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Differential genotypic and growth regulators response in androgenesis of rice (*Oryza sativa* L.)

Arpita Das[1]*, Nutan Verma[2], GS Mandal[1], Bholanath Saha[3] and AK Singh[2]

[1]Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia- 741252, India

[2]Birsa Agricultural University, Kanke, Ranchi, Jharkhand - 834 006, India

[3]Bihar Agricultural University, Noorsarai, Nalanda, Bihar - 803 113, India

Corresponding author e-mail: arpitacoh@gmail.com

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ABSTRACT

Effect of genotype, pollen developmental stage, synthetic plant hormones in influencing androgenesis of rice were assessed. Response regarding androgenic callus induction as well as green plantlet regeneration was superior in the genotype Rajshree in comparison with other genotypes like Sita, Jaya and Kanak. It was observed that the late uninucleate stage of pollen development is the best for calli formation from cultured anthers of rice. Among the growth regulator tested, MS medium fortified with 1.5 mg l^{-1} 2,4-D was most encouraging for callus induction and proliferation. However, regeneration medium supplemented with BAP (1.5 mg l^{-1}), NAA (0.5 mg l^{-1}) and KIN (2.0 mg l^{-1}) was found to be the most suitable for green plantlet regeneration. For root induction and elongation, MS medium fortified with BAP (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}) performed most dynamically. In vitro generated androclones were successfully acclimatized. The callus cytology showed the presence of different levels of cytodifferentiation and variant cells among the regenerated plants.

Key words: *Androgenesis, genotypes, growth regulators, androclonal variation, rice*

Rice is the most important food crop and around half of the World's population eats rice every day and about 70 % of the World's poor depend on rice as their staple food and also as source of energy (Khush *et al.* 2000 and Zeigler 2010). The demand for rice is continuously growing with the increasing population, thus genetic improvements of this crop have been targeted to increase productivity through suitable biotechnological approaches.

In the recent past among the various biotechnological approaches, anther culture in rice has been improved substantially. However, detailed study on various factors and identification and screening of useful cultivars for androgenesis and subsequent plant regeneration through in vitro system are vital steps in rice genetic improvement programme (Hoque *et al*. 2004 and Islam *et al.* 2005). Plantlets developed directly from microspores provide ample scope in developing homozygous doubled haploids (DH) without interference of heterozygosity. More over, it is also useful for increasing selection efficiency, widening of genetic variability through the production of gametoclonal variants and allowing early expression of recessive genes (Zapata 1982). Androclonal variation seems to have immense prospect albeit it was quantified in a very few cases in rice (Mandal *et al*. 2000). However, many difficulties of anther culture applying in rice breeding have been counted: genotype dependent, low frequency of calllus induction, low frequency of plant regeneration, low ratio of green plants to albino, and high frequency of haploid plants. Owing to the importance of anther culture in speeding up the breeding process, several experiments have been conducted to study the influencing factors such as genotypes of the explants (Shen *et al.* 1982; Li 1991; Roy and Mandal 2005), growth condition of the donor

plants (Chen 1988; Gioi and Tuan 2004; Niroula and Bimb 2009), developmental stages of microspores (Genovesi and Magil 1979; Park 2013), pre-treatment (Qu and Chen 1983; Bisnoi 2000; Gueye and Ndir 2010; Herath and Bandara 2011), culture methods and media (Sun *et al*. 1990; Mandal and Gupta 1997; Gioi and Tuan 2004; Ali 2004) and culture condition (Chen 1988; Silva 2010).

Considering the above aspects of anther culture, the present study was aimed at increasing the green plantlet regeneration frequency by optimizing proper pollen developmental stage, phytohormones and to assess the variation in genotypic response in androgenesis. In vivo and in vitro pollen cytology was also carried out to assess the pollen developmental stage as well as for histological observation.

MATERIALS AND METHODS

This experiment was conducted at department of Plant Breeding & Genetics, Rajendra Agricultural University (RAU), Pusa, Samastipur, Bihar, India. During this study field grown immature panicles of four cultivars of *indica* rice, namely Sita, Jaya, Rajshree and Kanak were selected for anther culture.

Explant sterilization

The immature panicles consisting of spikelet of 1.5 cm length and containing pollen at the mid to late-uninucleate stages of four cultivars were taken from experimental paddy field of RAU, Pusa and sterilized by following the standard explants sterilization protocol, previously described by different researchers (Zobayer *et al*. 2011 and Sikdar *et al*. 2012). The panicles were thoroughly washed with distilled water for three times. Then the explants were washed with 70 % ethanol for a duration of three minutes. In the laminar air flow cabinet, the explants were treated with 0.1 % $HgCl_2$ with the addition of few drops of Tween-20 for a duration of 4 to 6 minutes. Finally, the explants were washed with sterile distilled water for several times to remove all the sterilizing agent. Under the laminar air flow cabinet anthers were taken out from the surface sterilized panicles with the help of two pre-sterilized dissecting needles for inoculation. The developmental stage of the pollen grains was determined.

Callus Induction

The surface sterilized anthers were plated aseptically on to radiation-sterile test tube containing callus induction basal medium MS supplemented with different concentration and combination of growth hormones. 2,4-D, IAA and NAA were tried singly (1.5 mg l^{-1}) or in combination (2,4-D or IAA: 1.5 mg l^{-1} + KIN 1.0 mg l^{-1}). Thus five profiles of hormonal combination were constituted to initiate and promote callus induction. The innoculated culture tubes were maintained in an environmentally controlled growth room at temperature of 25 ± 2^0C and 50 -80 % relative humidity in dark condition. Callus induction frequency of the cultivars were observed at regular interval of one week.

Regeneration

For plant regeneration, the embryogenic part of calli was cut into small pieces of at least 2 mm diameter by removing non-embryogenic part and transferred to regeneration medium consisting of MS basal medium supplemented with 3 % (w/v) sucrose, 0.8 % (w/v) agar and different phytohormones. For regeneration, all together four different combinations of treatments consisting of BAP (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}) and BAP (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}) + KIN (1.0 to 3.0 mg l^{-1}) were attempted. The culture tubes were plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The culture tubes were maintained at 25 ± 2^0 C under a cycle of 16 hours light and 8 hours dark for 4 weeks.

Acclimatization and Field transfer

Healthy regenerated plantlets, with 2 to 2.5 cm root length and 4 to 6 cm shoot length were taken out along with agar and washed thoroughly with distilled water to remove all the traces of the adhering agar. The regenerated plants are transferred in small plastic cup containing sterilized 1:1 mixture of sand and compost and transferred to a poly house, accompanied with intermittent water spraying for a period of ten days. All the cups were covered with polythene sheet to provide the maximum humidity. After primary acclimatization, the plantlets were transferred into black poly bag filled with sterile pot mixture (FYM, sand , soil 1:1:1 v/v) along with optimum water supply to retain a high humid condition till transplantation in the field.

Cytological Studies

Pollen cytology of all the cultivars was done before inoculation to assess the pollen developmental state of anthers to be cultured and from the cultured anthers to

trace the different pathways of androgenesis. In vivo and in vitro anthers were fixed in 3:1 ethyl alcohol : acetic acid in separate capped sampling tube followed by staining in 2 % aceto carmine solution and finally observed under microscope. For cytological examination of calli, 6 weeks old primary calli of yellow green colour from each cultivar were taken and stained with 2% acetocarmin and kept for 6-10 days. The stained calli tissue were squashed in 45 % acetic acid solution on microslide and observation was taken regarding ploidy and cellular and chromosomal abnormalities.

Data Analysis

Complete Randomized Design (CRD) was followed for the in vitro culture experiments. Each single explant was considered as an experimental unit. The experiments were carried out in six replications including 20 explants in each and standard deviation was calculated from accumulated data (Gomez and Gomez 1984).

RESULT AND DISCUSSION

Experiments were conducted to optimize anther culture protocol in rice with special emphasis in determining the optimum stage of pollen for inoculation, effect of genotype, chemical composition of the medium for callus induction as well as for regeneration.

Appropriate media manipulation for maximum callus induction, callus proliferation and healthy plantlet regeneration is immensely important to develop an efficient androculture system.

Effect of pollen developmental stage

The anthers of four cultivars, namely, Sita, Jaya, Rajshree and Kanak were analysed cytologically and grouped into five sizes representing pollen mother cell, early uninucleate, late uninucleate, early binucleate and late binucleate stages of pollen development. Different sized anthers differing in pollen developmental stages were inoculated on medium containing MS +2, 4-D (1.5 mg l^{-1}) and anther callusing frequency was scored (Data was not shown). The best callusing frequency was observed at late uninucleate stage in all the four genotypes. The pollen developmental stage of the cultured anthers is one of the most important factors for the success of anther culture (Roy and Mandal 2005). Gioi and Tuan (2004) have found the late uninucleate stage of pollen development, the best for callus formation from the cultured anthers in rice.

Effect of genotypes on callus induction and regeneration

The genotype of the plant is an important factor that affects transformation of cultured anthers or microspores into calli and plants (Shen *et al*. 1982). Significant genotypic differences in callus initiation response were observed among the four rice genotypes investigated (Table 1). Among the different genotypes inoculated for callus induction in the present study, the maximum callus induction was observed in Rajshree (72.82 %) followed by Jaya (61.00 %); while Kanak showed the minimum callus induction (43.04%) in the best phytohormone combinations. Primary calli mass were white and globular comprising numerous proembryos and embryo initials in all the varieties. Sterio microscopic observations showed that such calli were consisted of embryogenic cells (whitish, compact, dry surfaced, friable, cytoplasm rich) and non-embryogenic cells (yellowish, brownish, vacuolated, elongated, tubular) as observed in rice (Mandal and Gupta 1995). All the genotypes were able to induce calli most of them were whitish green with friable and compact nature. When the calli were directly transferred into regeneration media after two subcultures, the maximum plant regeneration was observed again in Rajshree (60.85 %) followed by Jaya (54.38 %) (Table 2). Rajshree was also proved as the best variety regarding rooting through producing maximum rooting response (65.16 %). Likewise calli induction and regeneration, in case of rooting also Kanak showed a dawdling response. Plantlet regeneration is a genotype specific character (Biswas and Mandal 2007). The absolute growth factor requirements for both callus and plantlet development from cultured anthers are genotype dependent. This finding corroborated with the works of several scientists during androgenesis of rice (Mandal and Gupta 1997; Faruque *et al*. 1998 and Bishnoi *et al*. 2000)

Effect of phytohormone on callus induction and plant regeneration

Earlier (Yang *et al*. 1999) as well in the present study, it was observed that callus induction was hindered in the absence of 2,4-D when IAA, NAA and KIN were used singly or in combination. In all the varieties the most prominent result regarding callus induction was

Table 1. Effect of genotype and embryogenic induction medium on callus induction of rice in MS basal medium

Genotype	PGR (mg l^{-1})				% of culture showing callus formation	Callus colour	Callus nature	Callus growth
	2,4-D	IAA	NAA	KIN				
Sita	1.5	-	-	-	52.00±0.8f	White green	Friable, compact	++
	-	1.5	-	-	38.00±0.5j	White yellow	Friable, loose	+
	-	-	1.5	-	40.83±0.4i	White green	Friable, compact	++
	1.5	-	-	1.0	46.66±1.3g	White green	Friable compact	++
	-	1.5	-	1.0	36.00±0.0k	White brown	Friable, rough	+
Jaya	1.5	-	-	-	61.00±1.3c	White green	Friable, compact	+++
	-	1.5	-	-	43.00±0.5h	White yellow	Friable, loose	++
	-	-	1.5	-	47.50±1.5g	White green	Friable, compact	++
	1.5	-	-	1.0	55.00±0.0e	White green	Friable compact	+++
	-	1.5	-	1.0	41.00±0.5i	White brown	Friable, rough	++
Rajshree	1.5	-	-	-	72.82±0.9a	White green	Friable, compact	+++
	-	1.5	-	-	53.83±0.2ef	White yellow	Friable, loose	++
	-	-	1.5	-	60.72±0.3c	White green	Friable, compact	++
	1.5	-	-	1.0	66.00±0.0b	White green	Friable compact	+++
	-	1.5	-	1.0	58.39±0.4d	White brown	Friable, rough	++
Kanak	1.5	-	-	-	43.04±0.2h	White green	Friable, compact	++
	-	1.5	-	-	38.69±0.8j	White yellow	Friable, loose	+
	-	-	1.5	-	40.00±1.0i	White green	Friable, compact	++
	1.5	-	-	1.0	42.50±0.5h	White green	Friable compact	+
	-	1.5	-	1.0	31.34±0.8l	White brown	Friable, rough	+

+++: Prolific; ++ : Good, +: Fair

Data represents mean ± SD of 20 replicates per treatment in six repeated experiments. Mean within column separated by Duncan's Multiple Range Test (p=0.05)

Fig. 1. Plated anthers showing androgenic callus induction

Fig. 2. Androgenic calli harbouring regenerated plantlet

Fig. 3. Regenerated plantlet in rooting media

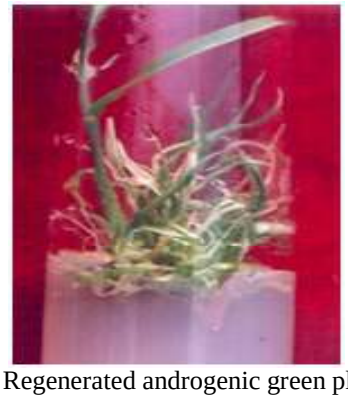

Fig. 4. Regenerated androgenic green plantlet

obtained when MS medium fortified with 2,4-D at the concentration of 1.5 mg l^{-1} (Table 1). Calli were whitish green in colour with friable and compact nature having good to prolific growth. The monitored performance

Table 2. Effect of genotype and growth regulators in MS basal medium on plant regeneration

Genotype	PGR (mg l^{-1})			Plantlet regeneration %	No. of plantlet/ callus	% response of rooting	Root growth
	BAP	NAA	KIN				
Sita	2.0	0.5	-	42.60±0.6h	5.00±0.5de	**50.09±0.2f**	++
	2.0	0.5	1.0	45.04±0.2fg	5.27±1.0d	48.24±0.4g	++
	2.0	0.5	2.0	**46.60±0.4f**	**5.32±0.7d**	47.85±0.2g	++
	2.0	0.5	3.0	44.00±0.6g	5.00±0.3de	45.34±0.5h	++
Jaya	2.0	0.5	-	45.39±0.3f	5.56±0.4d	**58.67±0.3c**	+++
	2.0	0.5	1.0	50.00±0.8e	6.00±0.7c	55.54±0.8d	++
	2.0	0.5	2.0	53.26±0.9cd	6.65±0.3c	53.09±0.6e	++
	2.0	0.5	3.0	**54.38±0.1c**	**7.23±0.8b**	52.47±0.6e	++
Rajshree	2.0	0.5	-	52.70±0.8d	7.52±0.5b	**65.16±0.9a**	+++
	2.0	0.5	1.0	57.68±0.4b	7.87±0.8b	64.00±0.0a	+++
	2.0	0.5	2.0	**60.85±0.2a**	**9.27±0.9a**	63.87±0.2ab	+++
	2.0	0.5	3.0	58.65±0.3ab	9.00±0.0a	61.25±0.6b	++
Kanak	2.0	0.5	-	30.49±0.2k	3.14±0.8g	40.83±0.2i	++
	2.0	0.5	1.0	32.86±0.4j	3.88±0.2f	38.47±0.6ij	+
	2.0	0.5	2.0	32.04±0.1j	3.66±0.4f	36.90±0.1j	+
	2.0	0.5	3.0	**34.23±0.7i**	**4.42±0.6e**	34.00±0.0k	+

+++: Prolific; ++ : Good, +: Fair

Data represents mean ± SD of 20 replicates per treatment in six repeated experiments. Mean within column separated by

on callus induction clearly pointed out that either IAA (1.5 mg l^{-1}) or NAA (1.5 mg l^{-1}) alone or a combination of IAA (1.5 mg l^{-1}) and KIN (1.0 mg l^{-1}) could not offer any satisfactory result. Whereas a combination of 2,4-D (1.5 mg l^{-1}) and KIN (1.0 mg l^{-1}) reflected some good result than when KIN was used in combination with IAA. In rice, it was reported that 1.5 to 2 mg l^{-1} 2,4-D was the optimum concentration for production of maximum embryogenic callus (Tan *et al*. 1999; Biswas and Mandal 2007). It was further reported that 2,4-D (2.0 to 3.0 mg l^{-1}) enhanced the callus induction but calli did not differentiate into green plantlets in rice (Anderson and Al-Khayri 1996). But it is well established fact that when 2,4-D used singly in callus induction media, better performance was displayed in respect of callus induction in rice (Kavikishor and Reddy 1986) and wheat (Redway *et al*. 1990). Four combinations consisting of combination of BAP (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}) and also different concentrations of KIN (1.0 to 3.0 mg l^{-1}) in combination with the same concentration of BAP and NAA were tried as regeneration media across rice varieties. The maximum plantlet regeneration was obtained in Rajshree (60.85 %) with more than nine plantlet/ callus in the MS medium fortified with BAP (1.5 mg l^{-1}) + NAA (0.5 mg l^{-1}) and KIN (2.0 mg l^{-1}). In case of Sita also maximum plant regeneration (46.00 %) as well as maximum number of regenerated plantlet/

Table 3. Frequency of different shaped callus cells

Sl No.	Shape	Total no. of cells	Frequency (%)
1	Round	34	29.56
2	Elongated	39	33.91
3	Sickle	33	28.69
4	Rectangular	9	7.82
Total		**115**	**100.00**

Table 4. Frequency of different sized rice callus cell

Sl No.	Size of cell/class interval (μm²)	Total no. of cells	Frequency (%)
1	000-1000	17	14.78
2	1000-2000	42	36.52
3	2000-3000	20	17.39
4	3000-4000	17	14.78
5	4000-5000	10	8.69
6	5000-6000	03	2.60
7	6000-7000	02	1.74
8	7000-8000	-	-
9	8000-9000	01	0.87
10	9000-10000	02	1.74
11	10000-11000	-	-
12	11000-12000	01	0.87
Total		115	100.00

Table 5. Frequency of rice callus as per number of nucleus

Sl No.	Type	Total no. of callus cells	Frequency of callus cells (%)
1	Uninucleate	91	79.13
2	Binucleate	10	8.69
3	Trinucleate	03	2.60
4	Tetranucleate	11	9.56
Total		115	100.0

Table 6. Ploidy of regenerant obtained through anther culture

Type of nucleus	Frequency of ploidy nucleus (%)
Haploid (n)	63.63
Diploid (2n)	25.45
Triploid (3n)	7.27
Tetraploid (4n)	3.63

Fig. 5. Acclimatized regenerated

callus (5.32) was obtained in the same media composition, whereas, in case of Jaya and Kanak the satisfactory result was obtained when KIN was used in the concentration of 3 mg l^{-1} instead of 2 mg l^{-1}. Invariably this concentration also stimulated maximum number of plantlet/callus. In cereals, high plantlet regeneration and development of more plantlets/calli through androgenesis largely depend upon medium and the type and quantum of growth regulators used. From earlier study, it was reported that, of the two cytokinins (BAP and KIN), KIN in combination with auxin produced more plantlets than BAP among the rice varieties (Peyachoknagul *et al*. 1994). However, in the present study, 2 mg l^{-1} BAP + 0.5 mg l^{-1} NAA + 2.0-3.0 mg l^{-1} KIN emerged as the best hormonal combination, which may be used to develop efficient anther culture in rice for high plantlet regeneration. For root induction and growth of root, as a growth regulator the effect of KIN was not satisfactory in the present study. With the increasing concentration of KIN the rooting percentage as well as root growth became sluggish. Irrespective of all the varieties, maximum rooting response was observed when MS medium fortified with BAP (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}).

Cytological Studies

The calli cells showed wide variation in shape, size, number of nucleus and ploidy status of nucleus (Table 3 to 6). Among the different shapes of calli cells, elongated large cells, which were mostly vacuolated with small or no nucleus, pointed towards the undifferentiated dying callus cells (Table 3). The maximum frequency of 33.91 % of such cells were found when rice calli were analysed for frequency of different sized callus. The round cells were the cells which underwent redifferentiation forming different organ and regenerating plant.

The analysis of rice calli cells for the number of nucleus has shown the maximum frequency of uninucleate cells (79.13 %). These cells were metabolically more active and represented the re-differentiating callus.

The presence of variant cells in callus can only be established through the study of ploidy status of its nucleus which was a reflection of its size. The haploid cells were the maximum (63.63 %), but the presence of diploid, triploid and tetraploid cells in good frequencies showed the high probability of variations.

Cytological studies of callus cells provided first hand information of calliclonal variation (Mandal *et al*. 2000; Roy and Mandal 2005). The increased number of nucleus and higher ploidy in the callus cells may have been produced due to the presence of phytohormones in the media (Stanilova *et al*. 1994). The phytohormones particularly the auxin which have been used in most of the media combinations, have been implicated for inducing genetic variations in tissue culture (Shepard 1981; Kumar *et al*. 1983).

In the present study, emphasis is given to haploid induction through anther culture. In vitro

Fig. 6. In vitro culture derived plantlet at milking stage under glasshouse condition

androgenesis via anther-microspore culture is most preferred techniques for obtaining haploids. The production of haploids and DHs provides a particularly attractive biotechnological tool, and the development of haploid technology and protocols to produce homozygous plants has had a significant impact on agricultural systems. Research efforts on the enhancement of response to anther culture have been confined mostly on manipulation of callus induction and plant regeneration protocols (Gioi and Tuan 2004, Niroula and Bimb 2009).

In conclusion, the aforesaid protocol of androgenesis assures a colossal prospective for exploitation in large scale. From the experiment, it was reflected that the late uninucleate stage of pollen development is the optimum stage for good callus induction and proliferation. Further, among the different genotypes, Rajshree proved its potential as a prominent explant in androgenesis of rice. Among the growth regulator tested, MS medium fortified with 1.5 mg l^{-1} 2,4-D was most encouraging for callus induction and proliferation. On the other hand, regeneration medium supplemented with BAP (1.5 mg l^{-1}), NAA (0.5 mg l^{-1}) and KIN (2.0 mg l^{-1}) was found to be most suitable in the best genotype for plant regeneration. Further, irrespective of all the varieties, maximum rooting response was observed when MS medium fortified with BAP (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1}). The callus cytology showed the presence of different levels of cytodifferentiation and variant cells which ultimately depicted the presence of variant somaclones among the regenerated plants, reflecting the possibility of use of androgenesis for the improvement of rice.

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