Tropical Agricultural Research & Extension 25 (3): 2022

SHORT COMMUNICATION

ANTHER CULTURE PERFORMANCES OF SELECTED *Indica* RICE VARIETIES IN SRI LANKA: ABILITY OF CALLUS INDUCTION AND PLANT REGENERATION

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Received: 09 January 2022, Accepted:14 July 2022

ABSTRACT

Doubled-haploid plant production through anther culture provides an efficient and appropriate system for rapid production of homozygous lines. Various culture conditions influence callus induction and plant regeneration efficiency. In this study, the optimization of media requirements and culture conditions for high-frequency callus induction and plant regeneration for selected *indica* rice varieties were evaluated using improved anther culture media. Panicles of ten selected rice cultivars (Bg 358, Bg 379-2, Bw 361, At 362, Bw, 364, Bw267-3, Bw 272-6b, At 306, Bg 357 and Kahata wee) were cold pre-treated at 10ºC for 7-10 days. After that anthers were cultured in agar solidified N6 and L8 media where hormonal combinations were modified. Calli were transferred to three different Murasinge and Skoog media for plant regeneration. Green and Albino shoots were observed. Best callus induction frequencies (6.9-11.1%) were acquired in the L8 medium containing 6% (w/v) maltose, over the N6 (3.6—5.7%) medium. Highly responsive genotypes for callus initiation were AT362, BG 379-2, BW 267-3 and AT 306. BG357 and BW 364 were the least responsive genotypes. The best callus induction frequency of 11.1% was observed in the L8 medium supplemented with 2.0mgl^{-1} 2, 4-D and 0.5mgl^{-1}Kinetin (KN) for AT 362. Maximum green plant regeneration could be observed in Kahata wee (29.2%) and AT362 (18.27%) varieties in MS media supplemented with 2mg/l BAP, 1mg/l NAA and 0.5mg/l KN. After 7-10days of callus initiation, they were transferred to MS medium to regenerate plants. Plant regeneration was observed after two weeks after the transfer. This information could be used in the development of homozygous plants with high yields and good grain quality from the best hybrid varieties.

Keywords: Anther culture, *Indica*, N6&L8media, Plant regeneration

INTRODUCTION

Rice is one of the staple food crops in the world (Kush, 2004). Being a highly Self-pollinated crop, the development and selection of elite breeding lines with superior phenotypes are the utmost objective of genetic improvement.

In this context, anther culture is an efficient process that can be utilized for the speedy development of homozygous plants. Pollen within the cultured anthers may be induced to form callus which will subsequently regenerate haploid genotypes. Haploids upon chromosome doubling yield fully homozygous in many plant species. In *Zea mays*, some cultivars were completely unresponsive in anther culture, while few produce haploid plantlets (Bhojwani and Razdan, 1996). In Triticum aestivum, many genotypes did not respond to anther culture and only in a few genotypes green plants were regenerated at low frequency (Bhojwani and Razdan, 1996; Ren et.al. 2017). Niizeki and Oono (1968) were the ones who regenerated

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rice plants for the first time through anther culture. Homozygous breeding lines in japonica rice have been made through this Anther culture technique and it can be called a successful process (Brar and Kush 2006).The use of anther culture in making *indica* rice has not been successful due to many constraints. For instance, the genetic background of *indica* varieties (He *et al*. 2006). But some local *indica* rice varieties have got successful results in several efforts (Kumari *et al*. 2006).

The less morphogenetic skill of anther-derived calli and a higher number of albino plant regeneration (Talebi et.al. 2007; Ren et.al. 2017) are the major constraints for doubled-haploid breeding. There are several other factors such as the composition of culture medium, pretreatment of buds or anthers, the growth stage of microspores, culture density, anther wall factor and effect of light and temperature also affect the success of anther culture (Guha-Mukhejee, 1973). Cultivars of *indica* rice reveal less androgenic response than the japonica cultivars (Hu, 1985).Gueye and Ndir (2010) reported that 79 were albinos out of 93 regenerants in their study. Therefore, culture variables being optimized can be presented as a vital move that indicates the effectiveness of using anther culture.

In Sri Lanka, there has been tremendous improvement in rice production through conventional breeding. However, the use of biotechnology to develop varieties, of biotic and abiotic stresses resisting has been limited. This study was undertaken to identify the optimum culture conditions for callus induction and green plant regeneration of ten *indica* rice genotypes.

MATERIALS AND METHODS

Plant materials and growth conditions:

Ten different rice varieties were selected from Rice Research and Development Institute, Batalagoda, Regional Rice Research and

Table 1: Selected Indica rice varieties with their specific characteristics

Variety	Desirable characteristics
Bg 358	3 - 3 ½ months samba variety, Resistant to BPH, BL and BLS, Moderately tolerant to iron toxicity, Recommended for general cultivation
Bg 379-2	4 – 4 ½ months variety, Have white pericarp/ Nadu, Resistant to BPH and BB Recommended for general cultivation.
Bg 357	3 ½ months variety**,** Have white pericarp/ Nadu, Resistant to BPH, Gall midge, Moderately resistant to BL, Thrips, Iron toxicity.
Bw 361	3 ½ months variety, Have red pericarp/ Nadu, Moderately resistant to GM, BPH, Recommended for dry and intermediate zones.
Bw 364	3 ½ months variety, Have red pericarp, iron toxicity tolerance variety, Moderately resistance to Blast, BLS,BPH and GM, Recommended for wet zone
Bw 267 – 3	3 ½ months variety, Have white pericarp and long slender grains, Resistance to Blast Iron toxicity and seed spotting, Recommended for thewet zone
Bw 272 - 6b	2 ½ months samba variety, red pericarp, Resistant to Blast, Resistant to lodging, Iron toxicity tolerance, Recommended for the low country wet zone, Suitable for bog soils.
At 306	3 months basmati variety, Long grains with high quality, Moderately resistant to BLS and GM, Resistant to blast and BPH, Recommended for general cultivation
At 362	3 ½ months variety, Have red pericarp/Nadu, Moderately resistant to Blast, BPH and GM, Recommended for general cultivation.
Kahata wee	6 months traditional variety, tolerance to Iron toxicity.

BPH-Brown Plant Hopper,BB- Bacterial Blight, BL–Rice Blast, GM–Gall Midge
BLS–Bacterial Leaf Streak

Development Institute, Bombuwala and egional Rice Research and Development Institute, Ambalantota according to farmer preference and their favourable characteristics (Table1).They were grown in pots under greenhouse conditions following the standard agronomic practices recommended by the Department of Agriculture.

Anther pre-treatment and preparation for culture:

Panicles were collected from all the varieties when the gap between the flag leaf auricle to the penultimate leaf (panicle length) was between 5-11cm. Anthers of the uni-nucleate stage (microscopic observation) were obtained from the spikelets of the middle part of the panicles. Collected panicles were wrapped in Aluminum foil, placed in polythene bags, and kept in an incubator at 10^{o}Cfor 7-10 days (Zapata-Arias 2003).

After cold pre-treatment, the intact panicles were rinsed using 70% (v/v) ethanol for the 20s.To remove the Spikelets from panicles were sterilized with 30% (v/v) commercial bleach solution (Clorox) for 20 minutes and washed thoroughly using sterilized distilled water. After that, the anthers were gently squeezed out by cutting the spikelets at the base. In a 100x15 mm petri dish, 100 anthers were placed on an agar solidified induction medium with maltose 6%.The pH of the media was adjusted to 5.8. Twelve different culture media were tested, which have been prepared by modifying the N6 medium (Chu, 1978) and L8 medium (Linsmaier and Skoog, 1965) added with different concentrations of 2 -4 D, kinetin and NAA (Table 2).For each genotype, on average ten replicates were cultured and a single petri dish represented one replicate. The cultures were kept in the dark at 28 °C (Chen *et al*. 1991) for callus induction and the percentage of anthers forming calli (callus induction frequency) was noted after six weeks.

Table 2: Concentration combinations of growth regulators in the N6 and L8 medium for callus induction

Type of Medium	Medium No.	2, 4-D (mg/l)	NAA (mg/l)	Kinetin (mg/l)
	1	2.0	0.0	0.5
	2	2.0	0.0	1.0
	3	0.0	2.0	0.5
	4	2.0	2.0	0.5
	5	2.5	2.0	0.5
N6	6	0.0	2.5	0.5
	7	2.0	2.5	0.5
	8	2.5	2.5	0.5
	9	0.0	2.5	1.0
	10	2.0	2.5	1.0
	11	2.5	2.5	1.0
L8	12	2.0	0.0	0.5

Table 3: Concentrations of growth regulators in the MS medium for regeneration

Medium	KN mg/l	NAA mg/l	BAP mg/l
R_1	2.0	1.0	2.0
R_2	2.0	1.0	1.0
R_3	0.5	1.0	2.0

KN=Kinetin, 2, 4-D =2,4-Dichlorophenoxy acetic acid, NAA= α-Naphthaline acetic acid, BAP= Benzyl amaino purine

Plant regeneration:
The induced calli were transferred to test tubes containing agar solidified 8g/l Murasinge and Skoog (MS) medium added with different concentrations of growth regulators (Table3) and 30g/l sucrose. Transplanted calli were incubated in a growth chamber at 28 ± 2 °C with 16h of light, at a light intensity of about 2000Lux. The pH of the media was adjusted to 5.8. Anther response and green plant regeneration were recorded in all treatments by counting the number of calli/anther and the number of green shoots/callus.

Data analyses:
All experiments were arranged in Complete Randomized Design (CRD) and Statistical analysis was carried out using the SAS (SAS Institute, Cary, NC, 2013).

RESULTS AND DISCUSSION

Callus induction started at four weeks of culture. The frequency of callus formation varied between 0 to 11.1% depending on the genotype and culture medium (Table 4).Callus induction was more in AT362 and BG379-2in comparison to others. Maximum callus induction (11.1%) was observed in AT362 when the L8 medium was supplemented with 2.0 mg l^{-1} 2, 4-D, 0.5mg/l $^{-1}$ Kinetin and 6% maltose. BG 379-2 (7.3%) and BW 267-3 (6.9%) varieties had high callus induction frequency than the other *indica* varieties. The *indica* varieties BG 357 and BW 364had the least anther culture response in all media. This genotype dependency of the anther culture has been reported by Chen *et al*. (1991); Shih-Wei and Zhi-Hong (1992); Asaduzzaman, *et al*. (2003); Gueye and Ndir, (2010) in their previous studies.

Among the medium tested, L8 medium supplemented with 2.0 mg l^{-1} 2, 4-D, 0.5mg/l $^{-1}$Kinetin and 6% maltose gave higher callus induction percentage in AT362,BG 379-2and BW 267-3varieties. N6 medium supplemented with 2.0 mg l^{-1} 2, 4-D, 0.5mg/l $^{-1}$ Kinetin and 6% maltose was the best for callus induction from AT306 (5.7%), AT364 (5.4%), BG 358 (3.6%), BW 272-6b (4.8%),

Table 4: Mean calli frequencies of tested rice varieties in different callus induction media

Variety/ Medium	Mean calli frequency in each medium											
	1	2	3	4	5	6	7	8	9	10	11	12
AT 306	5.7^{a}	1.8^{bc}	1.0^{cd}	1.0^{cd}	1.0^{cd}	0.6^{d}	0.4^{d}	1.0^{cd}	0.6^{d}	1.6^{bc}	2.3^{b}	1.7^{bc}
AT 362	1.2^{bcd}	0.4^{de}	1.2^{bcd}	0.6^{cde}	0.2^{e}	2.0^{bc}	2.1^{b}	0.2^{e}	1.0^{bcde}	0.2^{e}	1.0^{bcde}	11.1^{a}
AT 364	5.4^{a}	1.8^{bc}	1.4^{bc}	1.4^{bc}	1.0^{c}	0.8^{c}	0.1^{d}	0.2^{d}	1.0^{c}	2.6^{b}	2.6^{b}	4.8^{a}
BG 357	1.4^{ab}	0.0	0.6^{abc}	0.2^{cd}	0.2^{cd}	0.6^{abc}	0.3^{bcd}	0.3^{bcd}	1.6^{a}	0.6^{abc}	1.2^{ab}	0.4^{ab}
BG 358	3.6^{a}	1.3^{b}	1.6^{b}	0.2^{de}	0.0	0.4^{cd}	1.1^{bc}	1.0^{bc}	1.0^{bc}	0.6^{bcd}	0.2^{de}	0.6^{bcd}
BG 379-2	5.4^{ab}	4.4^{bc}	3.0^{bcde}	0.4^{f}	0.1^{f}	1.6^{c}	2.0^{de}	1.6^{e}	2.2^{de}	2.8^{cde}	3.6^{bcd}	7.3^{a}
BW 267-3	3.4^{b}	1.9^{bc}	0.4^{d}	0.2^{d}	0.1^{d}	2.3^{bc}	1.7^{bc}	1.4^{c}	1.4^{c}	1.5^{c}	3.4^{b}	6.9^{a}
BW 272-6b	4.8^{a}	1.2^{ab}	1.2^{ab}	1.2^{ab}	1.2^{ab}	2.1^{bc}	2.9^{b}	1.6^{c}	1.6^{c}	1.7^{c}	1.7^{c}	2.0^{bc}
BW 364	2.2^{a}	1.2^{ab}	1.2^{ab}	0.7^{bcd}	0.3^{cd}	1.4^{ab}	1.2^{ab}	1.0^{abc}	0.7^{bcd}	1.4^{ab}	1.2^{ab}	0.2^{d}
Kahata wee	4.2^{a}	2.0^{cde}	1.7^{def}	1.2^{defg}	0.0	1.2^{defg}	1.0^{efg}	0.8^{gf}	0.6^{g}	3.5^{ab}	2.2^{bcd}	3.4^{abc}

Means were compared across rows.
The composition of each medium equals the composition number code in table 2.

Table 5: Regeneration ability of calli on different regeneration medium

Variety	Frequency of callus induction	Frequency of Plant regeneration (Number of shoots per callus)			
		Medium	Green	Albino	Total
AT 306	5.7	R_1	8.11	9.51	17.62
		R_2	6.45	5.66	12.11
		R_3	**15.72**	**13.21**	**28.93**
AT 362	11.1	R_1	9.70	7.70	17.40
		R_2	8.50	7.60	16.10
		R_3	**18.27**	**22.43**	**40.7**
AT 364	5.4	R_1	6.7	4.3	11.0
		R_2	6.0	4.6	10.6
		R_3	**11.25**	**8.80**	**20.05**
BG 357	1.6	R_1	0	0	0
		R_2	0	0	0
		R_3	0	0	0
BG 358	3.6	R_1	0	0	0
		R_2	0	0	0
		R_3	0	0	0
BG 379-2	7.3	R_1	8.70	3.72	12.42
		R_2	7.20	4.15	11.35
		R_3	**10.25**	**7.11**	**17.36**
BW 267-3	6.9	R_1	9.25	7.10	16.35
		R_2	5.20	3.40	8.60
		R_3	**13.20**	**13.76**	**26.96**
BW 272-6b	4.8	R_1	10.31	9.30	19.61
		R_2	8.19	7.12	15.31
		R_3	**18.12**	**16.23**	**34.35**
BW 364	2.2	R_1	0	0	0
		R_2	0	0	0
		R_3	**9.18**	**9.60**	**18.78**
Kahata wee	4.2	R_1	11.22	8.91	20.13
		R_2	9.51	8.11	17.62
		R_3	**29.2**	**6.33**	**35.53**

BW364 (2.2%) and Kahata wee (4.2%) varieties.

Maximum green plant regeneration was observed in Kahata wee (29.2%)andAT362 (18.27%)in MS medium supplemented with 0.5mgl^{-1} Kinetin, 1mgl^{-1} NAA, 2mgl^{-1} BAP and 3% sucrose (R3medium).Among the *indica* genotypes AT306 (15.7%), AT364 (11.2%), BW272-6b (18.1%), BW267-3 (13.2%), BG379-2 (10.2%) and BW364 (9.1%) gave plant regeneration in R3 medium. BG 357 and BG 358 were not responded to for plant regeneration. All the tested rice varieties showed the highest green plant regeneration in MS media supplemented with 2mg/l BAP+1mg/I NAA+ 0.5mg/I KN (R3). Auxins, IAA and NAA promoted quickest androgenesis while 2,4- D aids rapid cell proliferation and development of callus. According to the observations that are done both 2, 4-D and NAA are not supported in their generation of plants and the cytokinins such as kinetin and BAP are used (Mandal and Gupta, 1995).According to Roy and Mandal (2005), it is clear that regenerating green plant

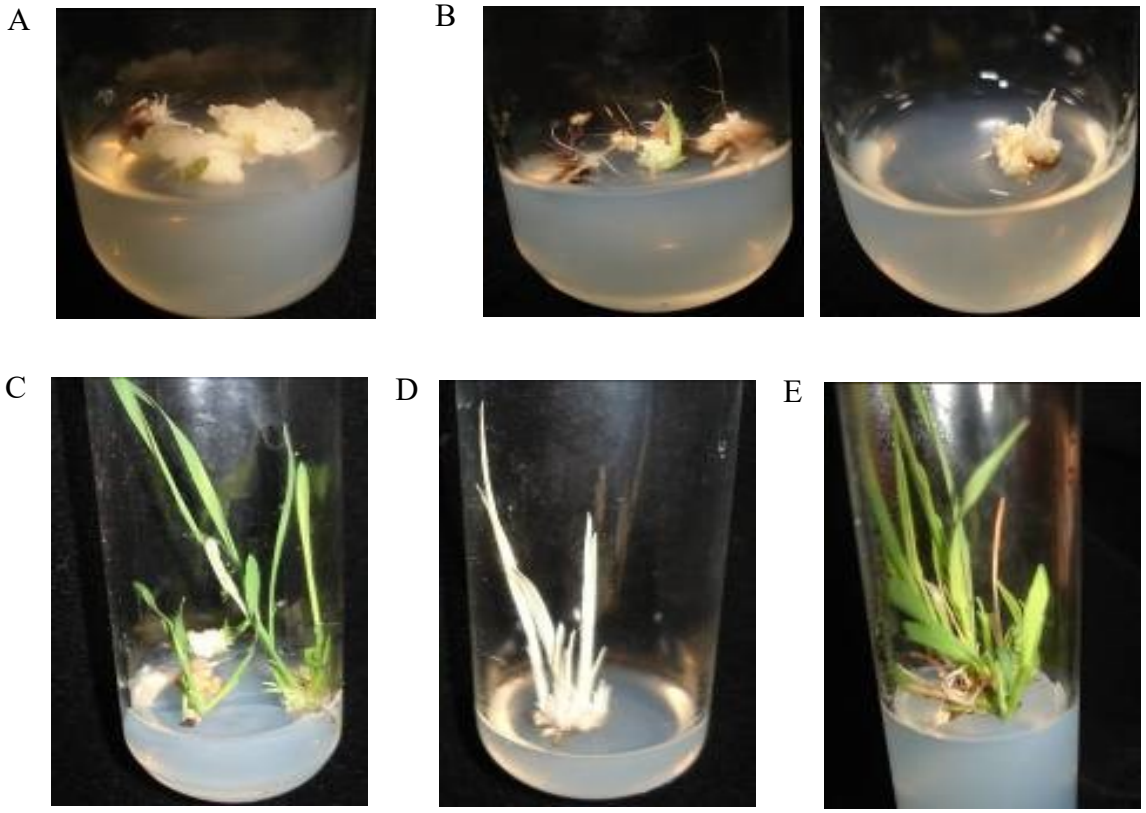

Figure 1: Plant regeneration from anther-derived calliof variety BG 379-2

(A) Callus on regeneration medium (B) Initiation of green/albino shoots (C) Re-generated green plantlets (D) Regenerated albino plantlets (E) Newly developed shoots and roots (3 months after plant regeneration)

by using androgenic calli is a very low and low reaction in anther culture; Albino plant regeneration in high percents are the major constraint in establishing successful anther culture in rice (Ren *et al*. 2017).

CONCLUSIONS

Our results clearly showed that the success of plant regeneration of *indica* rice by anther culture is highly specific to exogenous and endogenous factors. For example, the factors such as genotype, media components, hormonal combination, pre-treatment of anthers and culture requirements. The breeding process can be hastened by highly reproducible techniques of anther culture with optimized culture variables for the development of plant types with desirable and improved quality traits.

AUTHOR CONTRIBUTION

PLPS did the laboratory work and prepared the manuscript, DR conceived the research idea, supervised and edited the manuscript.

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