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Effect of cold pretreatment and different media in improving anther culture response in rice (*Oryza sativa* L.) in Bangladesh

Rokhshana Khatun[1], S M Shahinul Islam[1]*, Israt Ara[1], Narendra Tuteja[2] and M A Bari[1]

[1]Institute of Biological Sciences, University of Rajshahi, Rajshahi 6205, Bangladesh

[2]Plant Molecular Biology Group, International Center for Genetic Engineering and Biotechnology (ICGEB) New Delhi 110 067, India

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In vitro production of doubled haploid (DH) plants through anther-culture provides an efficient and convenient system for rapid production of homozygous lines. Various pretreatments have been reported to influence callus induction and plantlet regeneration efficiency. In the present study, cold pretreated anthers influenced the embryoid formation in five different induction media where hormonal combination was modified. Regeneration potential of 25 rice cultivars was assessed on the basis of anther response, embryo induction, plantlet regeneration and production of green and albino plantlets. Of 20 rice cultivars, embryoids were obtained from only five cultivars on media containing specific amino acids and different combination of phytohormones. IR43 produced maximum embryos (16.13%) and green plantlets (11.88%), followed by BRRI dhan33, IR54, Jaya and BR3 in SK3 medium. All the responding genotypes produced albinos in addition to the green plantlets. Cold pretreatment at 4°C for 3-7 d generated highest frequency of embryos and green plantlets. However, all the responding genotypes showed better induction from cold pretreated anthers in comparison to the control. 5-d (T_3) cold pretreatment was found to be the most effective compared to other treatments on SK3 medium.

Keywords: Albinos, anther-culture, cold pretreatment, embryoids, media, plantlet regeneration, rice

Introduction

Successful production of haploid plants through anther culture was first reported in *Datura innoxia* by Guha and Maheshwari[1] in 1964. Since then many androgenic lines have been developed in agricultural crops through anther and microspore culture[2]. Novel androclones of rice developed through anther-culture by Chen *et al*[3] had been shown to have higher quality and more yield. Moreover, haploids and doubled haploids provide useful tools in plant breeding of agriculturally important crops. Earlier, plant breeders used recurrent self-fertilization to obtain homozygosity, which had been a time consuming and tedious process taking about 5-10 years. On the other hand, complete homozygous plants can be produced within a year through anther and microspore culture.

Production of haploids in rice through anther culture was first reported in 1968 by Niizeki and Oono[4]. However, the spectacular progress in rice anther culture has been made only in the recent past[5-7]. Studies have shown that callus induction and anther culture response to plant regeneration were highly dependent on the genotype of donor plants[8] and the medium used for the culture[9]. The importance of culture medium in the induction of callus and plantlet regeneration of rice through anther cultures was also demonstrated by Oono[10]. Further, Ogawa *et al*[11] observed a higher degree of plantlet regeneration and green plant production in the presence of amino acids compared to their absence or presence of alanine in the culture medium. Raina[12] found that addition of maltose to the induction medium resulted in the enhancement of plantlet regeneration rate in rice.

Chaleff and Stolarz[13] reported better anther culture response in rice when anthers were pretreated at 7°C for 3 d in comparison to untreated ones. Zapata *et al*[14] observed that rice anthers pretreated by cold shock at 8°C for 8 d displayed best result. Further, Pande[15] found cold pre-treatment essential for androgenesis in the anther culture of indica cv. IR43 and 10°C for 10 d was the most conducive environment for the maximum culture response. Pre-treatments longer than 11 d resulted in the production of albino plants. However, regeneration of green plants is the most important factor to be considered to improve the efficiency of androgenesis.

*Author for correspondence:
Tel: +880-721-750928; Fax: +880-721-750064
Mobile:+88-01715209907
E-mail: shahin_ibsc@ru.ac.bd

The present investigation was undertaken to find out highly anther-culture-responsive genotypes, and to standardize the induction medium and cold pretreatment period for anther culture of rice in Bangladesh.

Materials and Methods

Seeds of 20 rice (*Oryza sativa* L.) cultivars, *viz.*, IR43, IR54, Jaya, BR3, BR4, BR5, BR9, BR10, BR11, BR22, BR23, BR25, BRRI dhan28, BRRI dhan29, BRRI dhan30, BRRI dhan31, BRRI dhan33, BRRI dhan34, BRRI dhan37 and BRRI dhan39, were collected from Bangladesh Rice Research Institute, Joydebpur, Gazipur and Regional Station, Rajshahi, Bangladesh. Plants were grown in the field of the Institute of Biological Sciences, Rajshahi University, Bangladesh during the growing seasons from November 2007 to June 2008. Spikes were harvested when the flag leaf had just emerged and microspores conditions were at the early to mid uninucleated stage, as observed by 1% acetocarmine staining. Harvested spikes were subjected to cold pretreatment at 4-7°C for 1-15 d in the dark.

Various genotypes, as mentioned earlier, were evaluated on different induction media, *viz.*, N_6[16], R2[17], He2[18], MO19[19] and SK3, for their androgenetic callus induction ability. N_6 medium contained (mg/L): KNO_3 (2830), $(NH_4)_2SO_4$ (463), $CaCl_2.2H_2O$ (166), $MgSO_4.7H_2O$ (185), KH_2PO_4 (400), $MnSO_4.4H_2O$ (4.4), $ZnSO_4.7H_2O$ (1.5), KI (0.8), H_3BO_3 (1.6), $FeSO_4.7H_2O$ (27.8), Na_2EDTA (37.3), glycine (2), thiamine-HCl (1), nicotinic acid (1), pyridoxine-HCl (0.5), 2,4-D (2.5), sucrose (3%) and phytagel (4). SK3 medium was a modification of N_6, in which sucrose was substituted with maltose (60 g/L); in addition, it also contained (mg/L): NAA (2.5), L-proline (500), L-glutamine (500) and yeast extract (1000). *p*H of all media was adjusted to 5.8 to 6.0.

To optimize the cold pretreatment duration, harvested spikes containing early to mid uninucleate microspores were stored for 1-d (T_1), 3-d (T_2), 5-d (T_3), 7-d (T_4), 9-d (T_5), 11-d (T_6), 13-d (T_7) and 15-d (T_8) at 4-7°C in the dark chamber. The spikes with no treatment (grown under normal condition) were harvested on the day of culture initiation. For this study, SK3 medium was employed as it gave best performance on the embryo induction in comparison to other media employed in the earlier study. Spikes were sterilized with 70% ethyl alcohol, washed in sterile distilled water, kept for 2-3 min in 0.1% $HgCl_2$ solution and then again washed 4-5 times with sterile distilled water. An average of ~ 40-50 anthers were cultured per Petri dish (6 mm) containing semi-solid (4-5 mL) and liquid (2-3 mL) induction medium. The cultures were kept at 27±2°C for 4-10 wk in the dark for embryo induction. Anther derived embryos were transferred to suitable semi-solid regeneration medium and subsequently transferred to the culture room at 16/8 h light/dark regime for plantlet regeneration. Data were recorded on anther-culture-response, frequency of embryo induction, embryo regeneration, and green and albino plants per 100 anthers.

Results and Discussion

Varietal Response and Media Effect

In the present study, 20 rice cultivars were tested for assessment of their androgenic response. Each genotype was cultured separately on five induction media. Of these cultivars, good number of embryoids (Fig. 1A) and regenerated plantlets (Figs 1B & C) were observed in five genotypes, *viz.*, IR43, BRRI dhan33, IR54, Jaya and BR3, which were cultured on N_6, R2, SK3, He2, & MO19 media. Among the five responding genotypes, IR43 showed best response on

Fig. 1 (A-D)—A. Anther-derived embryoids in rice, B. Embryoids were transferred to the semi-solid regeneration medium, C. Regenerated green and albino plantlets developed in semi-solid regeneration medium, & D. Development of shoots and roots from anther-derived embryos of rice.

SK3 medium, where the frequency of embryo production was 50.63%, followed by BRRI dhan33, IR54, Jaya and BR3 (Table 1). In this study, Z1 medium was also tested but there was no callus induction response. It was observed that, on N_6, He2, MO19 and R2 media, the androgenic response was very poor in comparison to SK3. Genotypic mean response and the efficiency of induction media (N_6, R2, SK3, He2 & MO19) are presented in Table 1. On the basis of above results, it may be concluded that, out of five media, SK3 was the best induction medium for anther culture response in rice. The superior response of anthers in respect on callus induction in SK3 might be due to the presence of amino acids at a relatively higher concentration and substitution of maltose with sucrose. Ogawa *et al*[11] also found glutamine to be promotory in callus induction from pollens in microspore cultures of an *indica* cultivar. However, alanine was found better than glutamine for plantlet regeneration and green plant production. In comparison to sucrose, maltose has been found superior source of carbohydrate for androgenesis in several species, including cereals[20]. A significant increase in anther culture efficiency and green plant formation was reported in *indica* rice cultivars when sucrose was replaced by maltose[12]. These studies corroborate the findings of the present study. Grewal *et al*[21] reported that occurrence of albinism is a common phenomenon in anther culture in cereals and the same was also found to be true in the present study.

Table 1—Androgenic callus induction of genotypes, culture media and its productivity on androgenic response in rice

Medium	Genotype	Response of anthers (%)	Frequency of embryo induction (%)	Frequency of embryo regeneration (%)	Frequency of green plants (%)	Frequency of albino plants (%)
N_6[16]	IR43	15.63	26.25	16.05	11.38	2.88
	BRRI dhan33	11.25	14.38	9.38	5.63	4.38
	IR54	10.63	13.13	8.75	4.63	3.13
	Jaya	9.75	10.50	7.63	3.88	3.38
	BR3	7.25	7.88	4.88	1.75	1.88
	Mean	10.90	14.43	9.34	5.45	3.13
R2[17]	IR43	2.88	4.75	3.63	2.50	0.88
	BRRI dhan33	1.88	3.13	2.13	1.50	0.63
	IR54	1.63	2.63	1.88	1.25	0.63
	Jaya	1.38	2.38	1.38	0.88	0.25
	BR3	1.13	1.88	1.25	0.75	0.38
	Mean	1.78	2.95	2.05	1.38	0.55
SK3	IR43	23.63	59.63	16.13	11.88	3.63
(modified N_6)	BRRI dhan33	16.88	35.50	10.13	7.13	2.88
	IR54	15.38	34.75	11.38	8.50	2.63
	Jaya	14.88	28.38	10.63	8.63	2.75
	BR3	11.88	15.88	8.50	5.13	2.25
	Mean	16.53	33.03	11.35	8.25	2.83
He2[18]	IR43	5.13	10.38	5.75	4.25	1.25
	BRRI dhan33	3.38	7.38	3.88	2.75	1.13
	IR54	3.63	8.50	4.13	3.38	0.50
	Jaya	2.88	7.75	3.63	2.00	1.38
	BR3	2.13	5.38	2.38	1.38	0.88
	Mean	3.43	7.88	3.95	2.75	1.03
MO19[19]	IR43	3.88	7.63	5.13	4.13	0.75
	BRRI dhan33	2.75	5.75	4.13	2.63	1.25
	IR54	2.38	4.88	3.50	2.38	1.13
	Jaya	2.25	4.38	3.25	1.88	1.38
	BR3	1.50	2.88	1.63	1.13	0.50
	Mean	2.55	5.10	3.53	2.43	1.00

Cold Pretreatment

Spikes pretreated at 4-7°C for different durations were cultured on SK3 medium. The highest embryo production was found in a 5-d (T_3) induction period and the percentage of embryo production was 88.88, 77.17, 79.67, 78.88 and 73.50% for IR43, BRRI dhan33, IR54, Jaya and BR, respectively (Table 2). Moreover, the efficiency of green plantlet regeneration for the corresponding cultivars was 24.25, 19.67, 20.17, 19.13 and 17.33%, respectively (Fig. 1C & D). In all treatments, there was significant increase in embryo formation and plant yield in comparison to the control (Table 2). In T_3, frequency of embryos and green plantlet regenerants were better compared to all other treatments, including the control. Importance of cold pretreatment of panicle in the anther culture of

Table 2—Influence of cold pre-treatment (4°C) on anther response and percent plantlet regeneration in rice

Cold pre-treatment	Genotypes	Anther response (%)	Frequency of embryo induction (%)	Frequency of embryo regeneration (%)	Frequency of green plants (%)	Frequency of albino plants (%)
Control	IR43	3.13	6.75	3.63	2.63	1.25
	BRRI dhan33	1.83	5.67	1.83	1.33	1.83
	IR54	1.67	4.83	2.00	1.67	1.67
	Jaya	1.13	3.25	1.63	1.38	1.13
	BR3	1.17	2.83	1.33	1.17	1.33
	Mean	1.79	4.67	2.08	1.64	1.44
	Diff.	-	-	-	-	-
T_1	IR43	16.63	38.38	14.88	11.13	5.88
	BRRI dhan33	13.83	32.83	11.17	7.50	6.17
	IR54	12.67	31.17	11.50	7.83	5.67
	Jaya	11.25	29.50	9.88	6.88	4.38
	BR3	10.17	27.83	8.83	5.17	5.17
	Mean	12.91	31.94	11.25	7.70	5.45
	Diff.	11.12	27.27	9.17	6.06	4.01
T_2	IR43	39.63	73.13	28.38	21.13	8.13
	BRRI dhan33	35.83	70.83	25.33	18.83	10.50
	IR54	37.17	70.17	29.17	19.33	9.67
	Jaya	34.25	67.13	24.38	17.13	6.88
	BR3	31.33	65.33	22.33	15.50	8.67
	Mean	32.74	69.32	25.92	18.38	8.77
	Diff.	30.95	64.65	23.84	16.74	7.33
T_3	IR43	50.88	88.88	32.13	24.25	9.63
	BRRI dhan33	37.33	77.17	25.33	19.67	11.33
	IR54	40.50	79.67	28.50	20.17	10.17
	Jaya	39.75	78.88	27.25	19.13	7.25
	BR3	33.17	73.50	23.67	17.33	9.83
	Mean	40.33	79.62	27.38	20.11	9.64
	Diff.	38.54	74.95	25.30	18.47	8.20
T_4	IR43	25.13	42.25	20.13	16.13	7.13
	BRRI dhan33	21.33	38.33	17.33	13.33	6.33
	IR54	22.17	37.17	16.17	12.17	5.67
	Jaya	20.38	36.00	15.25	11.13	4.50
	BR3	18.50	32.50	13.50	9.33	4.83
	Mean	20.55	37.25	16.48	12.42	5.69
	Diff.	18.76	32.58	14.40	10.78	4.25

(*Contd*.)

Table 2—Influence of cold pre-treatment (4°C) on anther response and percent plantlet regeneration in rice (*Contd.*)

Cold pre-treatment	Genotypes	Anther response (%)	Frequency of embryo induction (%)	Frequency of embryo regeneration (%)	Frequency of green plants (%)	Frequency of albino plants (%)
T_5	IR43	20.38	37.13	14.25	9.38	3.38
	BRRI dhan33	18.17	33.17	10.33	7.33	5.17
	IR54	17.33	32.50	10.67	6.67	4.50
	Jaya	16.13	30.38	9.13	6.13	3.13
	BR3	14.50	28.17	7.17	4.67	4.67
	Mean	16.53	32.27	10.31	6.84	4.17
	Diff.	14.74	27.60	8.23	5.20	2.73
T_6	IR43	9.38	18.25	6.63	4.38	5.25
	BRRI dhan33	7.67	15.17	4.50	2.83	7.17
	IR54	6.33	13.67	4.33	2.67	5.50
	Jaya	5.25	12.25	3.38	2.25	4.25
	BR3	3.67	10.50	2.67	2.17	4.33
	Mean	6.46	13.97	4.30	2.86	5.30
	Diff.	4.67	9.30	2.22	1.22	3.86
T_7	IR43	7.50	15.38	5.63	3.25	6.13
	BRRI dhan33	5.67	12.17	3.83	1.83	6.33
	IR54	4.33	11.50	4.17	1.67	5.67
	Jaya	3.13	9.25	3.25	1.63	3.63
	BR3	2.50	7.17	2.33	1.17	4.83
	Mean	4.63	11.10	3.84	1.91	5.32
	Diff.	2.84	6.43	1.76	0.27	3.88
T_8	IR43	6.38	14.50	5.13	3.38	4.63
	BRRI dhan33	4.33	10.17	3.50	1.33	5.83
	IR54	3.00	8.83	3.83	1.17	4.83
	Jaya	2.63	7.13	3.13	1.13	3.75
	BR3	1.83	4.33	2.17	0.83	2.83
	Mean	3.63	8.10	3.55	1.57	4.37
	Diff.	1.84	3.43	1.47	0.07	2.93

Control=Without any cold treatment; T_1 =1-d, T_2 = 3-d, T_3 = 5-d, T_4 = 7-d, T_5 = 9-d, T_6 = 11-d, T_7 = 13-d and T_8 = 15-d cold treatment at 4°C to harvested spikes; Diff. = Mean difference of treatments performance was estimated compared to control; SK3 medium was used for its better performance.

cereals has also been realized in other studies[22,23]. Croughan and Chu[24] found cold pretreatment (5°C) of panicles very effective in rice, but the induction potential was decreased when immature inflorescences were pretreated for long time. The critical factor for cold pre-treatment of the panicles was reported to be at 10°C for 28 d before isolation of the microspores[11]. However, in the present study, the best pretreatment time of the anthers was reported to be 5 d at 4-7°C, which may be attributed to the difference in the genotypes of plant material. These results further indicate that cold pre-treatment, media and genotypes synergistically contribute to enhanced induction and regeneration of androgenetic plants.

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