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In vitro Plant Regeneration Through Anther Culture of Some Iranian Local Rice (*Oryza sativa* L.) Cultivars

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Abstract: In this study, effect of different genotypes, different callus induction and regeneration media were investigated using seven Iranian local rice. Anthers were cultured on N6, Fj and L8 media containing the same hormonal combination 3 mg L^{-1} NAA, 0.5 mg L^{-1} Kn and 0.5 mg L^{-1} 2, 4-D and incubated at 25±1°C in dark for callus induction. All varieties in L8 medium, five varieties in Fj medium and six varieties in N6 medium produced highest percentage of callus. Calli induced in different induction media were transferred to SK 11 and N 19 medium and incubated at 25±1°C in light for plantlet regeneration. Among seven varieties upon transfer to SK 11 medium, highest percentage (40%) of green plants were produced in Hassani and in N 19 medium the highest percentage (15.78) of green plants and albino plants (21.05) were produced in Anbarbo. The finding in the present investigation showed that the successfully emberyogenesis and green plant regeneration in rice anther culture dependent on medium culture components and are affected by the genetic mack-up of the plants.

Key words: Rice, anther culture, callus induction, regeneration, media

INTRODUCTION

Rice is the most important crop at the global level, as it is used as a staple food in most countries of the world. Indica type rice feeds more than two billion people, predominantly in developing countries (IRRI, 1992).

Production of callus and its subsequent regeneration are the prime steps in crop plant to be manipulated by biotechnological means. Different tissues have been used in rice as explants (Bhaskaran and Smith, 1990).

Haploid plant production has been reported in more than 200 species (Dunwell, 1986). Today, androgenic haploids have been developed in economically important plant including vegetable and cereals crop (Villenx, 1994; Cao *et al*., 1995). The production of haploid plants from anther culture technique offers a rapid achievement of homozygous lines for early release of new crop varieties. Besides, it allows the use of haploid cells or protoplast for the induction and selection of recessive mutants.

In the recent past anther culture in rice has been improved substantially. However, detailed study on various factors govering culture response of anthers under *in vitro* condition especially in indica rice is extremely limited. Generally green plant regeneration from androgenic calli is very low irrespective of race. Low anther culture response, high percent of albino plantlet regeneration and abundance of haploids are principal constraints in establishing successful anther culture in rice (Roy and Mandal, 2005).

Most of the *in vitro* morphogenic response are genotype-dependent (Bhojwani and Raza, 1996). Karim *et al*. (1991) showed that callus induction efficiency in Z_2 was higher than other media, although different varieties hold different degree of potentially of anther induction in different media. In general, indica cultivars of rice exhibit poorer androgenic response that the japonica cultivars (Hu, 1985; Raina and Zapata, 1997). Miah *et al.* (1985) reported that anther culture response varied from 41% for a japonica cultivars to 0% from an indica cultivar. Even among the indica cultivars a considerable variation for pollen callusing and plant regeneration has been observed. The occurrence of a large proportion of albinos in the pollen plant population is probably the most frustrating feature of androgenesis in its application to rice breeding. The frequency of albino may vary from 5 to 100%. Indica rice cultivars are more prone to this problem than japonica rice. Several factors, including pre-treatment, culture medium and the protocol, affect the frequency of albinos. Ogawa *et al.* (1995)

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reported that a significant increase in anther culture efficiency and green plant formation in otherwise highly recalcitrant indica rice cultivars occurred when sucrose was replaced by maltose. The literature on androgenesis in cereals suggests that albinism can be considerably reduced by shortening the culture period (Karim *et al*., 1991; Asaduzzaman *et al*., 2003).

Under present initiative attempts have been taken towards finding the best media and standardizing the condition necessary to stimulate callus induction and green plantlet regeneration from cultured anthers of different Iranian rice varieties.

MATERIALS AND METHODS

Anther donor plants of seven local Iranian rice varieties which were widely grown in the north of Iran and were popular for their high domestics and international market owing to high consumers preference, were taken from the field of rice research institute, Amol, during the main crop season (April and September, 2003). The panicles with boot leaf sheath were washed to roughly in tap water and spread with 70% ethanol. They were covered with moist tissue paper, kept in polyethylene bag and cold shocked at 6-10°C for 6 days in a BOD incubator perior to anther plating. On the day of culture, selected spikelets were surface sterilized in tissue culture bottles with 0.1% freshly prepared $HgCl_2$ solution for 10 min the $HgCl_2$ was drained off and the panicles were washed four times in sterile distilled water. Fifty to sixty spikelets were cut at a time on sterile petridishes under laminar airflow (LAF) bench. Individual spikelets were cut at the base to free the anthers from the filaments. On an overage 40 anthers were inoculated in 10 mL callus induction medium. Three callus induction media viz., N6, Fj and L8 (Table 1) supplemented with NAA and 2,4-D in 7 cm, petridishes were used for this purpose. The cultures were sealed with parafilm and kept in dark at 25±1°C[6]. The plates were examined periodically at weekly interval to observe the progress in respect of callus formation. Emberyogenic calli of at least ~2 mm diameter were transferred to 25×150 mm culture tubes containing 10 mL of tow modified MS regeneration media (Murashige and skoog, 1962) viz., SK11 and N19 were exposed to 16 h photoperiod for shoot regeneration (Table 2). The pH of all media was adjusted at 6.2 and all media were solidified with Difco-bacto agar. Anther response and green plantlet regeneration were observed in each experiment by counting the No. of calli/anther and green plants/callus, respectively. A complementary randomized design was used for all experiments. Each experiments was replicated three times with 40 samples in each replicate.

Table 1: List of components in N6 and modified N6 media (Fj and L8) for induction of callus from the anthers of seven cultivars of rice (Chu *et al*., 1975)

	(mg L^{-1})		
Components	Fj	L8	N6
KNO_3	3150.00	3000.00	2830.00
$(NH_4)_2SO_4$	0.00	0.00	436.00
KH_2PO_4	540.00	540.00	400.00
$CaCl_2.2H_2O$	150.00	150.00	166.00
$MgSO_4.7H_2O$	185.00	185.00	185.00
$MnSO_4.7H_2O$	22.330	22.330	4.40
$ZnSO_4.7H_2O$	10.00	10.00	1.50
H_3BO_3	6.00	6.00	1.60
KI	0.830	0.830	0.80
$Na_2MoO_4.2H_2O$	0.250	0.250	0.00
$CuSO_4.5H_2O$	0.025	0.025	0.00
$CoCl_2.6H_2O$	0.025	0.025	0.00
EDTA	37.250	37.250	37.30
$FeSO_4.7H_2O$	27.850	27.850	27.80
Inositol	100.00	100.00	0.00
Glycine	2.00	0.00	2.00
Thiamine HCl	2.50	2.50	1.00
Pyridoxine HCl	2.50	5.00	0.50
Nicotinic acid	2.50	3.00	0.50
Kinetin	0.50	0.50	0.50
2,4-D	0.50	0.50	0.50
NAA	3.00	3.00	3.00
Casein	500.00	0.00	0.00
Lactalbumin	0.00	300.00	0.00
Sucrose	40000.00	50000.00	60000.00
Maltose	0.00	0.00	0.00
Agar	8000.00	8000.00	8000.00

Table 2: Modified MS regeneration media (Murashige and skoog, 1962)

Culture medium	NAA (mg L^{-1})	Kin (mg L^{-1})	BAP (mg L^{-1})
SK11	1.0	1.00	1.0
N19	1.0	16.00	0.0

RESULTS AND DISCUSSION

Callus induction: Seven varieties of rice viz., Sangejo, Domsiah, Hassansaraie, Binam, Anbarbo, Sepidrod and Hassani were tested for callus induction in three basal media (N6, Fj and L8) supplemented with 3 mg L^{-1} NAA and 0.5 mg L^{-1} Kn and 0.5 mg L^{-1} 2,4-D. Callus was induced in all the varieties in L8 media; however, the induction frequency was varied. In N6 medium Sepidrod, Sangejo in Fj medium and only Domsiah in L8 medium produced low percentage of callus. Highest frequency (34.19) of callus and highest embryogenic structure (82.01) was observed in Domsiah in Fj medium (Table 3). Out of the three media tested L8 was found to be effective medium than N6 (Chu *et al*., 1975) and Karim *et al*. (1991) suggested that Z_2 and L8 media could be used for efficient callus induction of indica varieties of rice. Similarity, Lentini *et al*. (1995) reported that only one out of 35 indica cultivars exhibited pollen callusing on N6 medium. It was also observed that only Sepidrod induced very lower percentage calli in all three basal media used for this experiment. The result indicated that different

varieties hold different degree of potentiality of anther induction in different media. This finding are consistent with the results obtained by Raina and Zapata (1997) and Ogawa *et al.* (1995). Among the indica cultivars a considerable variation for pollen callusing and plant regeneration has been observed. Miah *et al.* (1985) reported that anther culture response varied from 41% for a japonica cultivar to 0% for an indica cultivar. Even among the indica cultivars a considerable variation for pollen callusing and plant regeneration has been observed. Guha-Mukejee (1973) reported that only 5 out of 18 indica cultivars showed pollen callusing and in only four cases did the calli differentiate plants. The result showed that different varieties hold different degrees of potentiality of anther induction in different media. The genotype of the pollen plant has the greatest influence on the frequency of pollen callus formation (Chu, 1982).

Table 3: Frequency of callus induction from anthers of different rice varieties in three induction media (supplemented with 3 mg L^{-1} NAA+ 0.5 mg L^{-1} Kn + 0.5 mg L^{-1} 2, 4-D)

Media	Genotype	Percentage of callus formation (Means±SE)	Percentage of embryogenic response (Means±SE)
N6	Sangejo	18.75±0.75d	26.2±1.62c
	Domsiah	9.85±0.36a	14.88±0.61ab
	Hassansaie	12.56±1.02c	16.02±0.7a
	Binam	-	-
	Anbarbo	20.41±1.23e	63.70±1.7d
	Sepidrod	7.08±0.31b	13.2±0.49b
	Hassani	25.89±1.42f	73.7±2.1e
Fj	Sangejo	8.95±0.27a	13.82±0.52a
	Domsiah	34.16±1.52e	82.01±2.4d
	Hassansaie	21.66±1.21d	62.97±1.82e
	Binam	-	-
	Anbarbo	17.77±0.93c	23.2±1.2c
	Sepidrod	-	-
	Hassani	14.38±0.63b	17.23±0.72b
L8	Sangejo	15.00±0.7c	18.12±0.74b
	Domsiah	6.66±0.11b	10.29±0.98a
	Hassansaie	22.91±1.41d	66.37±1.59c
	Binam	13.88±0.52c	17.43±0.7b
	Anbarbo	32.50±1.48f	79.71±2.2e
	Sepidrod	2.91±0.01a	9.12±0.31a
	Hassan	25.53±1.51e	74±2d

Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis ($\alpha = 0.05$)

Plant regeneration: For plantlet regeneration calli were transferred to two modified regeneration MS media, SK11 and N19 (Table 4). Among seven varieties upon transfer to SK11 medium, highest percentage (40%) of green plants and albino plants (18.18) were produced in Hassansaraie and in N19 medium the highest percentage (15.78) of green plants and albino plants (21.05) were produced in Anbarbo. Results showed that calli induced in L8 medium had better regeneration ability than other calli that induced in Fj and N6. This result indicated that the callus induction medium affected the rate of regeneration in different genotype. In regeneration medium some calli lost their ability to produce plants and died while the others differentiated into green and albino plants. Oono (1975) reported the differentiation of primarily green plant from micro spore derived callus of the japonica variety Minehikari. Occurrence of albino pollen plants seems to be a common phenomenon in pollen plants of *Gramineae*. The recover of primarily albino plants from micro spore derived calli has been a formidable obstacle to the utilization of rice anther culture.

Table 4: Frequency of plantlet regeneration from anther derived calli of different rice varieties in two different regeneration media SK11 and N19

Genotype	Callus induction media	SK11 regeneration media		N19 regeneration media	
		Green plant (%)	Albino plant (%)	Green plant (%)	Albino plant (%)
Sangejo	N6	-	-	-	-
	Fj	-	-	-	-
	L8	-	-	-	-
Domsiah	N6	-	-	-	-
	Fj	-	-	-	-
	L8	-	-	-	-
Hassansaraie	N6	0.00	18.18a	4.00a	8.00a
	Fj	10.75a	16.10b	0.00	2.81b
	L8	40.00b	0.00	12.50b	0.00
Binam	N6	-	-	-	-
	Fj	-	-	-	-
	L8	-	-	-	-
Anbarbo	N6	8.33a	4.16a	0.00	11.50b
	Fj	5.88b	5.88b	12.00b	8.00a
	L8	0.00	0.00	15.78c	21.05c
Sepidrod	N6	0.00	0.00	3.57a	10.71d
	Fj	0.00	0.00	0.00	0.00
	L8	0.00	0.00	0.00	0.00
Hassani	N6	2.77a	11.10a	11.10b	11.75a
	Fj	0.00	0.50b	1.75a	10.00b
	L8	22.2b	11.20a	14.28c	11.42c

Values sharing the same letter in each column are not significantly different from each other by protected LSD analysis ($\alpha = 0.05$)

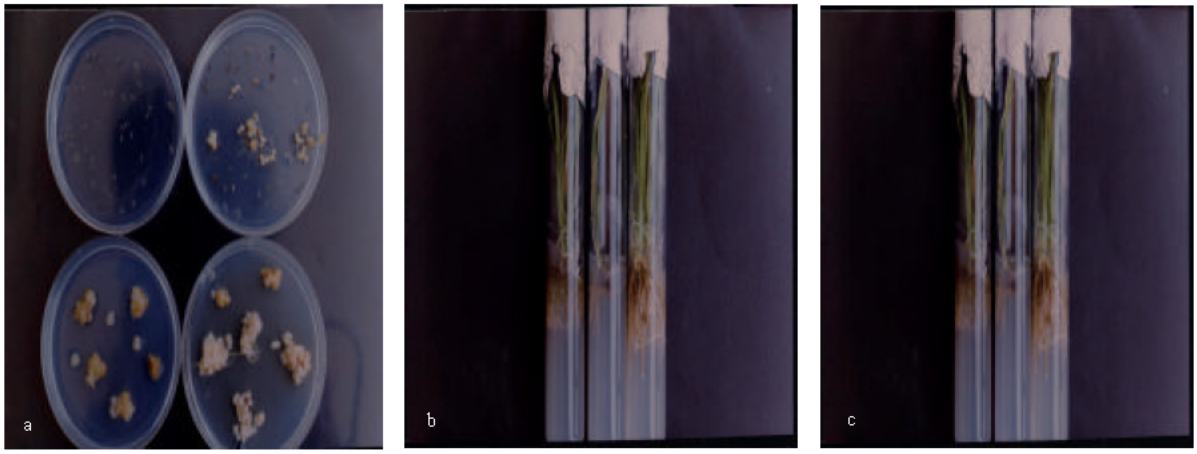

Fig. 1a: Induction of callus from cultured rice anthers: b and c) Regeneration of haploid rice plantlets from callus

Results from several laboratories have identified that the proportion of regenerated albino plants were genetically controlled (Oono, 1975; Chen and Li, 1976). Although considerable progress has been made to improve the *in vitro* androgenic response and feasible of *Ab initio* microspore culture of indica rice cultivars has been demonstrated (Fig. 1a), the routine application of this technique to rice breeding is still fraught with many problems. Dihaploid breeding has resulted in the production of several improved cultivars and breeding lines of rice but the success is largely restricted to japonica cultivars (Fig. 1b and c). Efforts need to be made to reduce the problem of albinism by manipulating the various *In vivo* and *In vitro* factors and achieving direct pollen emberyogenesis to reduce the culture period. In many treatments rice pollen produce rounded, smooth and shining Embryo-Like Structures (ELS) which eventually fuse with adjacent ELS' to form macrocalli. It has been possible to change the pollen callusing pathway to a pollen emberyogenesis pathway by manipulating the culture conditions (Kao *et al*., 1991). Present result showed that the SK11 regeneration media significantly was better than N19 media for percentage of green plantlet regeneration and callus induction result showed that L8 media was better than N6 and Fj. This study indicates that ability of callus formation from anthers of rice varieties are largely dependent on culture medium in addition choice of variety would be critical factor to ensure the recovery of a high frequency of green plants. In rice culture, the frequency of albino plants has been high. As long as we have to culture the pollen to obtain homozygous diploid plants, besides genotypes we must seek ways to improve the cultural conditions and the media to resolve the albino problems.

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