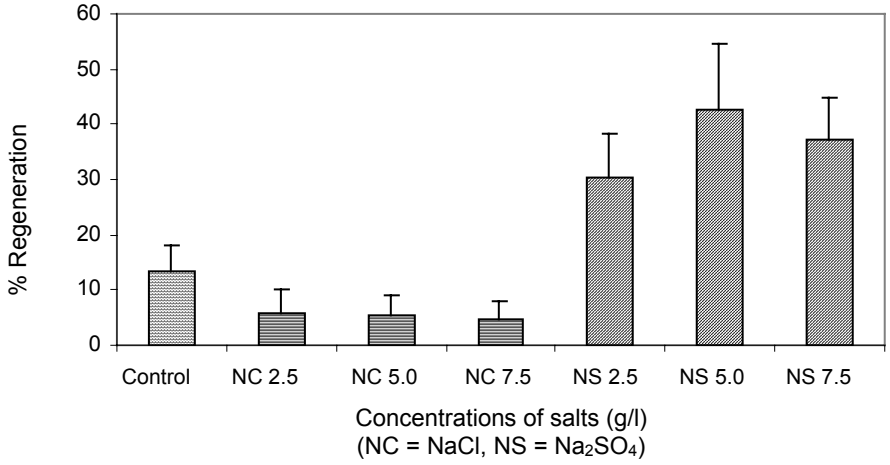

Fig. 2. Average regeneration rates of different varieties obtained in control and under salt stress.

Normal, healthy and green plants regenerated from all varieties cultured with either NaCl or Na_2SO_4 as a supplement. Only one albino plant regenerated from Taipei 309 at the concentration of 5.0 g/l Na_2SO_4. The absence or presence of either NaCl or Na_2SO_4 resulted in green plant regeneration from all varieties tested. The green plants were successfully acclimatised to ambient humidity level and transferred to soil in pots for agronomic study.

Our study revealed that Na_2SO_4 in a particular concentration is an important ingredient for better embryogenic callus initiation as well as better rate of regeneration and rooting ability. The findings would be useful for development of varieties through gene transformation technique.

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