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Effect of growth regulators on androgenesis and regeneration in rice (*Oryza sativa* L.)

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Abstract

Seven selected hybrids *viz.*, CR 1009/HA 891037, CR 1009/TNAU 841434, ADT 40/AS95035, CO 43/ADT 43, Improved White Ponni/CB 97033, Improved White Ponni /IET 15341 and Improved White Ponni /CB(DH) 95298 were anther cultured for the production of doubled haploid lines. Maximum callus induction was observed in Improved White Ponni /CB(DH) 95298 (32.67 per cent) and minimum in CR 1009/ HA 891037 (10.77 per cent). The cytokinin treatment with 2.0mg/l BAP and 2.0mg/l GA_3 accounted for maximum regeneration. The frequency of callus induction was distinctly influenced by the genotype and growth regulators. Callus induction occurred 20 to 25 days after inoculation and maximum number of calli per tube were formed between 30 to 75 days. The calli that emerged in 50 days had good competitive and morphogenetic potential for green plantlet regeneration and those calli emerging after 75 days had poor regeneration ability. Among the seven hybrids cultured, green plant regeneration was observed in only three hybrid combinations.

Key words : Rice, anther culture, androgenesis

Introduction

Diploids derived from pollen plantlets are uniform in both genotype and phenotype. This can accelerate the breeding process and shorten the breeding cycle. Since the pioneering work on the development of haploid embryoids *via* anther culture has received much attention worldwide after Niizeki and Oono [1] produced pollen plantlets in rice (*Oryza sativa* L.), scientists in China began research in rice anther culture in 1970. In the past three decades, the anther culture technique has been greatly improved and many pollen derived cultivars, mostly *japonica* have been bred and released for commercial use. Improving anther culture efficiency is a prerequisite for anther culture breeding. Anther culture breeding in *indica* rice, compared with *japonica* rice, is less efficient because it is difficult to obtain a large pollen plantlet population for breeding purposes. This challange has motivated many scientists to investigate and increase the efficiency of anther culture in *indica* rice. Anther culture efficiency was enhanced by many workers using various factors. The present study was undertaken to study the response of *indica* rice hybrids to anther culture.

Materials and methods

Seven superior hybrid combinations were selected based on earlier yield trails. At the flag leaf stage, two to three tillers from each healthy plant were selected and the panicles were taken. The inflorescence was dissected to observe the microspores in the immature anthers. The inflorescence containing microspores at mid to late uninucleate stage was selected. The panicles were covered with a polythene bag and kept for pre-treatment in the refrigerator at 9°C for 7-9 days. Based on the spikelet colour (light pale green) and position of the anther in the spikelet, the spikelets were chosen for mid-late uninucleate stage. Anthers having 1/3rd to 1/2 height of spikelets were used for culturing. The culture medium *viz.*, N6 [2] and MS [3] were used with modifications for the present study. In both the media, agar 0.8 per cent was used as gelling agent. The carbon source for callus induction was 6 per cent sucrose while the regeneration media had 3.0 per cent sucrose. The pH was adjusted to 5.8 (using 0.1 N HCl (or) 0.1 N NaOH). The medium was distributed to the test tubes and plugged with non absorbent cotton. They were then autoclaved at 15 psi at 121°C for 15 minutes. After pre-treatment, panicles (enclosed in flag leaf) were soaked in 0.1% $HgCl_2$ for 8-10 min and then rinsed in sterile distilled water thrice. Panicles were removed asceptically and using a small part of sterilised scissors, the end of floret was cut off and the anthers were tapped on the end of a test tube such that the anthers were placed horizontally on the media. The inoculated anthers were incubated in dark at 25 ± 2°C. After the induction of callus, the calli were transferred to MS regeneration media and incubated in light (5000 lux) for shoot development.Callus induction and regeneration response were studied in the *in vitro* cultured hybrids. The efficiency of different carbon sources separately and effect of their combination were studied with respect to callus induction. The anthers were inoculated in N_6 medium containing (2,4-D) at 1.0, 2.0, 3.0 and 4.0 mg/l in combination with kinetin 0.25, 0.50 and 0.75 mg/l.

The experiment constituted three replications with 10 tubes per replication. Each tube consists of 100 anthers. Callus induction per cent was worked out. The mean number of days taken for callus induction was calculated as a factorial one adopting randomised block design. The effect of cytokinin on regeneration was studied by using two cytokinins, *viz.*, BAP at 2.0, 3.0 and 4.0 mg/l and kinetin at 0.40, 0.60 and 0.80 mg/l in combination with GA_3 at 1.0 and 2.0 mg/l. The experiment was constituted at 10 tubes per treatment. The per cent regeneration was worked out. All data were subjected to factorial completely randomised block design analysis.

Results and discussion

Application of anther culture technique to highly heterotic hybrids may help in exploiting the advantage of superior genotypes. Theoretically it is possible to obtain homozygotes with fixed heterosis provided that partial to complete dominance predominates [4]. The influence of hybrids, auxin, kinetin and their interactions was highly significant (Table 1). Among the seven hybrids, maximum callus induction was observed in Improved White Ponni/CB(DH)95298 (32.67 per cent) followed by CO 43/ ADT 43 (22.42 percent) and minimum in CR1009/HA891037 (10.77 per cent). The reason for more callus induction in the hybrid Improved White Ponni/CB (DH) 95298 was either due to involvement of anther derived line CB (DH) 95298 or due to the involvement of Improved White Ponni which is an *indica-japonica* derivative. Japonica genotypes respond better than *indicas*. The frequency of callus induction was distinctly influenced by the cultivars, growth regulators and sugars as reported by many workers [5, 6-8]. The influence of genotype on callus induction has been studied by Zapata and others [9-11]. Phytohormone combinations in the medium had a major impact on callus induction from anthers of wild *Oryza* species [12]. Among the 12 treatments involving four levels of 2,4-D, and three levels of kinetin, it was observed that maximum callus induction frequency (26.43 per cent) was in the treatment with 2.0 mg/l of 2,4-D+ kinetin 0.5 mg/l. It was followed by 2,4-D 1.0 mg/l + kinetin 0.5 mg/l (23.47 per cent). Minimum level of callus induction (19.19 per cent) was observed in 2,4-D 4.0 mg/l with kinetin 0.25 mg/l. The influence of 2,4-D on callus induction was distinct with 2.0 mg/l accounting for a maximum callus induction of 23.27 per cent followed by 22.37 per cent at 1.0 mg/l. The least frequency of callus induction (19.98 per cent) was observed with 4.0 mg/l of 2,4-D. The influence of the level of kinetin application on callus induction was distinct with 0.5 mg/l accounting for maximum callus induction (21.48 per cent). The least frequency of callus induction (20.86 per cent) was observed with 0.25 mg/l kinetin. The interaction between the hybrids and the auxin levels was highly significant. Maximum callus induction frequency (35.78 per cent) was observed in the cross Improved White Ponni/CB(DH) 95298 with 2.0 mg/l of 2,4-D and it was minimum (9.18 per cent) in the hybrid CR 1009/HA 891037 with 3.0 mg/l of 2,4-D. The auxin 2,4-D 2.0 mg/l with Kinetin 0.5mg/l was the best treatment with a callus induction potential of 26.43 per cent among the 12 combinations cultured. Addition of 2,4,-D and kinetin to the medium resulted in differential response to callus induction [13]. On the contrary Mercy and Zapata [14] have reported that the presence of auxins alone will be sufficient for callus induction. The interaction between the hybrids, auxin and level of kinetin was significant in the hybrid Improved White Ponni/CB(DH) 95298 with 2.0 mg/l of 2,4-D and 0.50 mg/l of kinetin accounting for maximum callus induction of 39.00 per cent.

The interactions between cultivar, auxin and cytokinins were highly significant. This can be seen in the higher level of callus induction in Improved White Ponni/CB(DH) 95298 and low callus induction in CR 1009/ TNAU 841434 at the same level of treatment i.e 2,4,-D 2.0mg/l + Kn 0.5mg/l. A marginal balance of the hormonal concentrations with that of the endogenous levels present in the genotype is important for callus induction. The variation in response with genotypes was also reported by Paulas and Rangasamy [15] and Rangasamy *et al.* [16].

Results obtained on the effect of growth regulators on plantlet regeneration in MS medium are given in Table 2. The variance due to hybrids, treatments with different levels of growth regulators and their interactions were highly significant. The hybrid Improved White Ponni / CB(DH) 95298 had the maximum mean regeneration potential (42.83) and CR 1009/TNAU 841434 had the least (7.23). The cytokinin treatment with 2.0 mg/l of BAP and 2.0 mg/l of GA_3 accounted for maximum (35.71 per cent) regeneration followed by kinetin 0.6 mg/l with 1.0 mg/l - GA_3 (25.49 per cent) and 0.8 mg/l kinetin with 1.0 mg/l GA_3 (25.25 per cent). Effect of Giberellic acid on survival of dried callus was reported by Shin *et al.* [17]. There was maximum regeneration (68.40 per cent) when 3.0 mg/l of BAP was used in Improved White Ponni/CB (DH) 95298 along with GA_3 1.0 mg/l. Among all the hybrids cultured, green plantlets were regenerated only from three hybrids *viz.*, CR 1009/HA 891037, CO 43/ADT 43 and Improved White Ponni/ CB(DH)95298. Regeneration of albino plants was found to be more in all the hybrids. Among the three hybrids, CO 43/ADT 43 recorded more green plant regeneration than other two hybrids. The production of microspore derived green plants from anther culture of *indica* rice is generally very low compared with *japonica* cultivars. Hence, the need to search for new factors that would promote regeneration arises. So the effect of growth regulators on regeneration was tried utilising MS medium containing different levels of BAP and Kn in combination with GA3. The cytokinin treatment

Table 1. Response of genotypes to callus induction

Treatment		CR1009/	CR1009/	ADT 40/	CO43/ADT	I.W.Ponni/	I.W.Ponni/	I.W.Ponni/CB	Mean
Auxin mg/l	Kinetin mg/l	HA891037	TNAU 841434	AS 95035	43	CB 97033	IET 15341	(DH) 95298	
2.4.D	0.25	21.00	21.87	21.87	21.00	23.00	22.67	33.00	22.00
1.0	0.50	17.67	24.00	23.33	24.67	22.00	24.33	38.33	23.47
	0.75	21.00	19.00	22.67	21.67	21.66	25.00	30.33	21.62
	Mean	19.89	21.56	22.56	22.44	22.22	24.00	33.89	22.37
2.0	0.25	21.00	20.67	22.33	24.67	21.00	20.67	34.33	22.09
	0.50	28.33	27.00	23.67	25.67	23.67	27.67	39.00	26.43
	0.75	21.00	22.00	22.67	19.33	19.00	21.00	34.00	21.29
	Mean	23.44	23.22	22.89	23.22	21.22	23.11	35.78	23.27
3.0	0.25	18.67	20.00	20.00	22.00	20.00	19.00	31.33	20.14
	0.50	19.87	22.00	18.33	24.33	20.67	17.33	38.67	20.17
	0.75	19.00	19.33	19.33	24.00	21.00	20.67	31.00	20.62
	Mean	19.18	20.44	19.22	23.44	20.56	19.00	33.66	20.31
4.0	0.25	19.00	18.33	17.67	20.66	19.00	18.67	31.00	19.19
	0.50	19.67	19.33	19.67	22.00	19.33	18.33	30.67	19.86
	0.75	23.00	22.00	19.00	19.00	22.00	21.00	30.33	20.90
	Mean	20.56	19.88	18.77	20.58	20.11	19.33	30.67	19.98
	Grand Mean	20.77	21.21	20.86	22.42	21.03	21.36	32.67	21.48

	SE (d)	CD (P = 0.05)	CD (P = 0.01)
Hybrid (H)	1.2000**	2.3640	3.1320
Auxin (A)	0.1776**	0.3508	0.4627
Kinetin (K)	0.1538**	0.3036	0.4007
H × A	0.4698**	0.9276	1.2242
H × K	0.4069**	0.8033	1.0802
A × K	0.3076**	0.6073	0.8014
H × A × K	0.8138**	1.6067	2.1204

Table 2. Effect of growth regulators on plantlet regeneration (per cent)

(mg/l)	GA_3	CR1009/ HA891037	CR1009/ TNAU 841434	ADT 40/ AS 95035	CO43/ ADT 43	I.W.Ponni/ CB 97033	I.W.Ponni/ IET 15341	I.W.Ponni/ CB(DH) 95298	Mean
BAP									
2.0	1.0	20.70	8.00	18.10	20.30	13.30	41.00	51.10	24.64
	2.0	30.46	10.40	41.73	35.20	20.63	52.83	58.76	35.71
3.0	1.0	15.76	8.26	19.73	23.30	11.90	27.36	68.40	24.96
	2.0	18.33	11.06	11.56	17.50	13.83	19.76	62.60	22.09
4.0	1.0	9.54	6.36	15.70	12.43	9.63	15.86	28.60	13.94
	2.0	9.12	4.66	10.50	12.40	12.46	11.03	27.20	12.48
	Mean	17.31	8.12	19.55	20.18	13.62	27.97	49.44	22.30
Kinetin									
0.4	1.0	8.60	1.10	15.20	14.13	15.56	33.86	30.56	17.00
	2.0	8.73	2.30	16.26	15.26	18.10	16.33	31.20	15.48
0.6	1.0	10.33	11.40	36.03	40.20	10.40	30.50	39.60	25.49
	2.0	9.80	10.93	35.80	41.40	12.36	21.70	40.70	24.67
0.8	1.0	14.20	8.00	24.50	30.33	30.86	34.20	34.63	25.25
	2.0	18.30	4.36	9.56	32.20	30.93	30.66	40.53	23.82
	Mean	11.66	6.34	22.89	28.92	19.70	27.87	36.20	21.95
	Grand Mean	14.49	7.23	21.23	24.56	16.67	27.90	42.83	22.12

	SE (d)	CD (P = 0.05)	CD (P = 0.01)
Hybrid (H)	1.30**	2.561	3.393
Treatment (T)	1.20**	2.384	3.132
H × T	2.30**	4.531	6.000

with 2.0mg/l BAP and 2.0mg/l GA_3 accounted for maximum regeneration. Rohilla *et al.* [18] reported that MS medium with 1.0mg/l BAP and 1.0mg/l Kn was the best for regeneration. Regeneration of green plants were more in NAA induced calli as compared to 2,4-D according to Mandal *et al.* [19]. The hybrid Improved White Ponni/ CB(DH) 95298 recorded the maximum mean regeneration potential of 42.83 per cent while CR1009/TNAU 841434 had the least (7.23 per cent) regeneration potential. The hybrids showing high

regeneration potential gave only albino plants. Production of albino plants is one of the most frequent and conspicuous manifestations of somaclonal variation [20]. Factors which influence the emergence of albino plants were reported to be: genotype and physiological status of the anther donor plants, developmental stage of microspores [21], culture temperature for callus induction [22], cold pre treatment of anther [23], light intensity during culture [24], callus selection, growth regulator combination [5] and sucrose concentration in combination with growth regulators [25]. The frequency of regeneration of albino calli was significantly lower in the liquid regeneration medium [20]. A water loss of 11 and 16 per cent for *indica* rice calli and 39 and 59 per cent for *japonica* rice calli has been recorded for 24 and 48 hour dessication which improved the green plant regeneration [26]. Jain *et al.* [27] noted that the dry condition of tissues improved oxygen supply to the embroyogenic callus, which may also explain the enhancement of regeneration with dessication treatment. Only three hybrids *viz.*, CR 1009/TNAU 891434, CR 1009/ HA 891037 and CO 43/ADT 43 recorded green plant regeneration and they were transferred to the rooting medium for the induction of roots. Rooting medium consisting of MS + NAA 2 mg/l gave a better response for induction of roots. Anther culture of hybrids was performed to produce doubled homozygous lines for fixing heterosis in a short period of time. Among the seven hybrids cultured, green plant regeneration was observed in only three hybrid combination. Response of *indica* genotypes to anther culture was very less. Factors affecting regeneration potential of *indica* rice must be studied in detail for further improvement in the future.

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