EFFECT OF CULTURE MEDIA AND TEMPERATURE TREATMENTS ON CALLUS FORMATION IN ANTHER CULTURE OF DIFFERENT RICE (*ORYZA SATIVA* L.) GENOTYPES[1]

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ABSTRACT

Haploids or sporophytes which possess the gametophytic number of chromosomes can be utilized to increase the efficiency of plant breeding programmes. In rice (*Oryza sativa L.*), anther culture technique is one of the efficient and practical methods to produce haploids *in vitro*. The *indica* sub species of rice has a much lower efficiency of haploid production when compared to *japonica* sub species. This study was designed to examine the response of some genotypes and cultural conditions which elicit maximum response for microspore callus prodution in anther culture. One *japonica* (M101) and 3 *indica* (Bg 34-8, Bg 400-1, IR 50) genotypes were used with B5, N6, H5, modified B5 and modified H5 media. The cultured anthers were subjected to two temperature treatments of 4°C and 12°C for 8 days separately before incubation at 28°C in darkness. A control without temperature treatment was also used. The cultivars M101 and Bg 400-1 produced calli significantly higher than IR 50. Response of Bg 34-8 was intermediate. The media N6 and H5 significantly increased the callus production of M101, but had no significant effect on callus production frequency of the *indica* genotypes. However calli of responsive *indica* grew better on B5 and N6 than other media. The cold treatment of 12°C for 8 days significantly increased callus induction of cultured anthers of responsive genotypes of both the *japonica* and *indica*. The media B5 and N6 combined with temperature treatment of 12°C for 8 days may be suitable for callus production of cultured anthers of responsive *indica* genotypes.

KEY WORDS : Anther culture, Callus formation, Culture media, Rice genotypes

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INTRODUCTION

The production and use of doubled haploids in crop improvement has become popular in many crop species including rice (IRRI, 1987). Anther culture is the principal doubled-haploid system used in rice. However low efficiency of haploid production through current *in vitro* procedures limits the employment of anther culture as a breeding technique in rice. Further haploid production efficiency of *indica* sub species of rice is much lower when compared to *japonica* sub species (Morinson and Evans, 1988). The successful utilization of anther culture in crop improvement is dependant on the reliable *in vitro* methods applicable to leading cultivars of the crop species (Powell, 1985). Many studies have shown that modification of culture parameters such as composition of the medium and inductive treatments are vital for the success of *in vitro* procedures (Chen *et al.*, 1986; Powell, 1985). Androgenesis of rice usually has an intermediate step of microspore callus formation (Chen *et al.*, 1986). Therefore responsive genotypes with culture conditions which give maximum microspore callus production are helped to increase the efficiency of anther culture procedure. Cold shocks have been used as an inductive treatment prior to culturing the anthers in most of the studies (Chen *et al.*, 1986). Also the few studies, done with cold treatment on cultured anthers were limited to *japonica* sub species of rice (Hu *et al.*, 1978 and Zapata *et al.*, 1983). Therefore this study was designed to examine the effects of media and different cold treatments, given on cultured anthers, on microspore callus formation of one *japonica* and three *indica* rice cultivars.

MATERIALS AND METHODS

One *japonica* cultivar (M101) developed by the United States Department of Agriculture (USDA), California, 2 *indica* cultivars (Bg 34–8, Bg 400–1) developed at the Central Rice Breeding Station, Sri Lanka and one *indica* cultivar (IR 50) developed at the International Rice Research Institute, Philippines were used for the study.

The plants were grown under greenhouse conditions at the Department of Agronomy, Colorado State University during summer, 1988. Seeds were sown in 25 cm diameter plastic pots containing potting mixture (Fisons Sunshine mix. No. 1, pH adjusted) and sand (2:1, v/v), and watered twice a day until germination. After germination, pots were placed in

water—filled plastic trays and watered regulaly to maintain the soil moisture. At two weeks, seedlings were thinned to 3 plants/pot. Fertilizer was applied as required. During the experiment the temperature ranged from 20° to 37°C and a maximum relative humidity of 75% was observed.

At heading, panicles were collected and anthers were taken midway along the panicles for cytological determination of developmental stages of uninucleate microspores. Observations were made to identify the tillers containing the appropriate panicles of each genotype for anther collection.

All the leaves, except the flag leaf sheath, were removed from the collected tillers. Panicles with flag leaf sheaths were dipped in 70% alcohol and dissected under aseptic condition to get the middle half of the panicles. These were sterilized again by immersing in a 20% commercial bleach (Clorox) solution for 5 min. followed by rinsing three times in sterilized distilled water. During anther plating spikelets were kept in sterile distilled water to avoid desiccation.

The media used were B5 (IRRI, 1987), N6 (IRRI, 1987), H5 (Chen, 1986), modified B5 (MB5) and modified H5 (MH5) (Table 1). Both MB5 and MH5 comprised of 20% chopped potato boiled water in addition to compounds of B5 and H5. Glucose (5g/l) and yeast extract (1g/l) were added to MB5 and MH5 respectively. The pH of the media was adjusted to 5.6—5.8 before placing in the autoclave for sterilization.

About 50 anthers were plated in each plastic petri dish (90mm × 15mm) containing about 30ml of solid medium. Cultured dishes sealed with stripes of parafilm—M to avoid desiccation and contamination, were wrapped with aluminium foil to avoid light and sudden temperature changes.

Cultured plates were subjected for two cold treatments: T1, 4°C for 8 days, T2, 12°C for 8 days. The temperature for T3 (control) was maintained at 28°C. After temperature treatments these were incubated at 28°C in darkness. Although petri dishes were sealed with parafilm, a water pan was kept in the incubator to avoid possible desiccation of cultured anthers during long incubation period. After one month of culturing aluminium wrapping was removed and continued at the same temperature in darkness. Each treatment was replicated 4 times.

After 60 days of culturing, number of callus producing anthers and total number of cultured anthers per petri dish were counted to obtain percentage of anther responded by forming calli. Transformed data (square root of percentage plus 0.5) were analyzed using split — split plot design to compute the effect of genotype, medium and temperature treatments on the callus forming ability of cultured anthers.

Callus sizes were measured for all the genotypes on different media and for different temperature treatments.

RESULTS AND DISCUSSION

Approximately 4 weeks after plating, some anthers responded by calli emergence from interior of the anthers. It was observed that anthers became necrotic prior to forming the calli. At the end of 60 days of incubation, 365 out of 12,904 anthers responded by forming calli. Cultivar M101 gave the highest response (23.1%) on N6 medium, with the temperature treatment of 12°C, 8 days (T2) (Table 2). Among the *indica* genotypes, Bg 400 — 1 (14.4%) and Bg 34 — 8 (12.0%) gave the highest response on B5 and N6 respectively with T2 temperature treatment. Cultivar IR 50 had the lowest response among all the genotypes with the maximum of 6% at B5 with T2 temperature treatment.

Analysis of variance indicates that there was a highly significant difference between genotypes and a significant difference between media (Table 3). It also indicated a highly significant effect of temperature treatment and its interactions with genotype and media. There was a strong interaction between replications and treatments as shown by previous studies on anther culture (Anderson *et al.*, 1987). This had been attributed to the high sensitivity of callus formation to environmental changes.

Effect of genotype on callus formation

A significantly higher callus forming ability was observed for M101 and Bg 400 — 1 than IR 50 (Table 4). This higher response of *japonica* (M101) agrees with the previous reports (Chen, 1986).

However Bg 400—1 has responded similarly to M101. This may be due to genotypic differences within *japonica* as well as *indica* sub species (Guha Mukherjee, 1973). Possibly M101 is a low responsive *japonica,* while

Bg 400 — 1 is a high responsive *indica*. Another reason may be the beneficial effect of higher temperature in the greenhouse during the summer on *indica* genotypes for *in vitro* anther callus production than on *japonica* genotypes (Raina *et al.*, 1987).

Overall response of *japonica* genotype as well as *indica* genotypes was relatively lower than those reported previously (Karim *et al.*, 1985; Siva Reddy *et al.*, 1985). This could be due to the exposure of donor plants to stress conditions such as low humidity and high temperature in the summer. Collections of anthers from late tillers of weak plants for culturing may also be a reason for the lower response (Chen *et al.*, 1986).

Effect of culture medium on callus formation

The N6 medium showed significantly higher callus forming ability than MH5 (Table 5). Modified B5 which is composed of chemically undefined substances had no significant advantage over B5 medium. Therefore, in general N6, B5 and H5 may be equally suitable for anther callus production of rice.

Effect of temperature treatment on callus formation

Temperature treatment of 12°C for 8 days (T2) had a beneficial effect on the number of anthers responding compared to 4°C for 8 days (T1) and the control (T3). This favourable effect was significant for all but IR 50, the lowest responsive genotype in most of the media (Table 2). These findings agree with the observations of Hu *et al.* (1978) and Zapata *et al.* (1983), where only *japonica* genotypes with temperature levels of 10 to 13°C and 8°C respectively were used.

However these findings do not agree with the beneficial effect of 4°C cold treatment reported by Chen (1986). Instead, it showed some deleterious effect on callus formation of cultured anthers. That may be related to the fact that temperature treatments were given on cut tillers containing panicles in the previous study whereas in the present study, cultured anthers were subjected to temperature treatments. These results indicated a possibility of increasing the callus induction of cultured anthers significantly by a constant temperature treatment of 12°C for 8 days for both the *japonica* and *indica* genotypes. This is particularly important for low responsive *indica* genotypes except for recalcitrant genotype as IR50.

General remarks

The media effect was important only for M101, not for *indica* genotypes within T2 temperature treatment (Table 6). Cultivar M101 had significantly higher callusing on N6 than B5, MB5 or MH5. These findings suggest the suitability of N6 medium for anther callus production of *japonica* sub species as previously reported (Genovesi and Magill, 1979). In contrast to previous studies H5 medium did not show any significant advantage on callus formation of *indica* genotypes (Loo and Xu, 1986; Siva Reddy *et al*, 1985). The medium H5 composed of 2, 4—D (2mg/l), Naphthalene acetic acid (NAA) (2mg/l) and kinetin (3mg/l) has been recommended for anther culture of *indica* rice (Chen, 1986). Suitability of a medium depends not only on callus formation but also on its contribution to green plant production. Therefore it is necessary to study the green plant production of calli derived on H5 medium for a conclusion. However B5, N6 and H5 are composed of chemically defined substances and easier to maintain the standard of the media compared to MB5 and MH5.

Not only the callus forming ability but also the callus size differences were noticed among the genotypes. Callus size differences were present even in different media within the genotype. This genotype and medium effect on callus size was clearly showed by the data within the T2 temperature treatment which resulted, in a reasonable number of calli for each treatment (Fig. 1). Cultivar M101 had a higher callus growth on all the media compared to *indica* genotypes. This higher capacity of callus growth of *japonica* genotypes has also been reported in previous studies (Mikami an Kinoshita, 1988). Among *indica* genotypes, IR50 had the lowest growth while Bg 34—8 and Bg 400 —1 showed relatively higher growth.

Significant differences in callus forming ability within *indica* genotypes suggest the importance of screening genotypes for responsiveness of microspore callus formation to apply the technique effectively in *indica* rice. The callus growth of both Bg 34—8 and Bg 400—1, responsive *indica*, was relatively better in B5 and N6 than other media. If the calli are too small in size (<1mm) their growth is generally very slow and gradually become necrotic after transferring to the differentiation medium (Loo and Xu, 1986). Hence B5 and N6 may be more suitable for microspore callus production in anther culture of *indica* rice. Therefore, when applying anther culture

technique in *indica* rice, B5 and N6 media combined with temperature treatment of 12°C for 8 days could be used on cultured anthers for microspore callus production. However regeneration studies must be carried out for the calli derived under different treatment combinations to conclude their suitability for haploid production.

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Table 1. Composition of culture media

Component	*Concentration (mg/l)*		
	B5	*N6*	*H5*
KNO_3	2500	2830	3181
$(NH_4)_2SO_4$	134	463	231
KH_2PO_4	—	400	600
$CaCl_2.2H_2O$	150	166	166
$MgSO_4.7H_2O$	150	185	35
Na_2—EDTA	37.3	37.3	74.5
$NaH_2PO_4.H_2O$	150	—	—
$FeSO_4.7H_2O$	27.8	27.8	55.5
$MnSO_4.4H_2O$	—	4.4	4.4
$MnSO_4.H_2O$	10.0	—	—
$ZnSO_4.7H_2O$	2.0	1.5	1.5
H_3BO_3	3.0	1.6	1.6
KI	0.8	0.8	0.8
$CuSO_4.5H_2O$	0.025	—	—
$NaMoO_4.2H_2O$	0.25	—	—
$CoCl_2.6H_2O$	0.025	—	—
Inositol	160	—	—
Glycine	—	2.0	2.0
Thiamine HCI	10.0	1.0	0.6
Pyridoxine HCL	1.0	0.5	0.6
Nicotinic acid	1.0	0.5	3.0
2, 4—D	1.0	2.0	2.0
BAP	0.5	—	—
IAA	0.5	—	2.0
NAA	—	—	2.0
KT	—	—	3.0
Sucrose	20,000	60,000	60,000
Agar	8,000	8,000	8,000

BAP = Benzyl Amino Purine
IAA = Indole Acetic Acid
NAA = Naphthalene Acetic Acid
KT = Kinetin
2, 4 - D = 2, 4—Dichlorophenoxy Acetic Acid

Table 2. Callus forming ability of four genotypes on different media with different temperature treatments*

Medium	Callus forming ability (%)**		
	T1	*T2*	*T3*
		M101	
B5	0.0 b	5.57 a	4.20 ab
MB5	0.0 b	7.03 a	1.55 b
N6	0.47 b	23.13 a	2.18 b
H5	1.13 a	15.68 a	2.15 a
MH5	1.73 a	4.55 a	0.83 a
		Bg 34—8	
B5	0.0 b	3.85 a	0.0 b
MB5	0.0 a	4.30 a	1.45 a
N6	0.0 b	12.03 a	0.50 b
H5	0.0 b	6.13 a	0.0 b
MH5	0.0 a	2.33 a	0.50 a
		Bg 400—1	
B5	1.85 b	14.35 a	2.73 b
MB5	0.55 a	3.68 a	3.05 a
N6	0.52 b	6.80 a	4.45 ab
H5	1.45 a	5.45 a	1.63 a
MH5	0.50 a	3.35 a	1.00 a
		IR 50	
B5	0.0 b	6.00 a	0.0 b
MB5	0.0 a	1.40 a	0.0 a
N6	0.0 a	0.60 a	0.5 a
H5	0.0 a	0.48 a	0.5 a
MH5	0.0 a	0.53 a	0.0 a

* Average of 4 replicates

** Means of each row followed by the same letter are not significantly different at the 0.05 level according to Duncan multiple range test (DMRT)

T1 = 4°C for 8 days; T2 = 12°C for 8 days; T3 (control) = 28°C

Table 3. Analysis of variance of anther callus forming ability of four genotypes on different media with different temperature treatments

SV	*DF*	*MS*	*CALC.F*
Main plot			
Replication	3	0.647	
Genotype (Geno.)	3	10.283	6.995*
Error (a)	9	1.470	
Sub plot			
Medium (Med.)	4	1.949	3.480‡
Geno. X Med.	12	0.704	1.257 ns
Error (b)	48	0.560	
Sub — sub plot			
Temperature (Temp.)	2	43.405	107.172†
Geno. X Temp.	6	2.555	6.309†
Med. X Temp.	8	1.366	3.373*
Geno. X Med. X Temp.	24	0.966	2.385*
Error (c)	120	0.405	

Significant level ‡ p = 0.05 * p = 0.01 † p = 0.001

Table 4. Anther callus forming ability of different genotypes

Genotype	*Callusing ability (%)***
M101	4.68 a
Bg 400 — 1	3.47 a
Bg 34 — 8	2.07 ab
IR 50	0.62 b

* Average of 60 experimental units

** Means with same letter are not significantly different at 0.05 level according to DMRT.

Table 5. Anther callus forming ability of different media*

Medium	*Callusing ability (%)***
N6	4.26 a
B5	3.21 ab
H5	2.88 ab
MB5	1.92 ab
MH5	1.28b

* Average of 48 experimental units

** Means with same letter are not significantly different at 0.05 level according to DMRT

Table 6. Anther callus forming ability of four genotypes on different media with temperature treatment of 12°C for 8 days*

	*Callus forming ability (%)***			
Medium	*M101*	*Bg 34 — 8*	*Bg 400 — 1*	*IR50*
N6	23.13 a	12.03 a	6.80 a	0.60 a
H5	15.68 ab	6.13 a	5.45 a	0.48 a
MB5	7.03 b	4.30 a	3.68 a	1.40 a
B5	5.57 a	3.85 b	14.35 a	6.00 a
MH5	4.55 b	2.33 a	3.35 a	0.53 a

* Average of 4 replicates.

** Means with same letter are not significantly different at 0.05 level according to DMRT

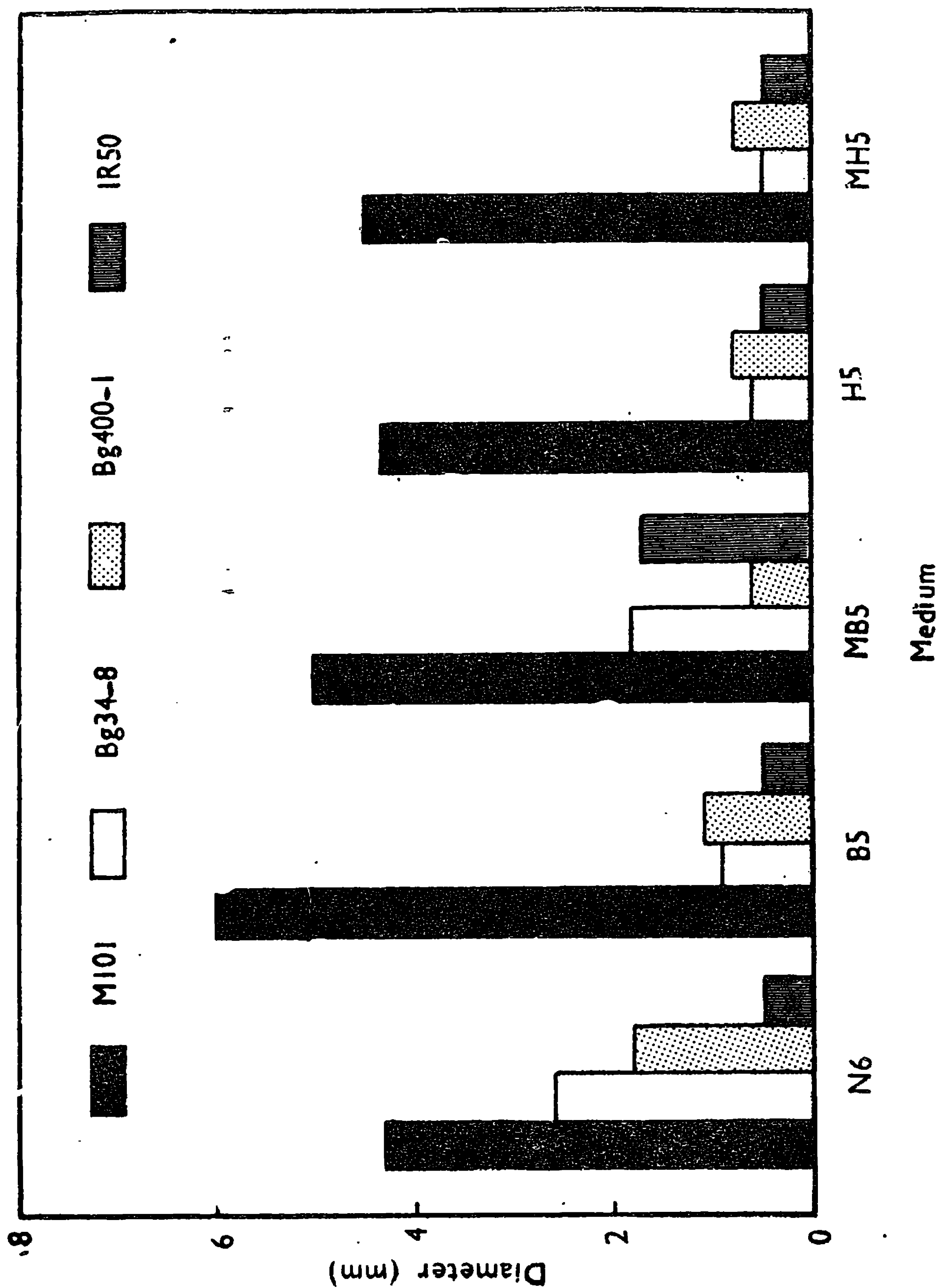

Fig. 1. Callus size of different genotypes on different media at temperature 12°C for 8days