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Regeneration Efficiency and Genotypic Effect of 15 Indica Type Bangladeshi Rice (*Oryza sativa* L.) Landraces

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Abstract

Genotypic effect was observed for embryogenic callus induction and subsequent plant regeneration in 15 indica type Bangladeshi rice (*Oryza sativa* L.) landraces. Different growth regulators were found to influence callus induction ability and green plant regeneration. The highest frequency (55.6%) of shoot regeneration was observed in Hashikalmi with the supplementary hormone concentrations and combination of 2 mg/l BA, 1 mg/l NAA and 1.5 mg/l Kn in MS. The embryogenic calli derived from mature seeds successfully produced shoots, and rooted well on MS basal salt with IAA. Five landraces which could be utilized in further manipulation, such as transformation and *in vitro* hybridization, were found to have higher regeneration ability (± 50%). In vitro regenerated plants were successfully established in soil and produced fertile seeds.

Introduction

Rice (*Oryza sativa* L.) is an important cereal grown in many countries of the world in wide range of agro-climatic conditions and a major source of calories for more than one-third population of the world (Khush 1997). The production of rice is affected by a number of biotic and abiotic stresses. Some of the major biotic and abiotic stresses, adversely affecting rice production, are bacterial bligh, blast, brown plant hopper, stem borer, drought, salinity, and submergence under water. The genetic variability for some of these traits is limited in cultivated rice germplasm. Thus, it is important to broaden the rice gene pool by introgressing new genes from diverse sources to meet the challenge affecting rice production. Wild species of rice are considered to be important source for such useful genes. However, several incompatibility barriers limit the transfer of useful genes

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(Brar and Khush 1986,1997). Recent advancement in biotechnology, such as transformation, and *in situ* and *in vitro* hybridization enhanced the introgression of new genes from different sources to the cultivated species. However, the aforementioned methods are effective for rice when an efficient and reproducible regeneration system is available. Therefore, identification of useful genotypes for callus growth and successful *in vitro* plant regeneration is a prerequisite for such genetic improvement of rice (Abe and Futsuhara 1986; Hoque and Mansfield 2004). In the present study, 15 *indica* type rice landraces were used for experimental purpose as they have some special characteristics including drought, saline and tidal submergence tolerance. Most of the landraces are moderately drought tolerant, and are grown in direct seeded condition. Some of the landraces are moderately tolerant to saline, submergence and non-saline tidal submergence. The latter were collected from the coastal region of Bangladesh.

The major problems of *in vitro* culture of *indica* rice are the low rates of callus production and subsequent plant regeneration (Chu and Croughan 1990). In general, it has been found that immature plant parts and meristematic tissues, which contain undifferentiated cells, are more suitable for plant regeneration than mature one (Morrish et al. 1987). In rice, immature embryos were found to be more responsive for tissue culture (Rueb et al. 1994). Since this explant is available only for a short period of the growth cycle of rice plant, other explants such as mature embryos, leaves and roots which are available year round, are more suitable for rice tissue culture, provided that a high frequency of plant regeneration can be achieved (Rueb et al. 1994; Hoque and Mansfield 2004). Therefore, the objective of this study was to screen *indica* type Bangladeshi rice landraces which were not used so far for experimental purpose to establish any efficient *in vitro* plant regeneration system for future use in genetic transformation and *in vitro* fusion studies.

Materials and Methods

Experiments were conducted at the Environmental Science Laboratory of the University of Southampton, UK during 2000 and 2001. Fifteen rice landraces received from Bangladesh Rice Research Institute (BRRI), Gazipur-1701, Bangladesh were included in this study. The landraces were collected from different ecotypes and rainfall distribution pattern of Bangladesh with some special characteristics are given in Table 1 (Bashar and Sarkar 1997; BRRI 1995-2004). Mature and healthy seeds were selected and dehusked. They were first surface sterilized in 70% (v/v) ethanol for three minutes with gentle shaking. Thereafter, they were washed with sterilized distilled water for three times. Then the seeds were again surface sterilized in 50% (v/v) commercial bleach (approx.

5% NaOCl) for 30 minutes with gentle agitation and subsequently rinsed with sterilized distilled water for five times.

Table 1. List of *indica* type rice landraces which were used in the present experiments.

Name of accession	BRRI accession number*	Growing season**	Collection district	Rainfall pattern[a]	Special attributes
Dular	0022 DA-22	Aus/ Upland	Dhaka	Moderate rainfall	Moderately drought tolerant
Panbira	4150	Aus/ Upland	Khulna	Moderate rainfall	Moderately drought tolerant
Boalia	2068	Aus/ Upland	Kishoregonj	Moderate rainfall	Moderately drought tolerant
Binamuri	2184	Aus/ Upland	Bogra	Moderate rainfall	Moderately drought tolerant
Bakee	2358	Aus/ Upland	Jamalpur	Moderate/ heavy rainfall	Moderately drought tolerant
Dudh Kalam	0278	T. Aman	Gaibandha	Low/ moderate rainfall	Submergence tolerant
Kumari	0203	T. Aman	Mymensingh	Moderate/ heavy rainfall	-
Hoglapata	3871	T. Aman	Barisal	Moderate rainfall	Tidal submergence tolerant
Kumragoir	3878	T. Aman	Barisal	Moderate rainfall	Tidal submergence tolerant
Kalamuna	0984	T. Aman	Khulna	Moderate rainfall	Moderately saline tolerant
Tilock Kachari	0758	T. Aman	Chittagong	Heavy rainfall	Submergence tolerant/photoperiod sensitive
Aus Kushi	3501	Aus/ Upland	Satkhira	Moderate rainfall	Saline/bacterial blight tolerant
Hashikalmi	0030 DA-23	Aus/ Upland	Dhaka	Moderate rainfall	Moderately drought tolerant
Hijolee	0571	Aus/ Upland	Rangpur	Low/ moderate rainfall	Moderately drought tolerant
Kumari Aus	2100	Aus/ Upland	Jamalpur	Moderate /heavy rainfall	Moderately drought tolerant

[a]Low rainfall < 1600 mm, moderate rainfall 1600-2500 mm, heavy rainfall > 2500 mm, - data not available. **DA means Dhaka Agricultural station collection number ** Rice growing ecosystem, Aus = Summer rice, T. Aman = Autumn/rainfed lowland rice, Boro = Winter/irrigated rice (Oka 1991).

MS basal salt supplemented with 3% sucrose (w/v) (Sigma); 1.5 - 2.5 mg/l, 2,4-D, 50 mg/l nicotinic acid and solidified with 0.5% agarose (w/v) (Sigma) was used to find out the callus induction ability of the genotypes. Three different concentrations *viz*. 1.5, 2 and 2.5 mg/l of 2,4-D were used in the media to evaluate their effect on callus induction ability. The pH of the media was adjusted at 5.75 before sterilization. Media were transferred evenly (25 ml each)

into Petri dishes and were sterilized by autoclaving at 121°C and at 1.1 kg per cm^2. Thereafter, sterilized seeds were placed in Petri dishes and incubated for the assessment of callus induction ability at 27 ± 1°C under darkness for four weeks. Experiments were replicated three times and eight Petri dishes with six seeds were used per replication for each genotype. After four weeks of incubation, calli were isolated from endosperms and other debris. Only embryogenic calli were selected and transferred on to fresh medium for multiplication, and subsequent shoot and root initiation. Subculture was carried out once in every two weeks with transfer of only the vigorously growing portions of calli. Browning calli were discarded. Percentage of induction ability for each genotype was calculated dividing the total number of seeds produced embryogenic callus by the total number of seeds cultured and multiplied by 100.

Regeneration ability was investigated at two stages i.e. at shoot initiation and at the time of root formation at different hormone concentrations and combinations.

MS basal salts were used for plant regeneration in four media. Media were supplemented with 3% sucrose (w/v) (Sigma), 100 mg/l myo-inositol and solidified with 0.5% agarose (w/v) (Sigma), and four different concentrations and combinations of hormones were used for shoot development. Compositions of regeneration media were as follows. RM I : 1 mg/l BA, 1 mg/l NAA and 1 mg/l Kn; RM II : 2 mg/l BA, 1 mg/l NAA and 1 mg/l Kn; RM III : 2.5 mg/l BA, 1 mg/l NAA and 1 mg/l Kn and RM IV : 2 mg/l BA, 1 mg/l NAA and 1.5 mg/l Kn. pH of the media was adjusted to 5.75 before autoclaving for sterilization. For regeneration, eight-weeks-old embryogenic calli were placed into glass jar containing 20 ml of medium and were incubated at 27 ± 1°C under light/dark cycle of 16/8 hrs with irradiance level of about 55 - 60 μ mol m^{-2} S^{-1}. Experiments were replicated three times and 12 calli were used per replication for each treatment for each genotype. Data on shooting ability/plantlet regeneration were collected after four weeks of transferring the calli on to media.

Regenerated shoots were transferred for root development to the rooting medium consisted of MS basal salt supplemented with 3% sucrose (w/v) (Sigma), 100 mg/l myo-inositol and solidified with 1% agar (w/v) (Sigma) instead of agarose, and only 1 mg/l IAA was used. When plantlets were big enough with healthy roots were transferred into soil.

The percentage of plant regeneration response for each genotype was calculated dividing total number of calli produced plantlets by the total number of calli cultured and multiplied by 100.

A completely randomized design with three replications per treatment for each genotype was followed in this study. ANOVA was integrated for assessment of the variation due to genotypes and media. LSD test was used to

show the variation between mean values of different parameters. Standard error of means was also calculated.

Results and Discussion

Mature dehusked rice seeds were used for callus initiation and after four weeks large calli were formed from the scutellum in all the three media. Both embryogenic and non-embryogenic calli were initiated (Fig. 1A). Embryogenic calli were found to be yellow to white, dry, compact and nodular (Fig. 1B). On the other hand, non-embryogenic calli appeared to be watery, light yellow to tan and non-nodular. After four weeks, the first subculture was carried out and calli were removed from seed endosperms and transferred onto fresh media.

Percentage of callus induction ability was significantly affected by the rice genotypes ($F = 95.25$, $p < 0.01$) and the media ($F = 8.98$, $p < 0.02$), but the interaction between media and genotype was not significant at 5% level on the basis of ANOVA. Callus induction ability highly varied between the landraces ranging from 0.69 to 83.80% (Table 2). The landrace Hijolee had the highest frequency of callus production ability (83.80%) among the landraces followed by Kumri Aus (77.08%) and Hashikalmi (75.69%). The landraces Panbira, Bakee and Auskushi also produced higher number of calli (Table 2). It appears from the results that high concentrations of 2,4-D produced higher number of calli in almost all the genotypes. Better callusing frequency was also observed in 2.5 mg/l 2,4-D (Table 2).

Due to poor callus induction and subsequent plant regeneration ability (%), two landraces, namely Dular and Boaila were discarded from the analysis of regeneration ability. Like the callus induction ability, plant regeneration ability (%) was also significantly affected by the rice genotypes ($F = 72.70$, $p < 0.01$) and the media ($F = 14.82$, $p < 0.01$) but media and genotype interaction was not significant on the basis of ANOVA. Table 3 shows the mean values of plant regeneration ability (%) of *indica* rice genotypes in four media. It appears from the table that the range of variability was high between the genotypes ranging from 8.0 to 56.0% which was also observed in callus induction ability. The landrace Hashikalmi had the highest frequency of regeneration ability (45 - 55%) between the rice landraces followed by Kumri Aus (44.5 - 52.8%) and Hijolee (47 - 50%). The regeneration efficiency was also high in the landraces Panbira, Bakee and Auskushi which ultimately produced considerable higher number of plantlets (Table 3).

As mentioned above, apart from genotypic effect, significant media effect was also observed in the present study. It appears from the results that the different combinations and concentrations of phytohormones produced different frequencies of green shoots (Table 3). In general, shooting media 3 and 4 gave

better results where considerably higher concentration of BA and Kn was used compared to other media. The plantlets having the height of more than 2 cm were transferred to the rooting medium for root development. All the plantlets produced healthy roots using the MS basal salt supplemented with 3% sucrose (w/v) (Sigma), 100 mg/l myo-inositol and solidified with 1% agar (w/v) (Sigma), and only 1 mg/l IAA was used (Fig. 1C). When plantlets were big enough with healthy roots these were transferred to soil. These subsequently developed mature plants (Fig. 1D).

Table 2. Effect of genotype and concentration of 2,4-D on callus induction ability (%) (mean ± SE) in rice.

Variety	CIM 1	CIM 2	CIM 3	Mean of 3 media
Dular	1.39 ± 1.39e	0.69 ± 0.69g	1.39 ± 0.69e	1.16 ± 0.61g
Panbira	73.61 ± 4.22ab	72.92 ± 5.51abc	80.56 ± 2.78a	73.39 ± 4.28b
Boalia	0.69 ± 0.69e	0.69 ± 0.69g	1.39 ± 0.69e	0.69 ± 0.41g
Binamuri	25.69 ± 1.84d	31.94 ± 3.03f	32.64 ± 0.69d	30.32 ± 0.83ef
Bakee	65.97 ± 7.35ab	73.61 ± 2.50abc	70.14 ± 3.67a	71.07 ± 2.21b
Dudh Kalam	53.47 ± 5.68bc	58.33 ± 6.70cd	52.78 ± 2.50b	55.79 ± 0.61c
Kumri	42.36 ± 8.45cd	52.78 ± 1.84de	52.08 ± 5.24bc	48.15 ± 3.41cd
Hoglapata	38.89 ± 4.86cd	37.50 ± 7.31ef	38.89 ± 3.67bcd	40.28 ± 4.62de
Kumragoir	34.03 ± 4.86cd	31.25 ± 4.81f	39.58 ± 3.61cd	34.95 ± 2.57ef
Kalamuna	31.94 ± 4.86d	36.11 ± 7.05ef	35.42 ± 4.17cd	36.34 ± 2.67ef
Tilock Kachari	27.78 ± 2.50d	31.25 ± 4.34f	31.94 ± 2.78d	29.63 ± 1.89f
Auskushi	65.28 ± 9.03ab	64.58 ± 5.24bcd	74.31 ± 3.03a	68.98 ± 6.03b
Hashikalmi	70.83 ± 7.88ab	79.86 ± 4.86ab	81.94 ± 3.03a	77.08 ± 4.18ab
Hijolee	84.03 ± 3.87a	84.72 ± 5.68a	82.64 ± 3.67a	84.72 ± 4.01a
Kumri Aus	72.22 ± 3.03ab	76.39 ± 4.55ab	75.69 ± 3.03a	75.69 ± 1.44ab
LSD (1% level)	21.40	19.10	14.67	10.24
CV (%)	20.71	17.33	12.76	17.00

Data were taken from average of three replications and each replication had 48 seed explants (Nine observations were used to calculate a mean). Means followed by a common letter are not significantly different at the 0.01 level of probability.

Embryogenic and non-embryogenic callus formation as well as plant regeneration are genetically determined factors (Maddock et al. 1983; Hodges et al. 1986). Several authors have also been reported significant genotypic variation in callus induction and subsequent plant regeneration potential in rice (Abe and Futsuhara 1985; Hartke and Lörz 1989; Lee et al. 2002; Hoque and Mansfield 2004). The present study also showed significant differences in callus induction ability between various rice landraces and the frequency of callusing ability (%) varied widely between the landraces (0.69 to 83.80%, Table 2). This observation

agreed with the findings of Hartke and Lörz 1989; Lee et al. 2002; Hoque and Mansfield 2004. After four weeks of incubation, different sizes of both embryogenic and non-embryogenic calli were observed in different landraces

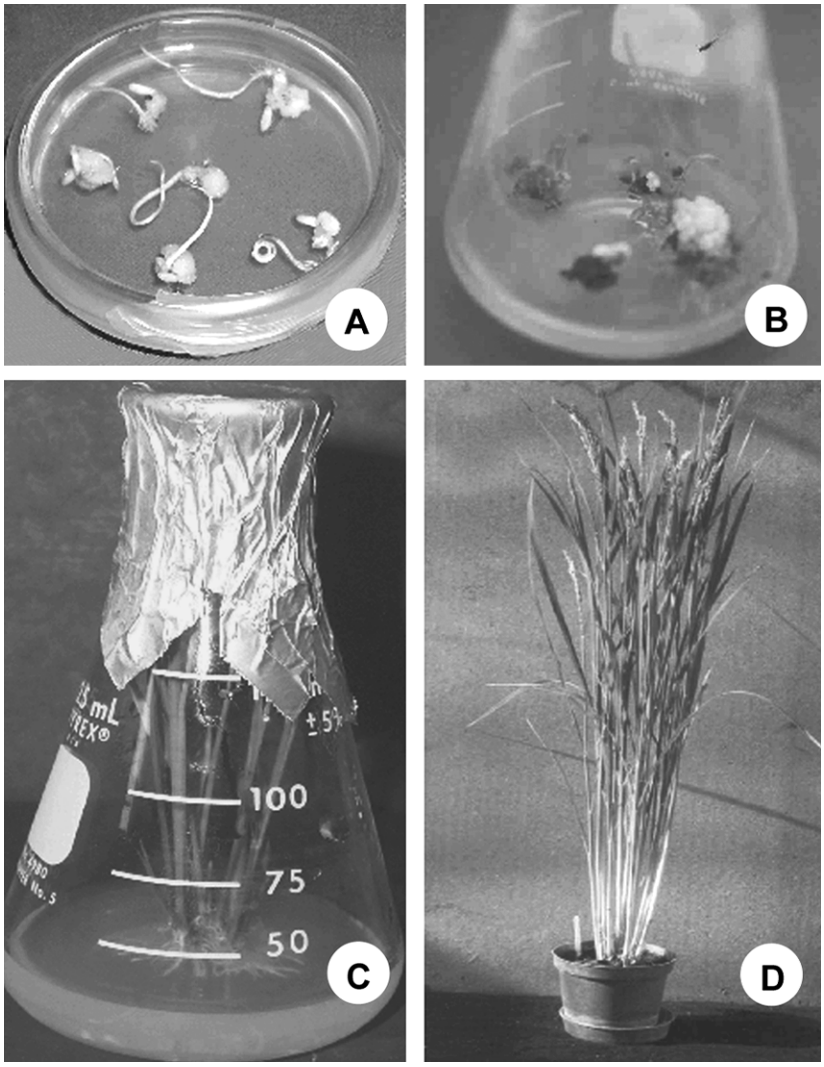

Fig. 1. Different steps of plant regeneration (callus induction to maturity) from mature embryos. (A) Callus induction from mature embryos (four-week-old calli), (B) embryogenic callus initiating green shoots, (C) regenerated plant from mature seeds derived calli with healthy root system and (D) mature rice plants in soil.

(Fig. 1A). The callusing ability was found to be directly proportionate to the concentration of 2,4-D. Reports are also available which show that the addition of adequate levels of 2,4-D, into basal medium resulted in proliferation of callus from variety of rice explants (Hartke and Lörz 1989; Lee et al. 2002).

Regeneration efficiency of rice plants was also affected by media composition, explants source and age, and culture environments (Torbert et al. 1998; Aditya et al. 2004; Hoque and Mansfield 2004). Several reports are available which show that genotype and nutrient composition of the medium are the most important factors for efficient rice plant regeneration (Jain 1997; Khanna and Raina 1998) and appropriate media composition can increase regeneration efficiency (Khanna and Raina 1998; Lee et al. 2002; Khatun et al. 2003). In this study, media RM III and RM IV produced more green plantlets where higher concentration of BA and Kn was used compared to other two media (Table 3). This higher rate of green plantlets production may be associated to the effect of higher concentration of BA, NAA and Kn. Similar reports have shown that some combinations of auxin and cytokinin along with the effect of basal salts played an important role for callus formation and subsequent plant regeneration (Prodhan et al. 2001; Lee et al. 2002).

Table 3. Effect of genotype and media combination with growth regulators on shoots formation (mean ± SE) in rice.

Variety	RM I	RM II	RM III	RM IV	Mean
Panbira	36.11 ± 2.78a	41.67 ± 4.81a	44.44 ± 2.78a	47.22 ± 2.77a	42.36 ± 0.69b
Binamuri	13.89 ± 2.78b	16.67 ± 4.81b	16.67 ± 4.81bc	19.44 ± 2.78b	16.67 ± 1.20de
Bakee	38.89 ± 2.78a	44.44 ± 2.78a	47.22 ± 2.78a	47.22 ± 5.56a	44.45 ± 1.84b
Dudh Kalam	19.44 ± 2.78b	25.00 ± 4.81b	27.78 ± 2.78b	25.00 ± 4.81b	24.31± 0.69c
Kumri	13.89 ± 2.78b	22.22 ± 2.78b	25.00 ± 4.81bc	16.67 ± 4.81b	19.44 ± 3.47cd
Hoglapata	16.67 ± 4.81b	22.22 ± 2.78b	25.00 ± 4.81bc	25.00 ± 4.81b	22.22 ± 0.69cd
Kumragoir	13.89 ± 2.78b	16.67 ± 4.81b	19.44 ± 2.78bc	16.67 ± 4.81b	16.67 ± 1.20de
Kalamuna	13.89 ± 2.78b	13.89 ± 2.78b	19.44 ± 2.78bc	19.44 ± 2.78b	16.67 ± 1.20de
Tilock Kachari	8.33 ± 4.81b	11.11 ± 2.78b	11.11 ± 2.78c	11.11 ± 2.78b	10.42 ± 1.20e
Auskushi	38.89 ± 2.78a	44.44 ± 2.78a	47.22 ± 2.78a	50.00 ± 4.81a	45.14 ± 0.69b
Hashikalmi	47.22 ± 2.78a	55.56 ± 2.78a	55.56 ± 2.78a	55.56 ± 2.78a	53.47 ± 1.84a
Hijolee	47.22 ± 2.78a	47.22 ± 2.78a	50.00 ± 4.81a	50.00 ± 4.81a	48.61 ± 3.67ab
Kumri Aus	44.44 ± 2.78a	47.22 ± 2.78a	47.22 ± 2.78a	52.78 ± 2.78a	47.92 ± 2.08ab
Mean	27.14 ± 2.44	31.41 ± 2.55	33.55 ± 2.51	33.55 ± 2.78	31.41 ± 2.46
LSD (1% level)	12.50	14.45	14.35	16.20	6.78
CV (%)		20.18	20.15	18.72	21.14

Data were taken from average of three replications and each replication had 12 calli (Nine observations were used to calculate a mean). Means followed by a common letter are not significantly different at the 0.01 level of probability.

The present screening test results showed that out of 15 landraces, two failed to produce green plantlets from callus and the rest produced plantlets with different rate of success, which clearly indicated the genotypic effect of plant regeneration (Tables 2 and 3). The identification of genotype(s) with superior

culture ability is a prerequisite for further experiments, such as transformation, *in vitro* hybridization and somatic hybridization. It appears from the present study that at least five indica rice landraces were found to be best for embryogenic callus induction and green plant regeneration. Such type of finding will prove useful for their further manipulation.

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