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Development of Highly *in vitro* Callogenesis and Regeneration System for Some Salt Tolerant Rice (*Oryza sativa* L.) Cultivars of Bangladesh

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Abstract: Embryogenic calli were initiated from mature seed scutellum (MSS) of salt tolerant Indica rice Cvs. BRRI dhan-40, BRRI dhan-41 and Binnatoa on LS 2.5 and MS 2.5 culture media supplemented with 2.5 mg L^{-1} 2, 4-D. Among the Cvs. used, high frequency callusing from mature seed scutellum was observed in Cv. BRRI dhan-40 (74%) on MS 2.5 medium and Cv. BRRI dhan-41 (79%) on LS 2.5 medium, respectively. In terms of plant regeneration, although all Cvs. responded well on the both RM-1 and RM-2 media but the response of different cultivar varied from medium to medium. The highest frequency (69%) of plant regeneration was observed in the variety Binnatoa on RM-1 medium. In contrast, Cv. BRRI dhan-41 produced the highest percentage (79%) of plant whilst plant regeneration frequency was 75% in Cv. Binnatoa on RM-2 regeneration medium, respectively. Embryogenic callus formation and efficient plant regeneration were influenced by culture media and plant genotype.

Key words: Salt tolerant rice, callogenesis, regeneration, protocol

INTRODUCTION

Salinity is a major inhibitor to agriculture practices. High concentration of salinity in soil and water has drawn attention of scientists now-a-days in Bangladesh as well as other many countries due to its harmful effects on rice production. For the geographical situation of Bangladesh, large acre of costal area (0.83 million ha.) is a parts and parcel for the our agriculture economy which contributes about 8% of the total rice area[1]. But soil and water salinity affects the growth of rice plants at all stages of it's life cycle and also restricts the expansion of the cultivation of modern rice. So to increase 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. Rice is moderately salt sensitive crop[2]. It exhibits considerable intraspecific variability in resistance to salinity[3]. Several research workers have attempted to increase the salt tolerance of rice species, both at the cellular and at the whole plant levels. *In vitro* selection of plants in salt-stressed culture medium is a potential tool to raise plants tolerant to saline environment[4,5]. In rice, Oono and Sakaguchi[6] observed that some mutant plants regenerated from calli grown on a salt enriched medium, were tolerant to high salinity. However, a high concentration of salt in the soil is a limiting factor for rice cultivation. Considering these, development of salt tolerant rice varieties is highly desirable that would permit the expansion of rice cultivation in saline areas. However, BRRI developed two HYV of rice for the costal belt of Bangladesh. BRRI recommended that Brridhan-40 and Brridhan-41 (HYV) can resist salinity which reduce the yield of almost all crops and the degree of reduction depends on the intensity of stresses.

The efficient *in vitro* regeneration of salt tolerant rice plant from cell to plant system via tissue culture is recognized as a prerequisite for nearly all the applications of most modern plant biotechnological approaches for improvement of rice plant. The purpose of this study was to develop an efficient *in vitro* culture system which will provide the basis for future studies on improvements of *in vitro* culture systems and *Agrobacterium*-mediated transformation to transfer gene of interest to salt tolerant Indica rice to increase rice production which is a increasing demand of present world due to the rapid growth of population and environmental changes.

MATERIALS AND METHODS

Seeds of two high yielding varieties (HYV) of rice (*Oryza sativa* L.) namely, BRRI dhan-40, BRRI dhan-41 and one local variety, Binnatoa were collected from Bangladesh Rice Research Institute (BRRI), Gazipur 1701, Bangladesh. Mature rice seeds were dehushed manually and immersed in 0.2% (w/v) $HgCl_2$ solution for 20 min.

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After surface sterilization, these sterilized seeds were rinsed with distilled water 5-6 times to remove $HgCl_2$ solution from surface of the seeds. Then the seeds were placed on the 70 mm sterile filter paper for about 8-10 min to remove excess water. After that, the sterilized seeds were placed in each petri-dish (8 seeds/petri-dish) containing 20 mL callus induction medium. Finally, inoculated petri-dish was sealed with Parafilm and incubated in the dark chamber at 26±2°C in the culture room for 14 days.

LS[7] and MS[8] media were used for callus induction. L.S medium was supplemented with 100 mg L^{-1} myo-inositol, 1.0 mg L^{-1} thaimine-HCI, 2.5 mg L^{-1} 2,4-D and 30 g L^{-1} (w/v) sucrose whilst MS was supplemented with 2.5 mg L^{-1} 2,4-D and 30 g L^{-1} (w/v) sucrose. The pH was adjusted to 5.8 using NaOH or HC1 (where necessary) before autoclaving for 20 min at 121°C. Media were semi-solidified with 0.8% (w/v) agar. Hereafter, these media were designated as LS 2.5 and MS 2.5, respectively. After 14 days, the number of calli induced per petri-dish were counted and recorded and elongated shoots and roots were removed and callus attached to the scutella containing seeds were sub-cultured on their fresh respective medium for another 14 days under the same growth conditions.

The number of total callus lines and embryogenic callus lines (dry, compact. nodular and white-pale yellow in colour) initiation frequencies for the different explants were defined as the percent of total explants forming callus and percent of explants forming embryogenic callus, respectively. Embryogenic callus (8-10 pieces, 5 mm diam. approx.) was transferred to 9 cm petri-dishes containing 20-25 mL of fresh LS 2.5 and MS 2.5 basal media. Sub-culturing was then carried out at 20-25 days interval with transfer of only the embryogenic calli. The later were maintained as mentioned above. Cultures showing slow growth, browning or organogenesis (such as rhizogenesis) were removed during each subculture.

Two types of plant regeneration media were used: designated as RM-I and RM-2. Both RM-1 and RM-2 media contained MS basal salts. RM-1 medium was supplemented with 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and 30 g L^{-1} sucrose (w/v). RM-2 medium was supplement with 2 mg L^{-1} Kn + 0.5 mg L^{-1} NAA and 30 g L^{-1} (w/v) sucrose. Some of the RM-1 and RM-2 media semi-solidified with 1% agar (w/v) for initial inoculation of calli in the dark at 26±2°C for 8-10 days prior to transfer in the light condition and some semi-solidified with 0. 8% agar (w/v) for plant regeneration in the light condition (a cycle of 14/10 h light and dark). The pH of both media was adjusted to 5.8 before autoclaving.

Twenty eight days old embryogenic calli were transferred to 20 mL aliquots of RM-1 or RM-2 regeneration media semi-solidified with 1.0% (w/v) agar and kept for 8-10 days in the dark. After that, calli were transferred to 0.8% (w/v) semi-solidified RM-1 and RM-2 media and kept in the light condition. The shoot regeneration frequencies were recorded 20 days after transfer of tissues to RM-I or RM-2 medium as the percentage of scutella derived-calli each produced one or more shoots. Two shoots (each 2-3 cm in height) were detached from individual calli and each shoot was multiplied by transfer to 175 mL capacity reagent bottle (glass Jars) for 20-25 days, containing 40 mL of rooting medium where MS-beasal medium supplemented with 0.5 mg L^{-1} NAA, 3% (w/v) sucrose and semi-solidified with 0.8% (w/v) agar. Well developed plantlets were transferred to soil conditions along with seed derived control plants. Agronomic characters were recorded after plant reached to maturity.

RESULTS AND DISCUSSION

From selected three varieties namely, BRRI dhan-40, BRRI dhan-41 and Binnatoa Mature Seed Scutellum (MSS) were cultured individually on MS 2.5 and LS 2.5 media, respectively. After 3-4 days, seeds of three varieties germinated on those media. After two weeks of inoculation calli developed at the scutelar region of seeds. During the 14 days prior to subculture, white to pale yellow embryogenic calli appeared on the surface of the scutella region (Fig. 2a). During the course of subculture,

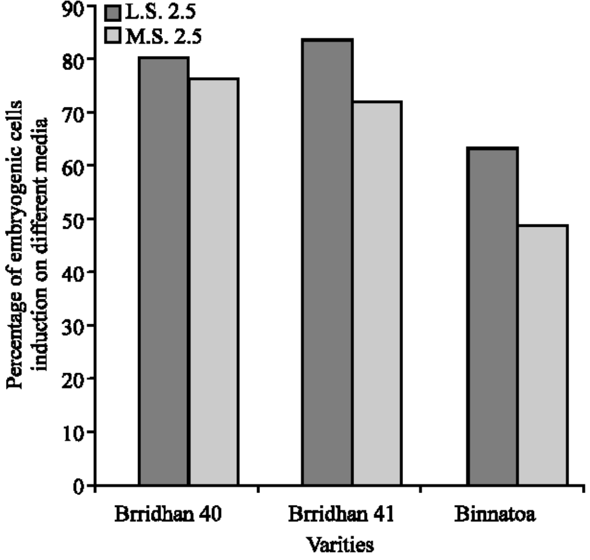

Fig. 1: Frequency of callogenoses on different callus induction media from salt tolerant cultivars

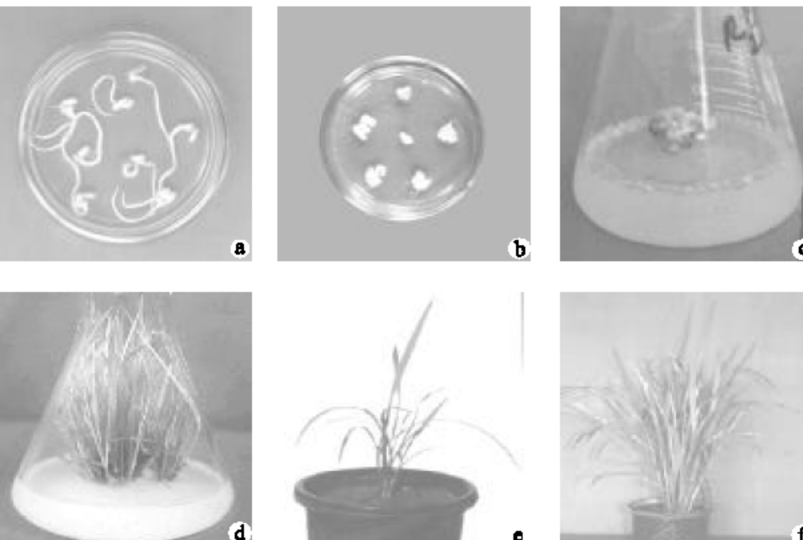

Fig. 2: (a) 14 day old callus, coleoptile, radicle attached with scutellum on MS 2.5 medium of Cv. BRRI dhan-40 (b) Well developed MSS-derived embryogenic callus after 28 days from Cv. BRRI dhan-41 on MS 2.5 medium (c) Embryogenic callus of BRRI dhan-40 on 1% (w/v) agar semi-solidified RM-2 regeneration medium (10 day old) (d) Well developed multiple shoots in RM-1 regeneration medium after 20-25 days from MSS of Cv. BRRI dhan-40 (e) Well developed multiple tillers in pot culture of Cv. BRRI dhan-40 (f) Somatic embryo derived plants of BRRI dhan-40 at the panicle stage under natural conditions

Table 1: Percentage of plant regeneration on different regeneration media

	% of plantlet regeneration	
Salt tolerant cultivars	RM 1 (Mean)	RM 2 (Mean)
Brridhan 40	53	65
Brridhan 41	63	79
Binnatoa	69	75

Table 2: Data for agromorphological characters of somatic embryo and seed-derived plants

	BRRI dhan-40		BRRI dhan-41	
Agromorphological characters	Seed derived plant (control)	MSS calli-derived plant	Seed derived plant (control)	MSS calli derived plant
Number of days to flowering	110	95	112	96
Plant height (cm)	82	75	89	77
Number of panicles	10	25	13	19
Number of tillers	29	28	20	21
Number of seeds/panicle	55	48	61	56

embryogenic calli were selected and transferred to new callus maintenance medium. No morphogenesis was observed under these culture conditions (Fig. 2b). Among the cultivars tested, Cv. BRRI dhan-40 produced the highest percentage (75%) of callus on MS 2.5 medium in contrast of that the callusing percentage of Cv. BRRI dhan-41 on LS 2.5 medium was 82% whilst Cv. Binnatoa responded poorly in terms of callus initiation on the both media (Fig. 1).

Following the two steps regeneration procedure, a initial culture of callus on RM-1 for 8-10 days in the dark were required where the medium semi-solidified with 1% (w/v) agar prior to transfer on the same regeneration medium except concentration of agar was 0.8% (w/v) and cultures were kept in the light condition. Within 14-20 days of culture on the regeneration medium, embryo-like structures were formed on the embryogenic callus surface (Fig. 2c). Three cultivars responded better on RM-2 regeneration medium compared to RM-1 medium in terms of plant regeneration where the highest (75%) percentage of plant regeneration was recorded in Cv. BRRI dhan-41 (Table 1 and Fig. 2d). Percentage of plant regeneration varied cultivar to cultivar (Table 1).

In Table 2, result shows that there are variations for several agromorphological characters depending on plant origin. A sample of two of each of the flowered plants of seed derived (control) and MSS calli-derived plants of Cvs. BRRI dhan 40 and 41 were used for collection of data to evaluate agronomic characters (Fig. 2e and 2f). The height of plants and the number of tiller were reduced compared to that of the seed derived plants. The number of panicle for MSS-calli derived plants was increased compared with control plants. The mean number of seeds/panicle of MSS-derived plants was significantly low compared with seed derived control plants.

The present investigation has demonstrated that mature seed scutellum is the best explant among the explants used for highly totipotent embryogenic callus initiation which is an agreement with earlier observation of a group of scientists. The earlier studies have shown that mature rice seeds were one of the most commonly used explants to initiate embryogenic callus due to their availability throughout the year[9]. In this study, the mature seed of three varieties were found to be capable of forming more embryogenic callus than the callus obtained from coleoptile and root segments of same Cv. at same conditions (data not included). Embryogenic callus formation is probably influenced by interaction between the plant genotype and media components. These observations are consistent with earlier reports of Abe and Futsuhara[10], Hartke and Lorz[11]. These reports have shown that somatic embryogenesis and shoot regeneration are genotype dependent and also strongly influenced by the composition of media.

In terms of callus production from two varieties on LS 2.5 and MS 2.5 medium all varieties showed a better response on LS 2.5 medium compared to MS 2.5 medium. It may be due to difference in medium composition among the two media. In LS 2.5 medium a higher concentration of thiamine-HCl 1.0 mg L^{-1} and absent of some vitamins including nicotinic acid, pyridoxine HCl, glycine could influence the better callogenesis.

There are also many other factors affecting embryogenic callus formation and subsequent shoot regeneration from rice callus[12-15]. Among these, it is well known that 2, 4-dichlorophenoxy acetic acid (2,4-D), an artificial auxin, is indispensable to callus growth in the callus proliferation step, while it inhibits shoot regeneration and embryogenesis[12]. The present investigation for callus initiation under *in vitro* condition could be attributed to the effect of 2, 4-D.

The carbohydrate source is one of the vital components of a culture medium for successful somatic embryo formation with subsequent plant regeneration, since sugar is know to function as an osmotic regulator as well as carbon source of cell growth[16,17]. Sucrose which is the main sugar transported in most plant has been used pre-dominantly as the major carbohydrate source in the culture medium.

In regeneration experiments, another important factor was the concentration of agar used for medium solidification. Shoot regeneration frequencies were tremendously increased when calli were cultured on 1% (w/v) solidified regeneration medium in the dark prior to transfer in the light condition where agar concentration was 0.8% (w/v). Agar, at higher concentrations, by virtue of its solidifying effect on the medium, may limit the water uptake by tissues[18]. Consequently, the tissue was growing on higher concentrations of agar are drier. This condition may also improve the oxygen supply to the embryogenic tissues which ultimately influenced to increase shoot regeneration. This findings is an agreement with the growth of callus and protoplasts of rice increased in oxygen enriched atmosphere[19].

In this study, two hormonal treatments were used for plant regeneration which are a combination of auxin with cytokinin (RM-1) and only cytokinin (RM-2). RM-1 medium based on MS salts supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA and RM-2 medium based on MS salts supplemented with 2.0 mg L^{-1} BAP and 0.5 mL^{-1} Kn. A higher frequency of plant regeneration was obtained on RM-2 medium compared to RM-1 medium for the cultivars used. This demonstrates that the combination of BAP with Kn is a more relevant growth regulator than a combination of BAP and NAA.

Comparison of agronomic characters between seed and MSS-derived plants within a breeding line showed positive shifts in some of agronomic characters which could improve crop production such as plant height, days to flowering, number of panicles in rice Cvs. BRRI dhan-40 and BRRI dhan-41. Previously, Sun *et al.*[20] have reported that such variation in morphological characters at a frequencies as high as 72 and 76% among progenies of large population of somatic tissue derived plants. Adkins *et al.*[21] analysed agronomically important characters in R_2 plants raised from seeds of tissue culture generated R_1 plants. They observed that R_2 somaclonal families on average produced fewer seeds and of a lower mass per seed when compared to the parental plants. However, a proportion of family members exhibited improved seed production, seed weight, shoot biomass and plant height. The present result follows the previous result of Adkins *et al.*[22].

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