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Effect of genotypes and different concentration of growth regulator on callus induction and plant regeneration through anther culture of rice

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Abstract
Rice (*Oryza sativa* L.) is one of the most important cereal crops. In rice, anther culture is an important biotechnological tool for immediate fixation of homozygosity thereby compressing the breeding cycle. In the present study, effect of plant growth regulators on callus induction and regeneration from anther derived callus in the two rice genotypes *indica* and *japonica* were studied. *Japonica* var. Moroberekan showed higher callus induction and regeneration ability compared to *indica* var. Jaya. The growth regulator treatment NAA 2 mg l^{-1} + Kinetin 1 mg l^{-1} induced highest percentage of callus (57.50%) in *japonica* var. Moroberekan, whereas the growth regulator treatment NAA 1 mg l^{-1} + Kinetin 0.5 mg l^{-1} induced highest percentage of callus(32.50%) in *indica* var. Jaya. The best treatment for plant regeneration in Moroberekan was NAA 0.5 mg l^{-1} + Kinetin 0.5 mg l^{-1}, plant regeneration percentage was 62.50 percent while for Jaya it was NAA 0.5 mg l^{-1} + Kinetin 1.5 mg $l^{-1}$with a regeneration percentage of 29.13 percent. The study showed that genotype and growth hormone concentration greatly influence *in vitro* androgenic callus induction and regeneration efficiency in rice.

Keywords: *Oryza sativa* L., anther culture, growth regulators

Introduction
Rice (*Oryza sativa* L. 2n=24) is a monocotyledonous angiosperm belonging to the family of grasses, Gramineae and genus *Oryza*. Rice is the second most widely consumed cereal in the world next to wheat. It is the staple food for two thirds of the world's population. Over 2 billion people in Asia alone derive 80% of their energy needs from rice, which contains 80% carbohydrates, 7–8% protein, 3% fat, and 3% fiber. Rice is a monocotyledonous angiosperm belonging to the family of grasses, Gramineae and genus *Oryza*. The cultivated species are *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* is grown all over the world while *Oryza glaberrima* has been cultivated in West Africa. *Oryza sativa* contains two major subspecies: *Japonica or sinica* and *Indica*. *Japonica* is a relatively short plant with narrow, dark green leaves, and the grains are short and sticky with low amylose content. Japonica varieties are usually cultivated in dry fields, in temperate East Asia, upland areas of Southeast Asia and high elevations in South Asia. Another subspecies is *indica* the plants of which tall with broad to narrow, light green leaves. The grains are long, slender, and non-sticky with high amylase content. *Indica* varieties are grown mostly in submerged conditions throughout tropical Asia.
The rice genome is small (about 430 Mb) compared to other cereal crops such as maize, barley and wheat and contains an estimated 32,000 to 62,000 genes (Bennetzen, 2002; Sasaki and Sedoroff, 2003) [3, 19]. This small genome size has contributed to rice becoming the prominent model system for cereal genomics as well as a model for monocotyledonous plants. In rice breeding programme and in the development of hybrid rice, emphasis has been given to improving the yield, increasing resistance to pests and diseases, and better adaptability to diverse growing conditions.
Considerable improvement has already been achieved by exploiting the natural variation through conventional breeding. Traditional breeding efforts alone cannot meet the increasing demand of rice consumers in the 21st century. At present various tissue culture techniques are being used for the genetic improvement of rice plant throughout the world (Raina. 1989) [17]. Among these anther culture, protoplast fusion and culture, leaf culture, root culture, immature embryo culture and mature seed culture are important in rice to create additional variation and novel rice varieties (Lutts *et al.*, 2001 and Sathish *et al.*, 1995) [12, 20].
Haploid plants are very important in various realms of research disciplines such as plant biotechnology, molecular genetics and traditional plant breeding (Chawla, 2002; Cuthbert *et al.*, 2008 and Touraev *et al.*, 2009) [4, 7, 21]. Production of haploid plants that inherit chromosomes from only one parent can greatly accelerate plant breeding.

Haploids generated from a heterozygous individual and converted to diploid create instant homozygous lines, by-passing the need for selfing for several generations. The use of completely homozygous doubled haploid (DH) lines would considerably shorten breeding processes. Haploids and DH lines can be obtained in various ways, including anther, microspore, and ovule culture, and DH lines have been used.

Anther culture is recognized as a valuable tool in plant breeding programs. A systematic anther culture approach with the use of suitable media and nutrients can lead to sufficient DH production for specific traits. The rate of success for callus induction and high regeneration can be enhanced by selection of explants, genotype, and improving the composition of tissue culture medium by manipulating the plant growth regulators (Mandal and Gupta, 1995) [14].

Among various factors associated with anther culturability, the most important one is the genotypic difference. Many researchers reported that different rice species, subspecies or varieties behaved quite differently in response to anther culture. With this background, the present investigation was undertaken with the effect of genotypic difference in callus induction and regeneration through anther culture in rice.

Materials and methods

The present investigations on Regeneration studies on anther derived callus of rice were carried out at the Plant Tissue Culture Laboratory, Department of Plant Biotechnology, University of Agricultural Sciences, GKVK Campus, and Bangalore-560065. Two Rice genotypes *indica* var. Jaya and *japonica* var. Moroberekan were used as the source of explants. These genotypes were grown in the field till the time of flowering. Recommended fertilizers and plant protection measures were adopted to raise healthy plant. Panicles were harvested at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Panicles with a maximum distance of 4-9 cm between the subtending leaf and the flag leaf were selected. Panicles were collected between 7.00 to 9.00 AM, washed with water and sprayed with 70 percent ethanol. One spikelet was randomly selected from each panicle and fixed in acetic acid-ethanol (1:3) as mordant for 24 h, then stained with 2 percent aceto-carmine and observed under 40X light microscope to identify the pollen development stage. These Panicles were wrapped in aluminium foil and sealed in a polyethylene bag. Cold pretreatment was given by placing them in the refrigerator at 4 °C for 5-6 days.

Necessary precautions were taken to avoid contamination. On the day of inoculation, selected panicles were taken out of the refrigerator and sterilized with 70 percent alcohol for 20 seconds, followed by 0.2 percent HgCl2 for 10 minutes in the laminar air flow chamber. After 10 minutes, the sterilant was drained off and the panicles were thoroughly rinsed with sterile distilled water for 3-4 times. After that, the anthers were isolated from spikelet avoiding any mechanical damage followed by inoculation on petri dish (60 mm × 15 mm) containing 10 ml of modified N6 solid basal medium with 3 percent of maltose. One petridish with 40 anthers constituted one replication for each treatment. Three replications were maintained for each treatment. Different concentrations of NAA (0, 0.50, 1.00, 2.00, 2.50 mgl^{-1}) and Kinetin (0.00, 0.50, 1.00, 2.00mgl^{-1}) in modified N6 as a basal medium with were the anther inoculation treatments.

Embryogenic calli were transferred into the MS basal medium supplemented with different concentrations of growth regulators and sucrose 3% (w/v). The cultures were incubated in a culture room maintained at 23±2 °C, with a relative humidity of 50-60 percent. Cultures were kept under continuous light for the first 2 days after which were kept in 16 hour photoperiod at a photon flux density of 3000 lux from white cool fluorescent tubes. All the experiments were conducted in the plant tissue culture laboratory, under uniform condition of temperature, humidity and light. For each treatment used in the experiment, three replications were maintained and data were analyzed by Factorial Completely Randomized Design (FCRD) method for callus induction and regeneration.

Results and discussion

Effect of genotypes and growth regulator concentration on callus induction

The study was carried out to find out the effect of different concentrations of growth regulators on callus induction in the two rice cultivars. Callus induction started 4weeks after culture. The callus induction frequencies of Japonica were higher with different concentration of growth regulators than in the Indica varieties. The effect of genotypes and growth regulator concentration on the callus induction frequency was significant at 1% level. Significant difference in callus induction was also observed between genotypes and treatments. In rice it has been demonstrated that *japonica* genotypes produce more androgenic callus than *indica* genotypes (Zapata *et al.,* 1990; Chen *et al.*, 1991; Mandal and Bonyopadhayi., 1997; Gosal *et al*., 1997; Yamagishi *et al.*, 1998) [24, 6, 9, 23]. In the present study it has been observed that the callus induction frequency *indic*a var. Jaya varied from 0.00 to 32.50 percent, which is lower than that observed in *japonica* var. Moroberekan which is varied from 2.5 percent to 57.5 percent. *Indica* varieties are known to produce either no or very low frequencies of callus because of early anther necrosis and poor callus proliferation Chen and Lin (1976) [5]. Callus induction ability of anthers in rice is a quantative trait controlled by nuclear gene. Miah *et al*. (1985) [15] showed that callus induction ability is inherited as recessive character and *japonica* appears to be a good combiner for callus induction.

Callus induction frequency high in the media supplemented with low levels of NAA and kinetin (Table 1). The auxins 2, 4-D and NAA are equally efficient in promoting microspore callus formation, but callus formed in the presence of 2,4-D is less capable of plant regeneration compared to that formed on medium supplemented with NAA (Ball *et al.*, 1993) [2]. NAA induces direct androgenesis, while 2,4-D promotes rapid cell proliferation and formation of non-embryogenic callus (Ball *et al.*, 1993) [2]. 2,4-D has inhibitory effects on callus differentiation and can be substituted by α-naphthaleneacetic acid (NAA) (Niizeki and Oono 1971). Hence in the present study NAA has been used for callus induction. Irrespective of genotypes, highest callus induction was observed in T8 treatment (45%), while lowest callus induction was observed in T6 treatment (9.58%). In T8 treatment the medium was supplemented with NAA 2 mg l-1 + Kinetin 1mg l-1 and T6 treatment the medium was supplemented with NAA 1 mg l-1+ Kinetin 2 mg l-1. The rate of success can be enhanced by manipulating the plant growth regulators (Mandal and Gupta, 1995) [14]. Trejo-Tapia *et al*., (2002) [22] studied callus induction from microspores and reported that combination of both NAA and Kinetin plant growth regulator was a better alternative than the use of auxin alone for callus induction. For efficient production of microspore callus of high morphogenetic potential, both auxin and cytokinin are needed in the callus formation medium.

Anther culture response in Moroberekan and Jaya

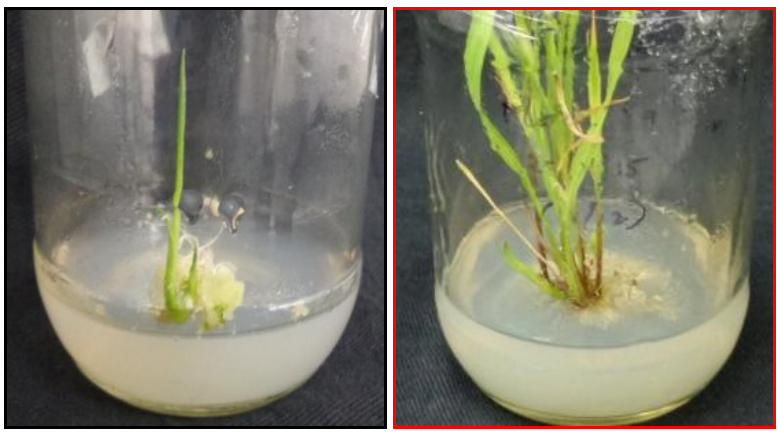
Table 1: Effect of different concentration of NAA and Kinetin in rice genotypes

Treatments	NAA (mg l $^{-1}$)	Kinetin (mg l^{-1})	Mean of Replication		Percent Of Callus Induction	
			Jaya	Moroberekan	JAYA	MOROBEREKAN
T_0	0	0	0	0	0.00(0.00)	0.00(0.00)
T_1	0.5	0.5	1.33	10.67	3.33(10.51)	26.66(31.08)
T_2	0.5	1	9.67	7	24.15(29.43)	17.50(24.73)
T_3	0.5	2	0	13.67	0.00(0.00)	34.15(35.76)
T_4	1	0.5	13	1	32.50(34.76)	2.50(9.15)
T_5	1	1	9.33	3.67	23.30(28.91)	9.15(17.61)
T_6	1	2	0	7.67	0.00(0.00)	19.15(25.95)
T_7	2	0.5	9.67	1.67	24.15(29.43)	4.15(11.75)
T_8	2	1	13	23	32.50(34.75)	57.50(49.31)
T_9	2	2	6.67	4.67	16.65(24.08)	11.65(19.96)
T_{10}	2.5	0.5	6.33	6	15.82(23.44)	15.00(22.79)
T_{11}	2.5	1	0	8	0.00(0.00)	20.00(26.57)
T_{12}	2.5	2	8.33	0	20.83(27.16)	0.00(0.00)

Plant regeneration from anther derived calli

Plant regeneration started two weeks after transfer of calli to the plant regeneration medium. The growth regulator combination used in the callus induction medium influenced plant regeneration significantly. There was no plant regeneration from the growth regulator free media.

Significant difference in shoot regeneration frequency was observed among the genotypes. Higher regeneration was recorded in *japonica* var. Moroberekan (21.15%) than *indica* var. Jaya (4.80%). Zhang and Hattori (1996) reported that regeneration of Japonica rice cultivars is controlled by a single dominant gene. *japonica* (Giza 171) or Japonica × Indica (Giza 171X Giza 175) cultivars are more responsive than *indica* × *japonica* (Giza 175 × Milyamg 49) cultivars (Rashed and EL-Sharkawy, 1998). The present results are in agreement with the above observations made by previous scientists that the genotype influences regeneration frequency. Irrespective of genotypes, highest shoot regeneration was observed in R1 treatment NAA 0.5 mg l-1 + kinetin 0.5 mg l-1 (31.25%) and lowest shoot regeneration was observed in R8 treatment NAA 0.50 mg l-1 + BAP 1 mg l-1 in (8.32%). The findings of the present investigation are in agreement with the observations made by Rukmini *et al*. (2013) that the regeneration medium supplemented with different combinations of the phytohormones, auxins and cytokinins (NAA, Kinetin, and BAP) stimulated highest regeneration. Bari *et al*. (2003) [1] and Paramasivam *et al*. (2010) were able to obtain a higher regeneration frequency when the callus was transferred to MS medium supplemented with BAP, kinetin and NAA.



Green plant regeneration from callus

Plant regeneration was comparatibly high in Japonica rather than indica. Most of the treatments regenerated plants were albino than green plant. Herath *et al*. (2008) [11] reported that high percentage of albino plant than the green plant occurred in both *japonica* and *indica* genotypes. Albinism is a major problem encountered in cereal anther and microspore culture. Many factors have been found to affect the degree of albinism such as the genotype and physiological state of the donor plants, the developmental stage of the microspores, culture temperature and cold pre-treatment. Herada *et al*. (1991) [10] discovered that albinism in rice is due to deletion of plastid genome. Similar observation has been made in wheat and barley by Day and Ellis (1985) [8].

Table 2: Plant regeneration from anther derived calli

Treatment	NAA (mg l $^{-1}$)	BAP (mg l $^{-1}$)	Kinetin (mg l $^{-1}$)	Plant regeneration frequency	
				Jaya	Moroberekan
R_0	0	0	0	0.00	0.00
R_1	0.5	0	0.5	0.00	62.50
R_2	0.5	0.5	0	0.00	33.38
R_3	0.5	0	0.75	16.63	33.38
R_4	0.5	0.75	0	0.00	45.88
R_5	0.5	0	1	0.00	0.00
R_6	0.5	1	0	0.00	37.50
R_7	0.5	0	1.5	29.13	0.00
R_8	0.5	1.5	0	16.63	33.38
R_9	0.5	0	2	0.00	0.00
R_{10}	0.5	2	0	0.00	0.00
R_{11}	0.5	0	2.5	0.00	25.00
R_{12}	0.5	2.5	0	0.00	0.00

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