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The Effect of Plant Growth Regulators on Anther Culture Response and Plant Regeneration in Selected Sri Lankan Indica Rice Varieties, Japonica Varieties and Their Inter- Sub Specific Hybrids

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ABSTRACT. *The effect of 2, 4-Dichlorophenoxy acetic acid (2, 4-D), ∝-Naphthalene acetic acid (NAA) and Kinetin in anther culture of Japonica rice variety Hu lo tao, Indica rice varieties BG 90-2, BG 379-2 and their F1 hybrids for high frequency of callus induction and plant regeneration was investigated. Anthers were cultured in N6 medium containing 5% sucrose and variable amounts of 2,4-D, NAA and Kinetin. The cultures were placed in the dark at 28± 2^0 C for callus induction. When the calli reached 1-2 mm in diameter they were transferred to half strength Murashige and Skoog (MS) medium supplemented with 2.0 mg l^{-1} Kinetin and 0.5 mg l^{-1} NAA. The effect of variety and the growth regulator combination for the callus induction frequency was significant at 5% significance level. Indica cultivars had the lowest callus induction (ranging from 0.0% - 5.0%) while Japonica and Indica × japonica F1 hybrids had comparatively high (ranging from 5.0% - 35.3%) callus induction. The F1 hybrid Hu lo tao × BG 90-2 had a significantly high level of callus induction than the other genotypes ranging from 13.6% - 35.3%. Both F1 hybrids had a high callus induction frequency than the Japonica and Indica parents. The addition of 2,4-D 2.0 mg l^{-1} and Kinetin 0.5 mg l^{-1} had the highest callus induction frequency for all the parental varieties and F1 hybrids.Plant regeneration (green, albino) was significantly high in Japonica parent and F1 hybrids. Highest plant regeneration was observed in Kinetin 0.5 mg l^{-1} + 2,4- D 2.0 mg l^{-1} (T12) treatment in all the genotypes.*

INTRODUCTION

The production of double haploids through anther culture represents a modern tool for the improvement of cultivated species enabling plant breeders to produce homozygous lines in a few months (Wojnarowiew *et al.*, 2002). Androgenesis results in homozygous progeny from a heterozygous parent in a single generation and provides excellent material for research, plant breeding and plant transformation (Wang *et al.*, 2000). Anther culture shortens the breeding cycle for producing homozygous lines to one generation rather than 8 - 10 generations and is a easier method to identify superior genotypes in a cross and produce new cultivars (Senadheera *et al.,* 2002).

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Androgenesis in many species is affected by factors such as genotype, growth of donor plants, pre treatments of anthers, composition of media and culture conditions (Ciner and Tipirdamaz, 2002). The capacity to regenerate a sufficient number of double haploid plants to produce adequate population sizes from which to select is an important aspect in the use of anther culture for varietal improvement (Chu *et al.*, 2002).

The practical application of androgenetic haploids in rice improvement is still limited by the low regeneration and low number of doubled haploids recovered, particularly in Indica types (Senadheera *et al.,* 2002). Further, in Indica rice, plant regeneration frequency is generally low and also highly genotype dependent (Raina *et al.,* 1996). As Zhang (1989) stated, making cross combinations among the parents with different anther culture abilities may contribute to overcome anther culture difficulties.

Plant growth substances, especially auxins in combination with cytokinins have been widely used in rice anther culture (Shigh-wei and Zhi-Hong; Chen *et al.,* 1991). The auxins 2, 4, dichlorophenoxy acetic acid (2, 4 - D) and α-naphthalene acetic acid (NAA) are equally efficient in promoting microspore callus formation but, 2, 4 - D alone has inhibitory effects on callus differentiation (Chen *et al.,* 1991). However, according to past work, if kinetin was used callus formation and plantlet formation were both stimulated (Shigh-wei and Zhi-Hong, 1991). Yi (1991) has reported that, N6 medium supplemented with 2.0 mg l^{-1} 2, 4-D + 0.5 to 1.0 mg l^{-1} kinetin results in high frequency of good calli. Similarly, there was direct regeneration of plantlets on the anther when the medium was supplemented with a low concentration of auxin and a high concentration of cytokinin.

High yielding commercially grown rice varieties and traditional rice varieties with resistance to pests and diseases and desirable grain quality characteristics that are grown in Sri Lanka were Indica types, and have poor response to anther culture (Senadheera *et al*., 2002). The transfer of anther culture response traits from Japonica to Indica rice varieties by crossing is important to improve Indica rice varieties. The aim of the present study was to investigate the effect of 2, 4-D, NAA and Kinetin in anther culture of some Japonica varieties, Indica varieties of rice and their F1 hybrids for high frequency of callus induction and plant regeneration.

MATERIALS AND METHODS

Plant materials

Seeds of the Japonica and Indica rice varieties were obtained from the Plant Genetic Resources Centre, Gannoruwa, Peradeniya, Sri Lanka (Table 1) and were grown in a green house providing all the standard agronomic practices.

Table 1. Characteristics of the selected parental varieties

Genotype	Characteristics
Hu lo tao	Japonica, traditional variety
BG 90-2	Indica, high yielding
BG 379-2	Indica, high yielding, resistant to brown plant hopper

Anther pre- treatment

Initially, two to three panicles were harvested from each genotype between 9.00 to 10.00 a.m. on sunny days. Panicles were harvested when the distance between collar of flag leaf and penultimate leaf was between 5-7 cm (Croughan, 1998) and the anthers were at uni-nucleate stage (microscopic observation). Anthers were obtained from spikelets of the middle part of the panicles. Panicles were wrapped in aluminum foil with moistened cotton plugs at the base. They were sealed in polypropylene bags and kept at 5^{o}C for 7 days (Croughan, 1998). The intact panicles were rinsed with 70% (v/v) ethanol for 20 seconds. Spiklets were then removed and surface sterilized with 30% (v/v) commercial chlorox solution for 20 minutes and rinsed thoroughly with sterilized distilled water.

Anther culture

Spikelets were cut at the base and the anthers gently squeezed out with a needle. One hundred anthers were inoculated in 100×15 mm petridishes with solidified agar N6 medium containing 5% sucrose and variable amounts of 2,4-D (2.0, 4.0, 8.0 mg l^{-1}), NAA (2.0, 4.0, 8.0 mg l^{-1}) and Kinetin (0.5, 1.0 mg l^{-1}) (Table 2). One petri dish with 100 anthers constituted one replicate and an average of 5 replicates were cultured for each treatment. The cultures were placed in the dark at 28$\pm$ 2^{0} C (Chen *et al.,* 1991) for callus induction. The cultures were examined at weekly intervals for six weeks and the percentage of anthers forming calli (callus induction frequency) was recorded after six weeks.

Plant regeneration

When the calli reached approximately 1-2 mm diameter they were transferred to 100 mm diameter × 15 mm height petri dishes containing 25 ml of half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2.0 mg l^{-1} Kinetin and 0.5 mg l^{-1} NAA (Croughan and Chu, 1991). The cultures were given a 16 hour photoperiod (50 µE m^{-1} S^{1}).

Counts were made at weekly intervals and the data on percentage calli regenerating green and/ or albino plants was recorded after 6 weeks of incubation. The experiment was repeated thrice and the data shown are per experiment mean.

The regenerated shoots were transferred to hormone-free half strength MS medium for rooting. Plantlets with well developed root systems were acclimatized and grown to maturity in a greenhouse providing standard agronomic practices.

Data analyses

Statistical analyses of data were performed using Statistical Analysis System (SAS Release 9.1). Data were subjected to analyses of variance and the mean comparison was done using Duncan New Multiple Range Test (DNMRT) at 5% significance level. All experiments were analyzed using the model of Completely Randomized Designs.

RESULTS AND DISCUSSION

Effect of plant growth regulators on callus induction

Callus induction started three weeks after culture (Figure 1, a). The callus induction frequency of Japonica and F1 Indica × Japonica hybrids were high in media with and without growth regulators than in the Indica varieties. The effect of variety and the growth regulator combination on the callus induction frequency was significant at 5% level. The interaction effect of the above two factors was also significant ($p>0.05$) for callus induction. Influence of genotype is considered to be the most important factor in anther culture whereas Indica cultivars have been reported to have lowest regeneration frequencies (Balachandran *et al.,*1999). In this study Indica cultivars had the lowest callus induction (ranging from 0.0% - 5.0%) while Japonica and Indica × Japonica F1 hybrids had comparatively high (ranging from 5.0% - 35.3%) callus induction. The F1 hybrid Hu lo tao × BG 90-2 had significantly high level of callus induction than the other genotypes ranging from 13.6% - 35.3%. Both F1 hybrids had high callus induction frequencies than the japonica and Indica parents.

Callus induction frequency was high in the media supplemented with low levels of 2, 4-D and NAA (Table 2). Roy and Mandal (2005) stated that N6 medium fortified with 2,4-D and NAA have promotive effects to induce callus in rice varieties. At high levels of 2,4-D and NAA, the callus induction frequency reduced significantly. Chen *et al.,*(2001) have reported that at higher or lower concentration of 2, 4 – D, the number of calli derived from anthers decrease. Hu lo tao and both F1 hybrids had highest callus induction frequency in the T12 (Kinetin 0.5 mg l^{-1} + 2,4- D 2.0 mg l^{-1}) treatment. BG 379-2 and BG 90-2 (indica) varieties had high callus induction frequencies in both T9 (Kinetin 0.5 mg l^{-1} + 2,4-D 2.0 mg l^{-1}) and T12 (Kinetin 0.5 mg l^{-1} + 2,4- D 2.0 mg l^{-1}) treatments. It can be concluded that, for efficient callus induction both auxins and cytokinins are required.

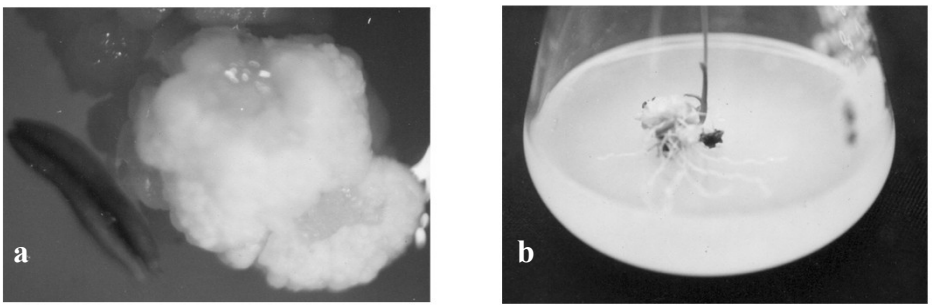

Figure 1. Anther culture response in Hu lo tao × BG 90-2 hybrid:
a: Callus development after three weeks in culture establishment
b: Green plant regeneration from callus three weeks after calli transfer

The addition of 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kinetin showed the best callus induction frequency for all the parental varieties and F1 hybrids.

Plant regeneration from anther derived calli

Plant regeneration started two weeks after from transfer of calli to the plant regeneration medium (Figure 1, b). Some calli regenerated only green or albino plants. Some regenerated both green and albino plants. The growth regulator combination used in the callus induction medium influenced plant regeneration significantly. There was no plant regeneration from the calli induced in the growth regulator free media. Callus formed in auxin free media became necrotic when they were transferred to plant regeneration medium.

Plant regeneration (green, albino) was comparatively high in Japonica parent and F1 hybrids (Table 3). Indica parents had very poor plant regeneration. Relatively high level of plant regeneration could be observed in F1 hybrids than in both Indica and Japonica parents. Datta (2005) stated that, the type of nutrient media, genotype and phytohormones are some of the important factors that influence microspore embryogenesis.

Parent genotypes had a high percentage of albino plant regeneration than green plant regeneration. However, the F1 hybrid genotypes had comparatively high level of green plant regeneration than albino plants. Generally green plant regeneration from androgenic calli is very low irrespective of race. Low anther culture response and high percent of albino plantlet regeneration are the principal constraints in rice (Roy and Mandal, 2005). According to Balachandran *et al.,* (1999), the parents that yield more calli exhibit a tendency to regenerate plants proportionately at a higher frequency.

Table 2. Effect of growth regulators on callus induction from anthers of selected Japonica, Indica parental varieties and their F1 hybrids

Growth regulator combination			Treatment	Percentage calli formation				
Kinetin	2,4-D	NAA		Hu lo tao	BG 90-2	Bg 379-2	Hu lo tao × Bg 90-2	Hu lo tao × Bg 379-2
0	0	0	T1	5.0^{n}	0.0^{h}	0.0^{g}	13.6^{j}	8.0^{j}
0	0	2	T2	11.3^{efg}	2.3^{cd}	1.6^{de}	19.6^{gh}	12.3^{fgh}
0	0	4	T3	9.6^{ijkl}	1.0^{f}	1.3^{def}	22.6^{e}	13.9^{e}
0	0	8	T4	9.3^{jklm}	1.3^{ef}	1.0^{ef}	19.3^{ghi}	11.6^{hi}
0	2	0	T5	11.6^{def}	2.6^{bc}	3.0^{b}	25.0^{cd}	17.0^{d}
0	4	0	T6	8.6^{lm}	1.6^{def}	1.3^{def}	20.3^{fgh}	13.6^{ef}
0	8	0	T7	8.3^{m}	1.0^{f}	0.6^{fg}	17.6^{i}	11.3^{hi}
0.5	0	0	T8	9.3^{jklm}	1.3^{ef}	1.3^{def}	20.0^{gh}	19.3^{c}
0.5	0	2	T9	12.6^{bcd}	3.3^{ab}	5.0^{a}	26.6^{bc}	14.0^{e}
0.5	0	4	T 10	10.6^{fghi}	1.6^{def}	2.0^{cd}	23.0^{e}	11.3^{h}
0.5	0	8	T 11	10.3^{ghij}	1.3^{ef}	1.6^{de}	19.0^{ghi}	10.3^{i}
0.5	2	0	T 12	17.6^{a}	3.6^{a}	4.6^{a}	35.3^{a}	25.6^{a}
0.5	4	0	T 13	13.6^{b}	2.3^{cd}	2.0^{cd}	26.0^{c}	21.3^{b}
0.5	8	0	T 14	11.3^{efg}	1.3^{ef}	1.6^{de}	22.0^{ef}	17.6^{d}
1.0	0	0	T 15	9.0^{klm}	1.0^{f}	1.3^{def}	20.6^{fg}	16.6^{d}
1.0	0	2	T 16	11.0^{efgh}	2.0^{cde}	3.0^{b}	25.3^{cd}	17.3^{d}
1.0	0	4	T 17	10.6^{fghi}	1.3^{ef}	1.0^{ef}	23.6^{de}	12.6^{efgh}
1.0	0	8	T 18	10.0^{hijk}	0.3^{g}	0.6^{fg}	19.3^{ghi}	14.0^{e}
1.0	2	0	T 19	13.0^{bc}	2.3^{cd}	2.6^{bc}	28.3^{b}	19.3^{c}
1.0	4	0	T 20	12.0^{cde}	1.3^{ef}	1.3^{def}	25.0^{cd}	13.3^{efg}
1.0	8	0	T 21	9.3^{jklm}	1.0^{f}	0.6^{fg}	18.6^{hi}	12.0^{gh}

* In each column the means with same letters are not significantly different ($p< 0.05$)

Highest plant regeneration was observed in T12 (Kinetin 0.5 mg l^{-1} + 2,4- D 2.0 mg l^{-1}) treatment in all the genotypes. Both 2,4-D and NAA at high concentration resulted in poor plantlet induction. Similarly, at high kinetin level (1.0 mg l^{-1}), plant regeneration was retarded. Roy and Mandal (2005) have reported that, the success of plantlet regeneration under *in vitro* culture system depends upon the type and dose of different growth regulators, especially auxins and cytokinins used in cereal anther culture. They have also reported that, among the auxins, 2,4-D is useful for callus induction and subsequently in green plantlet regeneration. Reddy *et al.,* (1985) stated that high concentration of auxins lower the ability of the callus to produce green plants. Thus instead of using high levels of auxins, addition of kinetin at low concentration encourages callus induction and subsequent plantlet regeneration.

CONCLUSIONS

The supplementation of N6 medium with 2.0 mg l^{-1} 2, 4-D and 0.5 mg l^{-1} Kinetin gives the best callus induction and plant regeneration in selected Indica and Japonica parents and their F1 hybrids.

Table 3. Green (G) and albino (A) plant regeneration from anther derived calli

Treatment	Percentage calli regenerating plants									
	Hu lo tao		BG 90-2		BG 379-2		Hu lo tao * Bg 90-2		Hu lo tao * Bg 379-2	
	G	A	G	A	G	A	G	A	G	A
T1	-	-	-	-	-	-	-	-	-	-
T2	2.1	3.3	-	-	-	-	3.5	2.7	-	3.2
T3	-	-	-	-	-	-	-	-	-	-
T4	-	-	-	-	-	-	-	-	-	-
T5	5.0	5.8	-	-	-	-	8.3	7.4	5.2	4.7
T6	-	2.2	-	-	-	-	2.1	3.2	-	2.3
T7	-	-	-	-	-	-	-	1.3	-	-
T8	-	-	-	-	-	-	-	-	-	-
T9	5.8	6.3	-	-	-	-	9.2	8.3	5.7	4.3
T 10	-	3.2	-	-	-	-	2.3	2.8	-	8.1
T 11	-	1.0	-	-	-	-	-	5.4	-	2.1
T 12	25.2	20.3	2.0	3.2	-	1.0	52.1	48.5	12.2	15.3
T 13	10.	17.2	2.0	4.0	-	2.3	40.3	38.5	8.7	6.2
T 14	-	-	-	-	-	-	-	-	-	-
T 15	-	-	-	-	-	-	-	-	-	-
T 16	8.2	10.7	-	5.8	-	3.8	18.7	12.3	10.1	9.3
T 17	3.2	5.9	-	-	-	-	9.7	8.3	2.1	3.8
T 18	-	-	-	-	-	-	-	3.0	-	-
T 19	10.1	13.5	2.0	4.7	-	3.2	29.8	26.3	8.2	7.9
T 20	4.2	6.3	-	-	-	-	6.8	5.9	2.1	1.3
T 21	-	-	-	-	-	-	3.1	2.8	-	

Data represents percentage plants (G or A) regenerated out of total calla (1 -2 cm diameter) transferred to the plant regeneration medium

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