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Role of Media, Plant Growth Regulators in Callusing and Plant Regeneration from Anthers of Indica Rice

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High frequency green plant regeneration from indica rice anther calli is one of the major problems in exploitation of anther culture in breeding programme. Four indica rice cultivars namely CR666-34-3, SR 26-B, Basmati Sd-10 and ptb.33 were evaluated to study the role of media, plant growth regulators and sucrose on anther callus induction and plant regeneration. Out of the four genotypes evaluated the ptb.33 exhibited higher frequency of green plant regeneration which included green (45%) and albino (30%) on MS media supplemented with a combination of NAA/IAA and BAP/KN.

Key Words: Anther, Media, Plant growth regulators, Plant regeneration, Indica rice and *Oryza sativa*

Introduction

Regeneration of haploids/dihaploids through anther culture is a potential tool for gene manipulation especially in selection of recessive genes and compression of breeding cycle. Efficient regeneration system is a pre-requisite for any genetic manipulation either through protoplasts or through microspore embryoids. Thus, it necessiates to understand the requirements and factors involved in anther culture differentiation. In indica rice cultivars, low frequency of callusing and green plant regneration as well as high frequency of albinos is still a major challenge, in successful exploitation of anther culture in rice improvement. The present paper deals with the study of various factors influencing high frequency callus induction and green plant regeneration from anthers of indica rice,

Materials and Methods

Four cultivars of indica rice were included after the preliminary screening for anther culture response in callusing and regeneration on Chaleff's R-2 medium (Chaleff & Stolarz 1981) supplemented with 2 mg-l-napthalene acetic acid (NAA) and 0.5 to 2 mg-l Kinetin. The cultivars, CR 666 36-4 and SR 26-B were the low responding types

while Basmati mutant semidwarf-10 (Basmati Sd-1) and Ptb-33 were high responding ones. The seeds were supplied from CRRI, Cuttack; DRR, Hyderabad and by our Plant Genetics Experimental Farm.

The fresh panicles enclosed within the sheath of the flag leaf were wrapped properly with aluminium foil to prevent the loss of moisture. Then the boots were kept at 10°C for cold pre-treatment for different days, starting from day one to day 12. Three cultivars, SR 26-B, Basmati Sd-10 and Ptb-33 were used to evaluate the effect of temperature pre-treatment on callusing of anthers.

Medium and Growth Regulators

Three basal media such as Chaleff's R-2, N6 (Chu et al. 1975) and MS (Murashige & Skoog 1962) were used for evaluation on anther culture response. On the basis of earlier studies from our laboratory (Aruna & Reddy 1988, Aruna 1990), initially 2 mg-1 NAA in combination with 0.5 mg-1 Kinetin was used for callus induction with 3% and 6% sucrose separately. To study the influence of different synthetic auxins on the frequency of callus induction from anthers with N6 media, NAA was replaced by 2,4-Dichlorophenoxyacetic acid (2,4-D) and the combination of 2 mg-1 2,4-D and 0.5 mg-1 Kinetin besides 3% and 6% sucrose. For further enhancement in the frequency of regeneration MS medium was used with both 3% and 6% sucrose fortified with low concentration of NAA or Indole acetic acid (IAA) 1 mg-1 and high cytokinins, such as combination of benzyle aminopurine (BAP) (0.2 mg-1) and Kinetin (0.6 mg-1). The type of plant growth regulators and the sucrose concentrations in plant regeneration were also evaluated with Basmati Sd-10 and Ptb-33.

Method

The panicles were collected from the first few tillers of the donor plant during the floral initiation period and subjected to cold pre-treatment (10°C) for various days. The cold pre-treated anthers sterilized with 0.1% mercuric chloride for 7 min. and washed thoroughly with sterile water. About 30-35 anthers were inoculated onto each test tube containing 15ml of medium having *p*H 5.8 and solidified with 0.8% agar-agar. For callus initiation, the tubes were kept in diffused light and at 25 + 2°C temperature, whereas for plant regeneration the initiated calli of 3-5mm diameter were transferred onto regeneration media and kept under constant illumination of 2000 lux with same temperature. The regenerated plants were transferred to 1/2 MS basal medium for root hardening for two to three weeks and transferred to pot culture, grown in net house.

Each experiment was repeated thrice and the callusing frequency was calculated from the ratio of the number of anther callused to that of the anthers inoculated. Similarly the regeneration frequency was ascertained from the ratio of number of callus differentiated to shoots and roots to that of calli inoculated.

Results and Discussion

Pre-treatment of Anthers and Callusing Response: About 2-4-fold increase in callusing frequency from anthers was observed in all the three cultivars after low temperature (10°C) pre-treatment, compared with control. However, the optimum period of treatment varied with the genotypes. The untreated and 3 day pre-treated anthers of SR 26-B and Basmati Sd-10 did not show significant variation in callusing frequency. While with 4 days of pre-treatment, SR 26-B exhibited maximum callusing (4%) and the frequency declined with 7 days of pre-treatment which was similar to control (2%). On the contrary, Basmati SD-10 exhibited maximum response (42%) with 7 days of pre-treatment

compared to control (13%) and no response was observed with 12 day pre-treated anthers. Similarly in Ptb-33, a 4-fold increase in callusing (33%) was observed with 8 day pre-tyreatment compared to control (8%). No callus induction was observed with 11 day pre-treatment. These observations indicated that, each genotype may require specific pre-treatment duration for optimum response. The longer period of pre-treatment may inhibit callusing response.

The microscopic observations indicated, that microspores initiated the division during the course of cold pre-treatment. Thus the possible role of low temperature treatment prior to inoculation may presumabily be attributed to the elimination of weak or non-viable microspores. Besides, the delayed anther wall senescence due to cold pre-treatment might be providing a suitable microenvironment to ensure supply of growth factors to trigger the embryogenesis from microspores (Cho & Zapata 1988, Tsay et al. 1988. Datta et al. 1990, Chen et al. 1991).

Influence of Medium, Growth Regulators and Sucrose on Callusing: Among the three media evaluated, both Chaleff's R-2 and N6 were equally efficient in callus induction while MS was least effective. High callusing frequency was observed with Ptb-33 (40-42%) and Basmati SD-10 (27-36%) with N6 medium. The other cultivars Rasi, SR 26-B, CR-666-36-4 exhibited almost similar frequency of callusing. The extent of response of different genotypes varied (table 1). Except Ptb-33, where the callusing response was considerably higher with NAA (42%), compared to 2,4-D (28%), the response was similar in other cultivars.

The callus initiation was expedited, 7-10 days earlier with NAA supplemented medium compared to 2,4-D. The induction medium (N6) with NAA and Kinetin promoted faster growth of the early formed calli whereas with 2,4-D and Kinetin, multiple calli (10-15 or more) per single anther was observed along with synchronous growth of developing calli. This improved efficiency may be attributed to the conversion of 2,4-D to relatively inactive glucosyl esters or active amino acid conjugates, which might have helped in DNA synthesis and mitosis (Skoog & Muller 1957).

Plant Regeneration from Anther Calli

Different media, plant growth regulators were studied to ascertain their role on plant regeneration from anther calli. In general, MS medium was found to be superior for plant regeneration in all the four cultivars compared to Chaleff's R-2 and N6. In Ptb-33, the callus upon transfer to MS regeneration medium devoid of any growth regulators, exhibited only rhizogenesis (30%). However, with the addition of NAA and Kn (2mg/l), both shoot and root differentiation was observed in all the three media (table 2). The plant regeneration response was genotype dependent in N6 and Chaleff's R-2. The frequency of plant regeneration was high on MS medium with 1 mg-1 either IAA or NAA, 2 mg-1 BAP and 0.6 mg-1 BAP

Table 1 *Role of auxins on callusing of anthers of indica rice*

Cultivar	Auxin	Anthers inoculated	Anthers responded	Callusing (%)
CR 66-36-4	NAA	500	13	3
	2,4-D	500	5	1*
SR 26-B	NAA	500	20	4
	2,4-D	300	8	3*
Rasi	NAA	400	32	8
	2,4-D	350	25	7*
Basmati Sd-10	NAA	720	260	36
	2,4-D	450	153	34*
Ptb-33	NAA	500	210	42
	2,4-D	450	127	28*

*Embryogenic

Table 2 *Role of media, plant growth regulators and sucrose on regeneration of anther calli of indica rice**

Cultivar	Media/growth regulators (mg-1)	Sucrose 3%		Sucrose 6%	
		Green	Albino	Green	Albino
CR 666-36-4	Chaleff's R-2 + 2 NAA + 2 KN	5	8	7	13
	N6 + 2 NAA + 2 KN	6	9	7	20
	MS + 1 NAA/IAA + 2 BAP + 0.6 KN	6	11	19	21
SR 26-B	Chaleff's R-2 + 2 NAA + 2 KN	0	18	0	22
	N6 + 2 NAA + KN	0	18	0	20
	MS + 1 NAA/IAA 2 BAP + 0.6 KN	0	20	0	28
Basmati Sd-10	Chaleff's R-2 + 2 NAA + 2 KN	0	18	0	24
	N6 + 2 NAA + 2 KN	0	15	0	20
	MS + 1 NAA/IAA + 2 BAP + 0.6 KN	0	35	27	28
Ptb-33	Chaleff's R-2 + 2 NAA + 2 KN	14	21	25	25
	N6 + 2 NAA + 2 KN	10	18	11	30
	MS + 1 NAA/IAA 2 BAP + 0.6 KN	18	24	45	30

Table 3 *Role of plant growth regulators and sucrose in callus induction and regeneration on anther calli of indica rice*

Cultivar	Auxins (Mg/l)	Sucrose (%)	Calli inoculated	Calli responded	Regeneration** (%)	
					Green	Albino*
Basmati Sd-10	2 NAA + 0.5 KN	3	40	6	0	15
		6	40	12	0	30
		9	20	0	0	0
	2-2,4-D + 0.5 KN	3	40	12	15	15
		6	40	25	38	25
		9	20	2	0	10
Ptb-33	2 NAA + 0.5 KN	3	30	6	10	10
		6	40	12	15	15
	2-2,4-D + 0.5 KN	6	60	45	45	30

*N6: **MS + mg1-1 MAA + 2 mg-1 BAP + 0.6 mg-1 KN + 6% sucrose

and 0.6 mg^{-1} Kinetin, ranging the 20% in both CR 666-36-4 and SR 26-B to 42% in Ptb-33. Among the different cultivars, Ptb-33 exhibited the highest frequency of plant regeneration with (16:19) green and albino plants on the MS medium whereas in CR 666 36-4, SR 26-B gave (2:3). The enhancement of sucrose concentration from 3 to 6%, the overall frequency of green plant regeneration was enhanced (table 2). The regeneration frequency was enhanced when the sucrose concentration was high. On N6 medium, maximum of 1.8 fold increase in regeneration frequency was observed in CR 666 36-4 with 6% sucrose. In Ptb-33, the regeneration frequency was increased to 75% when 6% sucrose was used in MS regeneration medium. Thus the higher cytokinin (BAP+KN) to auxin (IAA/NAA) ratio besides the probable interaction of BAP with high sucrose (6%) in favouring efficient plant regeneration was evident from the present study.

Role of Induction Media on Plant Regeneration

A distinct interaction between sucrose and plant growth regulators in the callus induction medium was observed in influencing subsequent plant regeneration. In general the calli induced on NAA supplemented medium were more friable and promoted only rhizogenesis in most of the cultures upon transfer to MS regeneration medium in comparison to induction media (2,4-D) with varying concentration of sucrose. In Ptb-33, callus induced on 2,4-D medium exhibited 42% and 75% regeneration on MS regeneration medium with 3% and 6% sucrose, respectively. On the other hand callus induced on NAA supplemented medium exhibited 20% and 40% regeneration with 3% and 6% sucrose, respectively. However, the calli induced with 6% sucrose gave 30% regeneration with 15% of green and albinos each. These results clearly suggest that the role of growth regulators and sucrose play a major role besides medium in determining the regeneration potential of the anther calli. The regeneration ability might probably be determined during the induction phase rather than regeneration medium (Szarejko & Kasha 1991).

The present study thus clearly suggests the role of cultural variables like media, plant growth regulators, sucrose and physiological factors temperature treatment besides the genotypic influence on successful exploitation of anther culturability in indica rice. Efficient green plant regeneration which is the limiting factor in indica rices has been accomplished by selecting a suitable genotype, media, hormonal concentration/combination besides carbohydrate levels. Out of five genotypes evaluated the Ptb-33 exhibited higher frequency of green plant regeneration (45) besides albinos (30%) on MS media supplimented with a combination of NAA/IAA and BAP/KN.

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