

Studies to Investigate the Interactions of Genotypes, Culture Media and Culture Temperatures on Androgenesis in Recalcitrant *Indica* Rice (*Oryza Sativa* L.)

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Article history

Received :1 August 2012
Received in revised form :7 Sept. 2012
Accepted :1 October 2012

Graphical abstract

Amino acid/Vitamins	SK I	N6	DKN	SK II
Aspartic acid	=	=	666	=
Glutamin	=	=	731	=
Myo-inositol	=	=	100	=
Casein H.	2000	2000	2000	2000
Glycine	2.0	2	=	2
Thiamine HCl	2.5	1	10	1
Pyridoxine	2.5	0.5	1	0.1
Nicotinic Acid	2.5	0.5	1	0.5

Abstract

Production of doubled haploids through androgenesis is well established in *japonica* rice and successfully used to develop high yielding varieties. However, *indica* rice is remaining recalcitrant to this *in vitro* technique. To understand the interactions of genotypes, culture media and culture temperature, two *indica* varieties (Pokkali and Nona Bokra) were cultured on three culture media under normal (25 °C) and alternate temperatures (30 °C/20 °C, 14 hours/10 hours). The responses of the recalcitrant varieties were examined at callus induction and regeneration stages. Pokkali was found responsive to culture media as well as culture temperatures. Although there was an increase in albino shoot production but green shoot productivity was recorded nine fold under alternate temperatures. Interactions of recalcitrant *indica* genotypes, culture media and culture temperatures were found significant at callus induction and regeneration stages. It is therefore reflected that to activate the recalcitrant genes for callus induction and regeneration of green shoots, interaction of genotypes, culture media and culture temperatures could play significant role. Pokkali responded well to SKI and SK II rather than others, indicated that alteration of culture media composition would be an important aspect in future investigation.

Keywords: Androgenesis, indica rice, culture media, alternate culture temperatures

Abstrak

Pengeluaran haploids dua kali ganda melalui androgenesis ini ditubuhkan pada beras japonica dan berjaya digunakan untuk membangunkan varieti yang memberikan pulangan yang tinggi. Walau bagaimanapun, beras indica baki rekalsitran ini dalam teknik *in vitro*. Untuk memahami interaksi genotip, media budaya dan suhu budaya, dua *indica* pelbagai (Pokkali dan Nona Bokra) yang diternak pada tiga media budaya di bawah biasa (25 °C) dan suhu silih ganti (30 °C/20 °C, 14 jam/10 jam). Jawapan kelainan rekalsitran telah diperiksa pada peringkat induksi kalus dan regenerasi. Pokkali didapati responsif kepada media budaya serta suhu budaya. Walaupun terdapat peningkatan dalam pengeluaran pucuk albino tetapi produktiviti pucuk hijau dicatatkan sembilan kali ganda di bawah suhu ganti. Interaksi genotip *indica* rekalsitran, media budaya dan suhu budaya didapati signifikan pada peringkat induksi kalus dan regenerasi. Oleh yang demikian, ia mencerminkan bahawa untuk mengaktifkan gen rekalsitran untuk induksi kalus dan regenerasi pucuk hijau, interaksi genotip, media budaya dan suhu budaya boleh memainkan peranan penting. Pokkali respons yang sangat baik untuk SKI dan SK II berbanding orang lain, menunjukkan bahawa perubahan komposisi media budaya akan menjadi satu aspek penting dalam penyiasatan masa depan.

Kata kunci: Androgenesis, indica beras, budaya media, budaya suhu silih ganti

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1.0 INTRODUCTION

Among rice, wheat, and maize, rice provides about 50 % calories consumed by the human population. There is an increase of 75 million people a year in the world population and 90% of this increase observed in the developing countries of Asia, Africa, and

Latin America. Providing for population growth, it requires an expansion in world grain production of 26 million tons per year (26). Plant breeding plays the key role in the process of food production. Among the modern biotechnology, haploidy breeding method is the most desirable supplement to plant breeding through androgenesis and gynogenesis. Since the first report on

haploid plant production in rice through anther culture (23), many early studies have been carried out on various aspects of rice anther culture including pollen ontogeny during culture (13, 17). More recently, anther culture technique applied to rice has been greatly improved and its scope expanded to facilitate other biotechnical approaches such as gene transformation technology. Very often gene incorporation results in heterozygosity of the transformed loci. Anther culture has considerable value in shortening the time required to convert the transgenic plants to homozygous breeding lines (1, 25) or as a technique to be adapted for direct one-step homozygous transgenic plant development (5). The potential of anther culture in producing marker-free transgenic rice, a vexed issue with the consumer, has also been recognized (37). However, most developments have occurred in temperate japonica cultivars while success with tropical *indica* types has remained poor (21, 27). As a result, anther culture is now used as a supplementary breeding tool in japonica rice (3, 21), but the potential of the technique for *indica* rice breeding is yet to be fully exploited (15) in spite of an initial report of the release of a salt tolerant *indica* variety through anther culture breeding (32). The poor response of *indica* types to anther culture has been clearly established in many studies. It is reported that anther culture response varied from 42% for a *japonica* cultivar to 0% for an *indica* cultivar (22). Another report described the comparison of *indica* and one *japonica* varieties for callus induction (34), *indica* were found extremely poor (1.7–4.4%) compared with the *japonica* variety (17%). A general trend has been observed in anther culture ability of *japonica* varieties,

indica varieties and their hybrids, in the decreasing order of japonica/japonica[japonica[indica/ japonica[indica[indica[indica (14, 36). Even among genotypes of a particular ecotype, *japonica* or *indica*, considerable variation in pollen callusing and green plant regeneration has been observed, the genotypic effect being greater among the indicas. For example, among seven *indica* varieties, callus induction frequencies varied from 3.6 to 51.7% while green plant regeneration efficiency ranged from 1.6 to 82.9% (15).

Recent studies on anther culture of *indica* rice varieties from countries as climatologically diverse as China, Bangladesh, Sri Lanka and Iran, using improved culture media, continue to highlight the genotype specificity of anther culture response within the *indica* subspecies (15, 18, 29, 35). The recalcitrance displayed by the *indica* types relates to poor callusing ability, limited morphogenetic potential or regeneration ability of the induced callus, and a higher percent of regenerated plants being albinos (2, 31, 35). The poor androgenic response of the *indica* rice thus limits the utilization of this technique as a breeding tool in areas predominantly planted with this ecotype such as the tropical and subtropical regions of Asia. However, several reports described the various factors and conditions that influence the haploid plant production, and critically examine different aspects that could improve this valuable technique (10, 12, 33).

Present studies were carried out to investigate the interactions of recalcitrant rice genotypes, culture media and culture temperatures to improve the androgenesis in *indica* rice.

Table 1 Concentration of macro and micro elements in culture media

Source Salt/s	Element	Callus induction mg/l			Plant regeneration mg/l		
		SK I	N6	DKN	SK II	N6	DKN
(NH ₄) ₂ SO ₄ ,KNO ₃	N	436.6	490.5	126.1	1371.4	490.5	126.1
NH ₄ NO ₃							
KH ₂ PO ₄							
NaH ₂ PO ₄ · 2H ₂ O	P	123.1	91	62	38.8	91	62
KNO ₃ ,KH ₂ PO ₄	K	1371.4	1208.2	312.4	782.7	1208.2	312.4
KI							
CaCl ₂ · 2H ₂ O	Ca	40.8	45.2	40	119.7	45.2	40
MgSO ₄ · 7H ₂ O	Mg	17.6	17.6	23.4	35.14	17.6	23.4
Na ₂ EDTA	EDTA	27.8	27.8	16.2	27.8	27.8	16.2
FeSO ₄ · 7H ₂ O	Fe	5.3	5.3	2.8	5.3	5.3	2.8
MnSO ₄ · 4H ₂ O	Mn	5.5	1.1	0.5	5.5	1.1	0.5
ZnSO ₄ · 7H ₂ O	Zn	0.3	0.3	0.5	1.9	0.3	0.5
H ₃ BO ₃	B	1.1	0.3	0.5	1	0.3	0.5
KI	I	0.8	0.6	=	0.6	0.6	=
CuSO ₄ · 5H ₂ O	Cu	0.05	=	0.05	=	=	0.05
Na ₂ MoO ₄ · 2H ₂ O	Mo	0.10	=	0.05	=	=	0.05
Sucrose	C	16842	25263	12631	12631	12631	12631
Sorbitol		=	=	11868	=	=	=

2.0 MATERIALS AND METHODS

Two indica land races, Pokkali and Nona Bokra, were used grown in pots in natural conditions. A short day treatment was given for three weeks for panicle initiation. Pollen grain developmental stage was examined to select appropriate panicles because mid uninucleate to late uninucleate pollen grain stage was reported the best for anther culture (19). Pre cold treatment was reported to be an effective practice (30), thus the selected panicles were given a pre cold treatment (10 °C for one week). Pollen grain developmental stage was examined after pre cold treatment to select appropriate spicklets. Surface sterilization of selected spicklets was carried out by dipping in 70% ethyl alcohol for 10 seconds and then soaked in 2% solution of NaOCl, including 2-3 drops of poly ethylene sorbitol, for 10 minutes. The spicklets were rinsed thoroughly with sterilized distilled water inside laminar flow. After cutting the 1/3 upper of spicklet, the anthers were excised by a fine forceps and placed on culture media.

Three culture media were selected based on the concentration of macro and micro elements as well as carbon (Table 1). SK I and SK II media were used for callus induction and regeneration of shoots, respectively (28). However, N6 (8) and DKN (9) having the same composition at both androgenesis stages. The concentrations of amino acids and vitamins used in each media are given in Table 2. The combination of axin and cytokinins in each media are described in Table 3.

Table 2. Concentration of amino acids/vitamins in culture media

Amino acid/Vitamins	SK I	N6	DKN	SK II
Aspartic acid	=	=	666	=
Glutamin	=	=	731	=
Myo-inositol	=	=	100	=
Casein H.	2000	2000	2000	2000
Glycine	2.0	2	=	2
Thiamine HCl	2.5	1	10	1
Pyridoxine	2.5	0.5	1	0.1
Nicotinic Acid	2.5	0.5	1	0.5

Table 3 Combination of plant growth regulators in culture media

PGRs	Callus induction mg/l			Plant regeneration mg/l		
	SK I	N6	DKN	SK II	N6	DKN
2,4-D	0.5	2.00	0.5	=	=	=
NAA	2.5	=	=	1	0.1	=
IAA	=	=	=	=	=	0.2
BA	=	=	=	1	=	0.5
Kinetin	0.5	=	=	0.5	0.5	=
Ratio A:C	2.5:1	2:0	0.5:0	1:1.5	1:5	1:2.5

Culture temperature is an important factor having considerable effects on the plant growth *in vitro*. Rice anther culture is usually carried out at constant temperature, day and night (25~28 °C), and was used as control alternate temperatures (20 °C/ 30 °C, 10 hours/14 hours as night and day temperatures, respectively) at callus induction and regeneration stages.

Randomized complete design was used with three replications. Four hundred anthers were cultured (20 petri dishes) in each treatment per replication. Petri dishes were placed to different culture temperatures in darkness. After four weeks, the response of recalcitrant indica rice cultivars was assessed by following parameters at callus induction stage:

Rate of responding anther (RA)

$$= (\text{No. of anthers yielded calli} / \text{No. of cultured anthers}) \times 100.$$

Calli per responding anther (C/RA)

$$= (\text{No. of total calli yielded} / \text{No. of responding anther}).$$

Callus productivity (CP)

$$= (\text{No. of yielded calli} / \text{No. of cultured anthers}) \times 100.$$

Calli were cultured on respective regeneration medium. Nine androgenic calli of 1-2 mm in size were transferred to each petri dish (3.5 cm) for regeneration and cultured in light (50 $\mu\text{E m}^{-2}\text{s}^{-1}$, 14 hours photoperiod) conditions with keeping the same respective culture temperatures. The effectiveness of culture media and culture temperature determine by recording following parameters at regeneration stage

Regeneration Frequency of albino shoots (RFAS)

$$= (\text{No. of calli regenerate green or albino shoot} / \text{No. of cultured calli}) \times 100.$$

Regeneration Frequency of green shoots (RFGS)

$$= (\text{No. of calli regenerate green or green shoot} / \text{No. of cultured calli}) \times 100.$$

Green shoots productivity (GSP)

$$= (\text{No. of green shoots regenerated} / \text{No. of cultured anthers}) \times 100.$$

Stat view was used for statistical analysis of data and presented in Table 4 (Javed et al. 2007), Table 5 and Table 6.

3.0 RESULTS AND DISCUSSION

The response of recalcitrant indica rice cultivars was found significantly different to experimental treatments. Pokkali performed well at callus induction as well as regeneration stage (Table 4). At callus induction stage varieties, callus induction media and culture temperatures exhibited significant differences for responding anthers (RA), calli per responding anther (C/RA) and callus productivity (CP). However, all the interactions were significant at $P < 0.01$ for RA and CP but C/RA exhibited significance for V x CIM only (Table 4). Although all factor showed significant differences all parameters at callus induction stage but interactions of these factors was found highly significant for RA and ultimately same for CP. The coefficient of variation (CV) for CP was lower than C/RA (Table 4) and represents the low degree of variation. It reflected that interactions of genotypes, culture media and culture temperature would be important for higher rate of callus induction. With respect to media compositions, SKI and N6 have higher doses of nitrogen, phosphorus and potash compare to DKN (Table 1) and showed a higher CP under normal and alternate culture temperatures (Table 4). It reflected that indica rice may need

higher doses of nitrogen, phosphorus and potash to initiate callus induction compare to japonica rice.

Table 4. Response of recalcitrant indica varieties to culture media and culture temperatures

Varieties	Culture Temperature	MEDIA	RA	C/RA	CP	Reg. Frequency		GSP
						Green shoots (G)	Albino shoots (S)	
Nona Bokra	25 °C	SK-I	2.5	1.2	3	0	4.2	0
		N 6	1.5	1.3	2	0	0	0
		DKN	1	1.5	1.5	0	0	0
	20/30 °C	SK-I	3	1.3	3.9	3.2	12.9	0.13
		N 6	2.5	1.2	3	0	0	0
		DKN	1	2	2	0	0	0
Pokkali	25 °C	SK-I	7.5	1.2	9	5.6	8.3	0.5
		N 6	4.5	1.3	5.8	0	6.5	0
		DKN	1	3.5	3.5	0	0	0
	20/30 °C	SK-II	12.8	1.9	24.3	18.6	14.4	4.5
		N 6	5.5	1.5	8.3	0	0	0
		DKN	1.5	4	6	0	0	0

(Source: MA Javed et al. 2007)

Rate of responding anther = RA

Calli per responding anther = C/RA

Callus productivity = CP

Regeneration Frequency of albino shoots = RFAS

Regeneration Frequency of green shoots = RFGS

Green shoots productivity = GSP

Table 5. Analysis of variance at callus induction stage

Source of variation	df	Mean Squares		
		RA	C/RA	CP
Varieties (V)	1	78.012**	4.002**	284.006**
Culture Temperature (CT)	1	12.427**	0.602**	83.030**
Callus induction media (CIM)	2	55.392**	5.145**	99.885**
V x CT	1	5.292**	0.135ns	54.722**
V x CIM	2	25.019**	2.112**	61.359**
CT x CIM	2	3.392**	0.112ns	28.857**
V x CT x CIM	2	3.274**	0.045ns	26.289**
Error	11	0.058	0.06	0.264
CV %		6.5	13.4	8.49

*, **: Significant at 5% and 1% level, respectively

ns: non significant

Albinism is counted a major problem in anther and microspore culture in rice, especially in *indica* rice (7). Although the primary cause of albinism has not yet been characterized, many factors have been found to affect the degree of albinism, such as genotype, physiological state of donor plant (4, 20), developmental stage of microspore (6), culture temperature (16), and pre cold treatment (11). In this study, there an increase in RFAS was observed but there was an increase of more than five folds in RFGS and GSP was improved nine folds in Pokkali under alternate temperatures

(Table 4). All three factors were exhibited high significance for RFAS, RFGS and GSP. The interactions of these factors were also highly significant for RFAS, RFGS and GSP. However, coefficient of variance (CV) was recorded quite high for GSP (Table 6), may be due to poor regeneration rate of Nona Bokra. These results reflected that interaction of culture medium and genotype is important for regeneration of green shoots. Presently, anther culture is a routine breeding method in *japonica* rice. N6 and MS media has been used intensively for anther culture of *japonica* rice. Koshihikari is a *japonica* rice variety cultivated on large scale in West Japan. It was found recalcitrant to existing anther culture media. DKN media, new formulation, was found effective (9) to improve the anther culture efficiency of this cultivar. Present study also reflected high amount of that nitrogen and calcium and relatively low phosphorus and potash could have an important role in initiation of regeneration (Table 1 and Table 4). However, a detailed investigation is needed to be carried out with respects to alteration of media composition for efficient shoot regeneration in indica rice. SK media might be optimum for Pokkali rather than Nona Bokra.

Table 6. Analysis of variance at regeneration stage

Source of variation	df	Mean Squares		
		RFGS	RFAS	GSP
Varieties (V)	1	72.802**	25.420**	3.920**
Culture Temperature (CT)	1	43.740**	11.070**	2.870**
Regeneration media (RM)	2	250.253**	456.08**	8.841**
V x CT	1	15.682**	14.570**	2.470**
V x RM	2	145.603**	13.041**	7.841**
CT x RM	2	87.480**	122.716**	5.741**
V x CT x RM	2	31.363**	11.591*	4.941**
Error	1	1.232	4.431	4.055
CV %		15.19	16.33	141.47

*, **: Significant at 5% and 1% level, respectively

An *in vitro* response is the results of a complex interaction involving the physiological state of ex-plant, medium and external environment etc. Remarkable effects of alternate temperatures have been reported in *japonica* rice on callus induction and regeneration of green shoots (24). Keeping in view of Pokkali, just a change of external environment (culture temperature) showed favorable effects on callus induction and regeneration in SK-I and SK-II media, respectively. These results reflected that alternate temperatures could be used to improve the anther culture efficiency of recalcitrant *indica* rice cultivars. The interaction of genotype, culture media and culture temperatures could also be an important factor in haploid plant production in indica rice.

4.0 CONCLUSIONS

To activate the recalcitrance of *indica* rice for androgenesis, the interactions of genotypes, culture media, and culture temperatures are important. The interaction of genotype and culture temperatures could play important role to improve the callus induction and regeneration of green shoots in anther culture of *indica* rice. However, high doses of nitrogen, phosphorus and potassium could be imperative at both stages of androgenesis in *indica* rice.

Acknowledgement

The first author thanks the Research Management Center (RMC), Universiti Teknologi Malaysia (UTM), Malaysia for the financial support under Foreign Academic Visitor Fund (Vote number: 4D044).

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