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Full Length Research Paper

Genotypic variability in callus induction and plant regeneration through somatic embryogenesis of five deepwater rice (*Oryza sativa* L.) cultivars of Bangladesh

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Experiments were carried out to induce embryogenic callus and plant regeneration from five different deepwater rice cultivars. Using mature embryos as explant, all the cultivars demonstrated high callus induction and plant regeneration frequencies. When coleoptile and root segments used as explants, they had low frequencies of embryogenesis. Depending on different genotypes, the best plant regeneration was obtained on LS (Linsmaier and Skoog, 1965) based medium supplemented with 2 mg l^{-1} BAP + 1.5 mg l^{-1} 2,4-D. Large variabilities in callus growth and plant regeneration potential were revealed among the cultivars tested. Cultivar HA-8 formed a high frequency (78%) of callus than that of other cultivars. In contrast cv. HA-1 produced the highest percentage (72%) of plant regeneration. The callus growth potential was not correlated with the plant regeneration potential. Coleoptiles and root segments produced calli, which did not develop any shoot bud in regeneration media. Moreover, the calli turned blackish, watery and translucent after 25-28 days of culture. It is clear that mature seed scutellum (MSS) is the best explant for callus induction and plant regeneration.

Key words: Callus, regeneration, embryogenesis, genotypic variability, deepwater rice.

INTRODUCTION

Establishment of an efficient tissue culture *in vitro* protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. There is no other more important crop in the world today than rice. There are about five million acres of land under deepwater rice in Bangladesh, representing nearly 20% of the total rice acreage. It is the main crop of nearly 18 million farmers of this country whose life is build on the expectations of a good yields that is rarely achieved. The application of advanced tissue culture techniques may lead to new avenues in crop improvements. Utilizing *in vitro* technique for hybrid embryo culture (embryo rescue) has led to success in the development of addition lines. This also led to production of rice plants, which are stable and fertile as well as carrying resistance to plant hopper, and comparable yield to the *O. sativa* parent (Jena and Khush, 1990). Another important trait of plants derived from somatic embryo is their uniformity and genetic stability. Protoplast fusion, gene transfer, induction of somaclonal mutation, cell or callus culture and subsequent plant regeneration may become routine procedures for crop breeders in future.

The successful application of the tissue culture technique to crop breeding, callus growth and plant regeneration potential of each crop must be determined. Even within the Indica rice group, there are significant variations in the *in vitro* cultures response among different genotypes (Ozawa and Komamine, 1989; Peng and Hodes, 1989). Recently, *Agrobacterium* mediated stable transformation of rice has been reported (Hiei et al., 1994; Al-Forkan et al., 2004). For both the microprojectile and *Agrobacterium* based transformation of rice, efficient regeneration systems, defining both highly responsive Indica genotypes as well as culture conditions need to be identified and standardized. The efficiency of plant recovery from somatic embryos of rice species continues to be unacceptably low because of our lack of understanding of basic developmental processes that underlie somatic embryogeny. Utilization of plant cell regeneration systems in biotechnology ultimately must

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Abbreviations: 2,4-D=2,4-dichlorophenoxy acetic acid; CH = Casein hydrolysate; NAA=1-napthalene acetic acid; BAP=6-benzyl amino purine; MSS=Mature Seed Scutellum; Kn=Kinetin

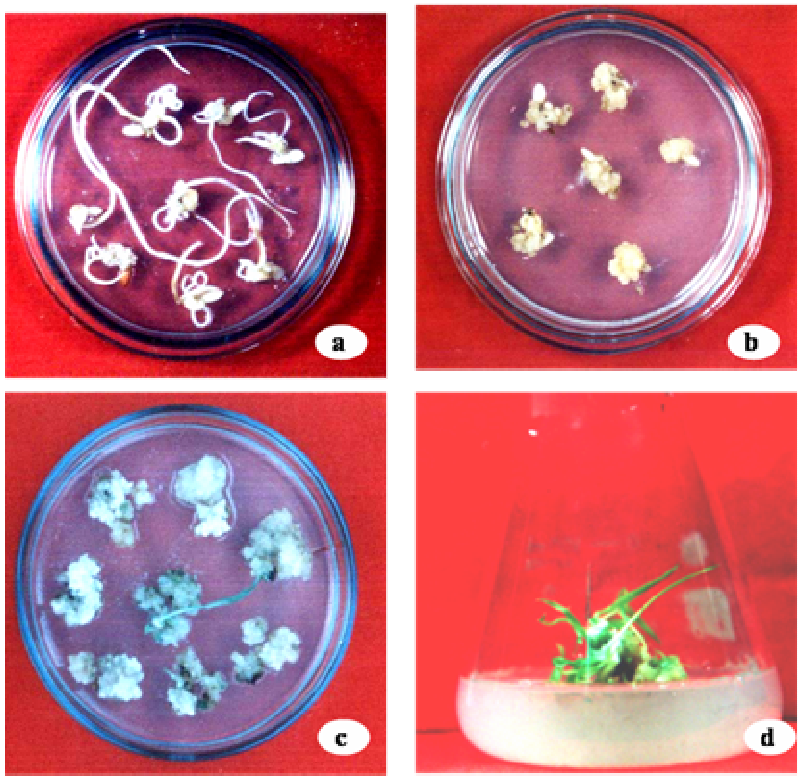

Figure 1. The different *in vitro* stages of inoculated deepwater rice cultivars. (a) 14-Days old callus, coleoptile and radicle attached with scutellum on MS based medium supplemented with 2.0 mgl^{-1} 2,4-D. (b) MSS-derived embryogenic callus from cultivar HA-2 on MS based callus induction medium. (c) 38-Days old greenish, compact and well proliferated embryogenic callus from MSS of cv. HA-1 on 1% (w/v) agar semi-solidified regeneration medium. (d) 44-Days old tiny shoots on the same medium with 0.8% (w/v) agar from cv. HA-1 in light condition.

depend on a deeper understanding of developmental control mechanisms. The development of a reproducible tissue culture system for efficient plant regeneration via somatic embryogenesis from mature seed scutella of deepwater Indica rice cultivars will be a base line to develop a universal approach for rice transformation. The deepwater rice cultivars used in this study were selected on the basis of commercial importance and availability.

The main purpose of this study is not only to develop a *in vitro* protocol for deepwater rice, but also the possibilities to understand and solve the most intricate problems related to *in vitro* culture of different genotypes. The influence and the role of different types and concentration of culture medium on the induction of somatic embryogenesis leading to plant regeneration from mature seed scutellum (MSS), coleoptile and root segments will also be determined.

MATERIALS AND METHODS

Rice cultivars

Five deepwater rice cultivars namely, Habiganj Aman-1, Habiganj Aman-2 and Habiganj Aman-8 released from BRRI regional station and Murabajal and Gheoch land races grown in the coastal saline region of Comilla and Hatiya were used in this study as source of explants.

Callus induction

Dehusked seeds from five deepwater rice were placed in two basal media MS (Murashige and Skoog 1962) and LS (Linsmaier and Skoog, 1965). Both the basal media were supplemented with 2 mg l^{-1} 2,4-D, 0.1% CH, 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin on the basis of requirement and also 30 g l^{-1} sucrose added and media were solidified with 0.8% (w/v) agar. Three explants, MSS, coleoptiles and root segments from different rice cultivars were used for callus induction. Prior to culture, dehusked seeds were sterilized with 0.2% (w/v) $HgCl_2$ solution. In case of coleoptile segments, germinating seedling in the hormone free MS medium were taken out from culture vessels for dissection after 4 to 5 days culture, and coleoptile segments were separated from endosperm (scutella) by cutting of the base of coleoptiles. The tips of the coleoptiles were removed. The separated whitish hollow and tubular coleoptiles (1-1.5 cm long) without any internal organs were used for culture. Seedling roots (10-12 days post germination) were excised and cut into 1-1.5 cm sections. For callus induction nine sterilized seeds per petri dish were inoculated in 20 ml callus induction medium and five replications for each treatment were maintained. Finally seed inoculated petri dish was sealed with parafilm paper and incubated in the dark chamber at 26 ± 2°C in the culture room (Figure 1a). After 14 days, shoots and roots were excised (for MSS) and the

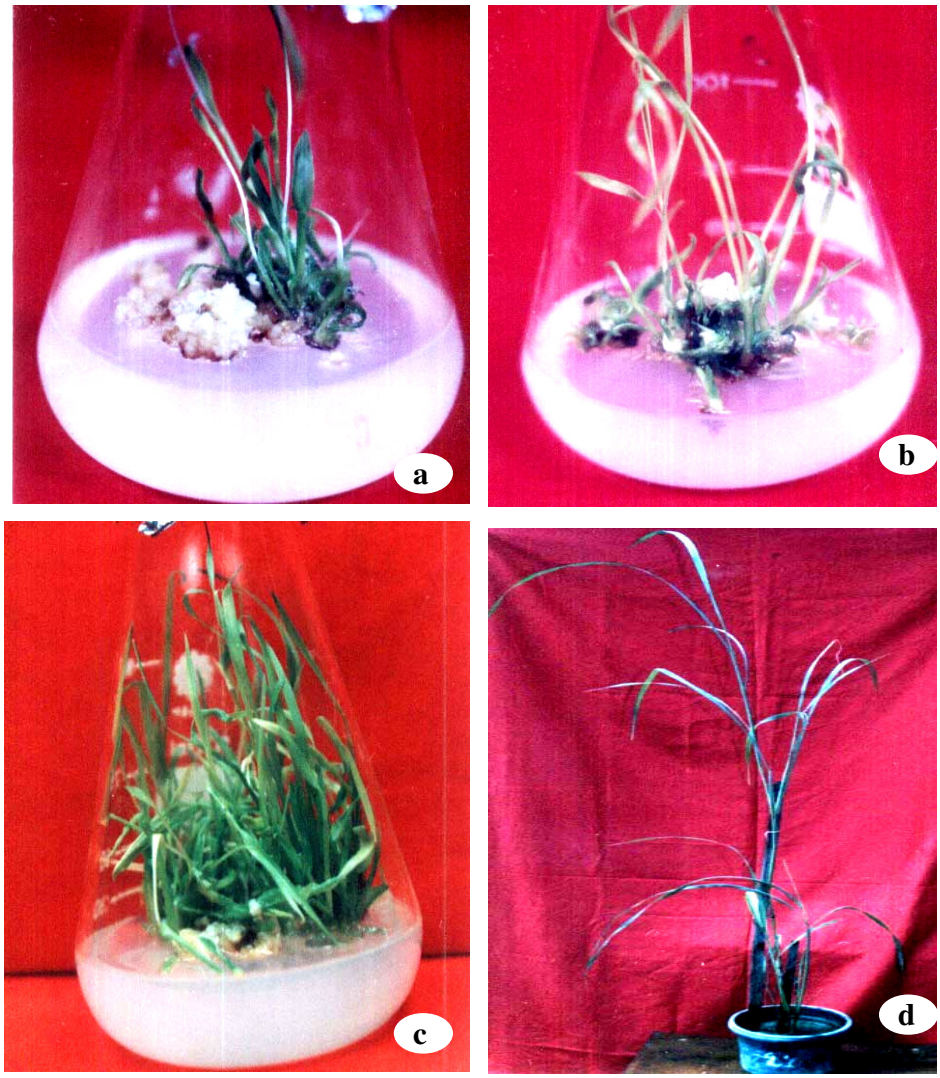

Figure 2. Following photographs are showing the different *in vitro* stages of plant regeneration. (a and b) Multiple shoots differentiated from the embryogenic callus after 13 days of transfer in the regeneration medium. (c) Well-developed multiple shoots showing rapid growth on regeneration medium. (d) MSS-derived plant grown in the soil conditions (4 months old).

scutella derived calli were transferred to fresh medium and maintained for a further 14 days under the same growth conditions (Figure 1b). On the other hand, after 21 days, induced callus from coleoptile and root segments were sub-cultured on the same medium for further 21 days.

Plant regeneration

Calli were transferred to MS and LS basal media supplemented with 2 mg l^{-1} BAP, 0.5 mg l^{-1} kinetin, 1.5 mg l^{-1} 2,4-D and 1.5 mg l^{-1} NAA as required and 30 g l^{-1} (w/v) sucrose. The medium was gelled with 0.8% (w/v) or 1% (w/v) agar. During the course of sub-culture, explants-produced embryogenic calli were selected and transferred to MS and LS based regeneration media with 1% (w/v) agar. The calli, which turned to pale yellowish in colour, watery, blakish and shown dry, were rejected. The shoot regeneration frequencies were recorded 20 days after transfer of tissue to regeneration medium. When regenerated plants (MSS derived) of five cultivars were 8-10 cm in height, they were cultured on MS + 1.5 mg l^{-1} NAA supplemented rooting medium. Well developed plantlets (after 20-25 days) were taken out from rooting medium, their roots were washed with tap water to remove excess agar (Figure 2c) and were grown in natural conditions (Figure 2d).

RESULTS

Two types of calli were formed namely, embryogenic (compact, yellowish and big in size, Figure 1b) and non-embryogenic (friable, translucent and slimy). Significant differences were observed among the MSS-derived embryogenic callus from five different genotypes. The highest percentage (78%) of callus was observed in cultivar HA-8 in MS medium supplemented with 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP, where the percentage of embryogenic

Table 1. Interaction between the genotype and growth regulators on callus induction from MSS of deepwater rice cultivars.

Cultivar	MS or LS based media supplemented with 2 mg l^{-1} 2,4-D +							
	0.5 mg l^{-1} BAP				0.1% (w/v) CH			
	% of MSS produced callus		% of embryogenic callus		% of MSS produced callus		% of embryogenic callus	
	MS	LS	MS	LS	MS	LS	MS	LS
HA-1	59	50	54	58	40	48	45	48
HA-2	43	37	42	45	43	40	40	43
HA-8	78	70	50	48	60	50	42	46
Murabajal	71	60	52	55	63	47	50	51
Gheoch	46	43	37	39	33	30	33	35

Table 2. Comparison of responsiveness for callus formation from the coleoptiles and root segments of five deepwater rice cultivars on different media.

Cultivar	MS or LS based media supplemented with											
	2 mg l^{-1} 2,4-D				2 mg l^{-1} 2,4-D + 0.1% (w/v) CH				2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kinetin			
	Coleoptile		Root		Coleoptile		Root		Coleoptile		Root	
	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS
HA-1	20*	16	60	58	26	21	68	65	30	27	77	71
HA-2	16	14	50	44	21	17	70	67	23	19	73	60
HA-8	10	13	53	51	15	17	62	60	25	23	64	62
Murabajal	15	12	58	51	18	13	62	53	19	17	65	58
Gheoch	21	19	40	37	22	18	56	50	25	22	70	65

*% of callus induction.

Table 3. Percentage of embryogenic callus formation from coleoptile and root segments of different cultivars on different media.

Cultivar	MS or LS based media supplemented with											
	2 mg l^{-1} 2,4-D				2 mg l^{-1} 2,4-D + 0.1% (w/v) CH				2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kinetin			
	Coleoptile		Root		Coleoptile		Root		Coleoptile		Root	
	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS
HA-1	50*	54	43	47	30	31	44	49	44	38	50	54
HA-2	45	49	35	39	28	33	49	56	37	39	51	53
HA-8	21	27	28	33	18	22	40	45	20	23	35	41
Murabajal	48	59	25	37	41	45	55	58	43	47	56	60
Gheoch	48	52	18	23	28	35	28	35	40	46	43	46

*% of embryogenic callus formation.

callus was 50% and was the highest percentage of callus (Table 1). On the other hand, callusing frequency in the genotype Gheoch was lower (46%), and the embryogenic callus was 37% in the same medium. This means cultivar HA-8 was better than other cultivars in terms of callus production. These results indicated a good deal of differences between genotypes. It is clear that callus growth varied widely among cultivars in the same medium. The cultivars HA-1, HA-2 and HA-8 produced the most prolific, compact, nodular, yellowish and large callus while cultivars Murabajal and Gheoch produced sometimes hardy, rooty and non-prolific callus. Among the cultivars, calli of the later two cultivars were slightly necrotic. In case of coleoptile explant, there were significant differences in respect of callus initiation among the cultivars. The genotype HA-1 demonstrated the highest percentage of callusing on MS based media (Table 2).

The highest embryogenic natures of calli were observed in cv. Murabajal (59%) and cv. HA-1 (54%) on LS medium supplemented with 2 mg l^{-1} BAP (Table 3). The lowest percentage (18%) of embryogenic calli induced from cultivar HA-8 was obtained on 0.1% CH (w/v) supplemented media (Table 3). A wide range of variations in

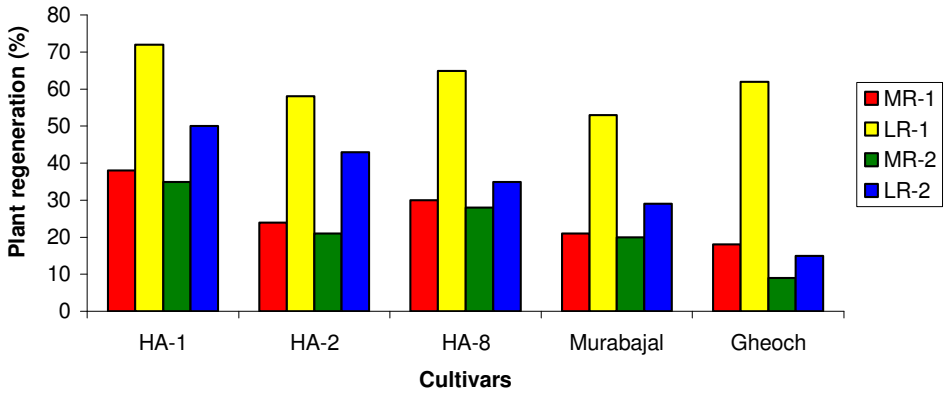

Figure 3. Percentage of plant regeneration from MSS on MS and LS based regeneration media supplemented with different additives.

terms of total callus induction frequency and percentage of embryogenic callus formation from coleoptile segments were observed. When the root sections were cultured on MS and LS based media contained with 2,4-D, CH or Kn; small, globular and grainy calli arose from lateral root primodia within about 20 days of culture. The highest percentage of callus formation (77%) was observed in root segments of cv. HA-1 on MS based medium supplemented with 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} kinetin, while cultivar HA-8 produced 64% of callus in the same medium (Table 2). The range of percent of embryogenic callus production from root segments were 18-43% and 23 -47% on MS and LS media (Table-3). When the embryogenic calli were transferred to the different combinations of MS and LS based regeneration media (after 10 days), green-pigmented spots were first observed at the middle part of callus tissue (Figure 1c), which developed in to tiny shoots (Figure 1d). The primodia which pigmented green, were surrounded by a highly organized leaf like structure (Figure 2a, b). It was observed that the plant regeneration ability of plated calli depended on the genotypes. Among the studied rice cultivars, cv. HA-1 regenerated maximum number of plants (72%) followed by HA-8 (65%), Gheoch (62%), HA-2 (58%) and Murabajal (53%) on LS based medium supplemented with 2 mg l^{-1} BAP + 1.5 mg l^{-1} 2,4-D (LR-1). It is apparent from Figure 3 that calli cultured on LR-1 medium showed better performance in plant regeneration for all the cultivars compared to others media such as MS + 2 mg l^{-1} BAP + 1.5 mg l^{-1} 2,4-D (MR-1), MS + 2 mg l^{-1} BAP + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA (MR-2) and LS + 2 mg l^{-1} BAP + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA (LR-2) combinations.

DISCUSSION

Remarkable genotypic variations in plant regeneration from MSS derived calli were observed (Figure 3). Cultivar HA-8 showed good callus growth 78% (Table-1) and comparatively lower (30%) shoot formation (Figure 3). Cultivars HA-1 and HA-2 showed poor callus growth 50 and 37%, respectively (Table 1) but produced high shoot regeneration 72 and 58%, respectively. Khanna and Raina (1998) reported that the genotypes influenced differently both on callus induction and plant regeneration. Among the five cultivars tested, it is clear that cvs. HA-1 and Gheoch produced significantly different percentage of plant regeneration that demonstrates the genotypic variation in terms of plant regeneration and also medium interaction. This observation recorded in the present study is the agreement with the findings of Pandey et al. (1994). The authors reported that the success of *in vitro* cultures largely depends on the nutrition, growth regulators, variety and the interaction between the variety and medium. Similar reports were also made by Gao and Cao (1982). Significant variation has been reported in the response amongst different plant genotypes with the Indica cultivars in terms of callus formation as well as plant regeneration (Seraj et al., 1997). The present study has confirmed that the high frequency of embryogenic callus formation is probably influenced by the interaction of plant genotypes.

In the present study, callus initiation was investigated from MSS, coleoptiles and root segments from five deepwater rice cultivars. Here, plant regeneration from cpoleoptile and root segments was unsuccessful. It seems that coleoptile and root derived calli were not totipotent for plant regeneration. However, the present investigation has demonstrated that mature seed scutellum is the best explants among the explants for highly totipotent embryogenic callus initiation, which corroborates with the findings of Al-Forkan et al. (2005). Plantlet regeneration dramatically increased when callus was subjected to culture on 1% (w/v) agar gelled medium rather than 0.8% (w/v) agar medium. Earlier it was reported that the same gelling agent as various concentrations have profound influence in retention of water and regulation of moisture regime of the medium,

which influences plantlet regeneration response immensely (Suprasanna et al., 2000). High concentration of gelling was found to improve plantlet regeneration in this study, which was similar with the report of Jain et al. (1996) in rice. In this study, large genotypic variations in callus growth and plant regeneration potential were observed in deepwater rice cultivars. The identification and screening of useful genotypes for callus growth and plant regeneration are prerequisite for the application of tissue culture techniques to new breeding programs of rice for genetic improvements. It can be concluded that the *in vitro* protocol described here could be used for efficient plant regeneration of deepwater rice cultivars followed by useful gene transformations.

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