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Identification of Callus Induction and Plant Regeneration Responsiveness in Presence of NaCl in *in vitro* Culture of Some Deepwater Rice (*Oryza sativa* L.) Cultivars

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Abstract: The experiment was conducted to identify the *in vitro* responsiveness for callus induction along with plant regeneration of deepwater rice in the presence of NaCl. In this study, callus induction and plant regeneration responsiveness were tested using different concentrations of 0.1-0.3% (w/v) NaCl which added in MS and LS based media. Marked variation was observed both in calli proliferation and plant regeneration among the six deepwater rice cultivars. In the presence of 0.1% (w/v) NaCl, the highest number of callus induction in all genotypes recorded on the media supplemented with 2 mg L^{-1} 2,4-D + 0.1% (w/v) NaCl. The highest dose of NaCl salt 0.3% (w/v) inhibited callus induction compared to 0.1 and 0.2% NaCl media combinations. Among the cultivars tested, cv. Murabajal produced the highest percentage (39%) of callus whilst cvs. Gheoch (23%) and BR224-2B-2-5 (8%) responded poorly in terms of callus production on the MS based 0.1% NaCl supplemented medium. NaCl had no clear promotive effect on regeneration percentage. In this study, green plant did not regenerate from cv. HA-8. Salinity strongly reduced regenerating capacities of callus obtained from all the cultivars. The mean number of shoots produced per regenerating calli was gradually decreased in all the cultivars when the concentration of NaCl increased in the medium. The apparent tolerance of NaCl in these deepwater rice cultivars may be at least partially related to its growth rate since it can dilute the contents of ion in the shoot. The results of the present study showed the decreasing trend in callus proliferation and plant regeneration with the increasing concentrations of NaCl.

Key words: Embryogenic callus, NaCl, salt-tolerance, plant regeneration, deep-water rice

INTRODUCTION

Considering it's importance as the most major crop in the world, a better understanding of stress signaling in rice would undoubtedly have an enormous impact. 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). For the geographical situation of Bangladesh, large acre of coastal areas (0.83 million ha^{-1}) is a part and parcel for the agricultural economy, which contributes about the 8% of the total rice areas. High concentration of salinity in soil and water are drawn attention of scientist now a day in Bangladesh as well as other many countries due to its harmful effects on rice production (Karim *et al*., 1990). During reproductive development, tolerant genotypes tend to exclude salt from flag leaves and developing panicles (Yeo and Flowers, 1986). Salinity tolerance at the seedling and reproductive stages are only weakly associated; hence, pyramiding of contributing traits at both stages is needed for developing resistance salt tolerance cultivars (Moradi *et al*., 2003). So increases the salt tolerance of rice plant both at the cellular and at the whole plant levels is an important phenomenon for the proper utilization of saline affected area.

For many years, *in vitro* tissue culture contributes a useful tool to study the cultural mechanisms of salt resistance since it allow a control of the homogenesity of the stress and its application at the cellular level, independently a regulatory mechanisms occurring at the whole plant levels. The salt tolerance mechanism involved at the whole plant level could however be quite different from those involved at the plant cell level and an ontogenic evolution of salt tolerance was clearly demonstrated (Adams *et al*., 1992). Application of salt stress during the regeneration process constitutes a convenient way to study the effects of salinity on the morphogenic steps of development. In rice, as in several other species, the presence of NaCl in the regeneration medium strongly reduce (Vajrabhaya *et al*., 1989) or even completely inhibited (Subhashini and Reddy, 1989) regeneration of plants. Several successful attempts to

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improve rice callus regeneration in the absence of stress are reported in the literature. Keeping in mind, the magnitude of the problem related to cultivation of deepwater rice and containing of suffering of millions of farmers in Bangladesh is necessity of research to develop cell to plant system in deepwater rice has been given priority. In this study, callus induction and plant regeneration frequencies were tested using different concentrations of NaCl, which added in different media. The aim of the present study was to analyze the effect of NaCl on rice callus obtained from six deepwater rice cultivars differing in salinity tolerance and on the subsequent survival percentage of regenerated plantlets.

MATERIALS AND METHODS

The experiment was carried out at the Tissue Culture Laboratory of Botany Department, University of Chittagong, Chittagong, Bangladesh during the period from 2004-05. Mature Seed Scutellum (MSS) of selected six cultivars namely; HA-1, HA-2, HA-8, Murabajal, Gheoch and BR224-2B-2-5 were used in the present experiments. At first seeds were dehusked and seeds were then washed thoroughly in the running tap water. The floating dehulled seeds were discarded. Then the seeds were taken in to a sterile tube and immersed into 0.2% (w/v) $HgCl_2$ solution for 15 min. Seeds were then rinsed 4-5 times with sterile deionished water. Then seeds were placed on Murashige and Skoog (1962, MS) and Linsmier and Skoog (1965, LS) based NaCl supplemented callus induction media. MS and LS basal media were supplemented with 2 mg L^{-1} 2,4-D and 0.1-0.3% (w/v) NaCl, 30 g L^{-1} sucrose and 0.8% (w/v) agar. Media were sterilized in an autoclave at a temperature of 121°C for 20 min at 1.16 kg cm^{-1} pressure. After 5-6 days of inoculation, seeds of six cvs. germinated on these media. A mass of callus was formed at the scutellar region after 20 days. After 20 days, calli were further inoculated on the same fresh media. After two sub-cultures, the calli showed variation in colour and texture. When the texture became

Fig. 1(a-d): Effect of NaCl on different stages of callus induction from MSS of different cultivars. (a) 20 days old callus from MSS of cv. Murabajal cultured on MS+2 mg L^{-1} 2,4-D medium supplemented with 0.1% (w/v) NaCl, (b) 35 days old callus from MSS of cv. Murabajal cultured on the same medium, (c) 35 days old calli from cv. Gheoch induced on MS based medium without NaCl treatment (Control) and (d) 35 days old callus on the same medium from cv. Gheoch which was watery and whitish colour

compact and colour became brownish, the calli were then transferred to MS and LS based regeneration media which were supplemented with only 2 mg L^{-1} BAP and 2 mg L^{-1} BAP + 0.1% NaCl, semi solidified with 1% (w/v) agar and incubated calli were kept in the dark for 10 days to produce shoots. After that, calli were then sub-cultured on the same medium, semi solidified with 0.8% (w/v) agar and incubated to the light condition (Fig. 2b). When regenerated shoots grew to about (7-8 cm) in height, they were separated aseptically from each other and transferred to freshly prepared rooting medium supplemented with MS + 2 mg L^{-1} BAP + 1.5 mg L^{-1} NAA + 0.1% NaCl to induce roots. Finally, well-developed plantlets with sufficient root system were successfully transferred to potted soil along with seed derived control plants.

RESULTS

After sub-cultures white to pale yellowish embryogenic calli appeared on the surface of the scutella region (Fig. 1a). At this stage, two types of calli were formed. Regenerable calli were compact, organized white in colour, smooth with knobby appearance, while necrotic along with friable, watery and non regenerable calli were yellow to translucent, wet and rough to crystalline in appearance. In the presence of 0.1% (w/v) NaCl, the highest 39% embryogenic calli induced on MS based medium from MSS of cv. Murabajal (Table 1). The explant MSS which did not produce callus, although a small coleoptile during the 8 days of culture on MS and LS based media supplemented with 0.2% (w/v) and 0.3% (w/v) NaCl with 2 mg L^{-1} 2,4-D of cv. BR224 2B-2-5 (Table 1). Results from different treatments with different concentrations of NaCl showed that the growth rate of calli decreased when concentrations of NaCl increased in the media. During the course of sub-culture, embryogenic calli were selected and transferred to new callus maintenance medium (Fig. 1b). Cultivar Gheoch was induced globular, yellowish, embryogenic calli, when it was transferred on MS supplemented with 2 mg L^{-1} 2,4-D, NaCl free medium (Fig. 1c). On the other hand, cv. Gheoch produced callus placed on regeneration medium, they

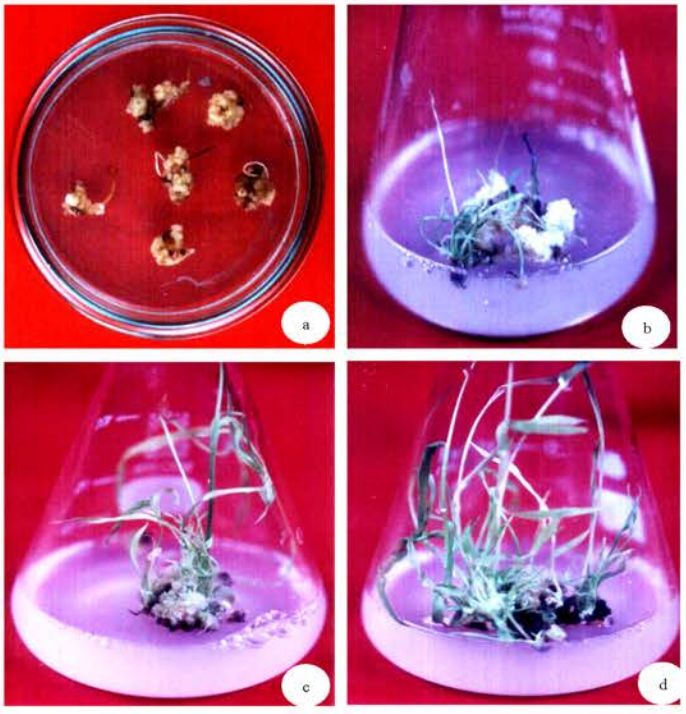

Fig. 2 (a-d): Effect of NaCl treatment on different stages of plant regeneration (a) Embryogenic callus from cv. Murabajal on LS-based regeneration medium supplemented with 0.1% (w/v) NaCl and semi-solidified with 1% (w/v) agar at dark condition, (b) Induction of plant regeneration from embryogenic callus on the same regeneration medium except medium semi-solidified with 0.8% (w/v) agar, (c) Development of shoots on the same NaCl added regeneration medium and (d) Development of multiple shoots on the same regeneration medium after 40 days

were found to be off-white and watery at first sight (Fig. 1d). They gradually became brown, watery and translucent and finally turned in to blakish (Fig. 2a) in colour on MS and LS based media supplemented with 2 mg L^{-1} BAP + 0.1% (w/v) NaCl.

After that the same embryogenic calli were transferred on the NaCl free regeneration media where cv. Murabajal produced 35% of plants on LS based R3M3 medium (Table 2). On the other hand same cultivar produced 33% of plants on 0.1% NaCl added LS based R4M4 medium (Table 2). In case of plant regeneration, all the cultivars responded better on LS based regeneration medium (R3M3 and R4M4, Table 2) compared to MS based regeneration medium (R1M1 and R2M2, Table 2). Interestingly, cv. HA-8 did not produce any plantlets on among the regeneration media tested. Some parts of calli grown in the light condition showed necrosis and finally did not produce shoots. This could be due to salinity stress on callus. Cultivar differences for regeneration ability from the callus was studied on the basis of number of shoots formed per callus. On average most of the calli developed multiple shoots (Fig. 2d), which were

Table 1: Percentage of MSS produced callus on MS or LS based media supplemented with various concentrations of NaCl

	% of callus induction							
	MS or LS based media supplemented with 2 mg L^{-1} 2,4-D and							
	0.1% NaCl		0.2% NaCl		0.3% NaCl		% of embryogenic callus on 0.1% NaCl supplemented media	
Cultivars	MS	LS	MS	LS	MS	LS	MS	LS
HA-1	38	29	27	23	22	18	35	44
HA-2	34	26	26	25	25	13	32	34
HA-8	35	33	24	23	17	14	00	00
Murabajal	39	35	30	33	14	16	5	18
Gheoch	23	21	21	19	10	12	00	15
BR-224-2B-2-5	8	6	0	0	0	0	0	0

Table 2: Comparison of plant regeneration percentage from MSS on MS and LS based regeneration media supplemented with only BAP and BAP+0.1%NaCl

	% of plant regeneration			
	MS based media supplemented with		LS based media supplemented with	
Cultivars	2 mg L^{-1} BAP (R1M1)	2 mg L^{-1} BAP + 0.1%NaCl(R2M2)	2 mg L^{-1} BAP (R3M3)	2 mg L^{-1}BAP + 0.1% NaCl (R4M4)
HA-1	10	7	33	25
HA-2	17	5	20	13
HA-8	0	0	0	0
Murabajal	12	5	35	33
Gheoch	13	8	6	5

Table 3: Average number of shoot produced per regenerating callus when embryogenic calli were cultured on MS and LS-based NaCl free and NaCl supplemented regeneration media

Culivars	MS or LS based medium supplemented with 2 mg L^{-1} BAP or 2 mg L^{-1} BAP + 0.1% NaCl (medium code)	Total No. of inoculated callus	Total No. of callus shoot produced	Average shoot/ regenerating callus
HA-1	R1M1	60	6	4±1
	R2M2	60	4	5±2
	R3M3	72	24	6±2
	R4M4	64	16	5±2
HA-2	R1M1	48	8	4±2
	R2M2	44	2	5±1
	R3M3	60	12	6±1
	R4M4	64	8	3±1
HA-8	R1M1	44	00	00
	R2M2	40	00	00
	R3M3	49	00	00
	R4M4	50	00	00
Murabajal	R1M1	51	6	4±1
	R2M2	57	3	4±1
	R3M3	50	5	4±2
	R4M4	56	4	5±1
Gheoch	R1M1	48	6	3±1
	R2M2	50	4	3±1
	R3M3	48	3	7±1
	R4M4	40	2	6±3

stimulated to be more than one shoots per callus piece. The mean number of shoots produced per regenerating callus (Table 3) was gradually decreased in all the cultivars when the concentration of NaCl increased in the regeneration media (Fig 2c). NaCl had no promotive effect on regeneration percentage and mean number of shoots per regenerating callus. When data were pooled for MS and LS based R1M1 and R3M3 regeneration media, the mean number of shoots produced per regenerating callus was higher compared to NaCl added R2M2 and R4M4 medium (Table 3). The present investigation has demonstrated that the presence of NaCl decreased the number of shoots produced per regenerating callus in all the cultivars. Noticeably, after the inoculation of callus in the regeneration media, in the presence of NaCl, the callusing behavior changed dramatically.

DISCUSSION

Marked variation was observed both in calli proliferation and plant regeneration among the cultivars. The probable reason of this variation could be due to the genetic characters influenced by heredity. The findings of regeneration decrements obtained in the present study were in agreement with many researchers who reported negative response of NaCl towards plant regeneration. Sathish *et al.* (1997) suggested that, soil salinity markedly suppresses the growth of rice (*Oryza sativa* L.). They established rice anther culture to select for rice callus lines adapted to NaCl stress and regenerated plant progenies resistant to a NaCl stress of embryogenic calli. Lutt's *et al.* (1999) reported that salinity strongly reduced regenerating capacities of callus obtained from all cultivars and on all regeneration media tested. According to Kishor *et al.* (1999), the regenerants could be obtained from calli under initial salt stress as a supplement to the callus induction medium. Bong *et al.* (1996) observed only 28 salt tolerant regeneration out of 4050 calli in the medium containing 1.5% NaCl. Binch *et al.* (1992) reported that high increase in salt concentration (up to 1.5%) with a total inhabitation of plant development. Vajrabhaya *et al.* (1989) studied that regeneration rates after stress (1 or 2% NaCl in culture media) were reduced to 0.07% or less as against regeneration rates of 8.3 to 30% normally obtained for non-stressed conditions. These findings were in compliance with present study. NaCl in the regeneration medium had a detrimental effect upon most parameters associated with plantlet regeneration. Several authors reported, a strong NaCl decreased in plantlet regeneration in rice (Vajrabhaya *et al.*, 1989; Binch *et al.*, 1992), which is an agreement with the present study. Salt stress simultaneously presents both an ionic component linked to the toxicity of high external amounts of Sodium and Chloride ions and an hydric component linker to the decrease in the external osmotic potential. Torrizo and Zapata (1986) who demonstrated that different genotypes have varied response to ABA under the same culture conditions. Lutt's *et al.* (1996) demonstrated that in the presence of NaCl, the osmotic potential of the roots and of the oldest and youngest leaves were lower in the salt resistant than in the salt sensitive genotypes, differences among genotype increasing with stress intensity. The apparent tolerance in these deepwater cultivars may be at least partially related to its growth rate since it can dilute the contents of ion in the shoots. From these findings it is concluded that the frequency of callus formation and efficient plant regeneration are influenced by the composition of cultured media in the presence of NaCl and also regenerating capacity of embryogenic callus influenced by the genotype. Peng and Hodges (1989) and Abe and Futsuhara (1991) previously demonstrated that the response of rice cultivars to *in vitro* tissue culture is under complete genetic control and that separate groups of genes are involved in the control of callus induction, callus growth and plant regeneration.

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