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Original paper

The role of alternating culture temperatures and maltose in enhancing the anther culture efficiency of salt tolerant *indica rice* (*Oryza sativa* L.) cultivars, Pokkali and Nona Bokra

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Abstract The effects of different culture temperatures, culture media and saccharides on the anther culture efficiency in two salt tolerant rice (*Oryza sativa* ssp. *indica*) cultivars, Pokkali and Nona Bokra, were examined. Comparison of two culture temperatures (constant temperature at 25°C and alternating temperatures at 30°C/20°C) indicated that alternating temperatures showed remarkable effects not only on the callus induction but also on the plant regeneration. Among the basal media examined, higher callus productivity was observed in SK-I medium and higher shoot productivity was obtained in SK-II medium than other basal media. When alternating temperatures and revised N_6 medium including maltose were used throughout anther culture, callus productivity and green shoot productivity showed the highest score in the salt tolerant two cultivars used in this study. Maltose and alternating temperatures increases the anther culture efficiency of recalcitrant *indica* rice cultivars. This is the first report suggesting high production of haploids or doubled haploids from anther culture of these salt tolerant *indica* cultivars.

Key words: Alternating temperatures, anther culture, *Oryza sativa* spp. *indica*, maltose, salt tolerance.

Soil salinity is one of the main obstacles to increasing rice production. Since rice (*Oryza sativa*) is rated as an especially salt sensitive crop (Mass and Hoffman 1977; Shannon et al. 1998), most of the modern high-yielding rice cultivars perform poorly in saline environments. In addition, as saline soils are usually waterlogged, it is not feasible to grow crops other than rice in such areas (Gregorio and Senadhira 1995). The rice germplasm has genetic variability for salt tolerance (Xie et al. 2000), and two traditional *Oryza sativa* spp. *indica* cultivars, Pokkali and Nona Bokra, possess salt tolerance sufficiently high for rice breeding programs (Akbar et al. 1985; Gregorio and Senadhira 1993).

Anther culture is a useful technique to produce lines with desirable combinations of required traits. It has been well integrated into rice breeding programs especially in Japan and China, where a number of high yielding, disease resistant and better quality rice cultivars have been selected from microspore derived plants (Loo and Xu 1991). However, *indica* cultivars respond poorly to *in vitro* techniques (Abe and Futsuhara 1986; Hartke and Lörz 1989), and in consequence the practical production of haploids from anther or microspore culture in rice breeding is limited to *Oryza sativa* spp. *japonica* cultivars. Anther culture is accomplished through two steps in rice; the first step involves the induction of embryogenic calli from microspores and the next is the regeneration of green plants from the calli. In the case of *indica* rice, early anther necrosis, poor callus proliferation and albino plant regeneration are currently recognized as the major problems (Chen et al. 1991). The genotype and the composition of the culture medium are of prime importance for *in vitro* culture to obtain green plantlets (Khanna and Raina 1998; Bishoni et al. 2000). Culture temperature is an important factor in the anther culture of *japonica* rice (Okamoto et al. 2001), and maltose instead of other saccharides is superior in studies of anther culture of cereal crops (Jähne and Lörz 1995; Lentini et al.1995; Raina 1997). Therefore, the effects of several combinations of culture temperatures, culture media and saccharides were investigated to improve anther culture efficiency using two salt tolerant *indica* rice cultivars, Pokkali and Nona Bokra.

Under natural summer conditions in Japan, the two cultivars used in this study do not produce panicles. Therefore, short day treatment (day length, 8 h) was carried out with nine-week-old plants for three weeks to induce reproductive growth. Since the mid to late

Abbreviations: BAP, 6-Benzyladenine; 2,4-D, 2,4-Dichlorophenoxyacetic acid; IAA, Indole-3-acetic acid; NAA, Naphthaleneacetic acid.
This article can be found at http://jspcmb.jp

Table 1. Compositions of modified anther culture media used in this study.

Component	Callus induction media (mg l^{-1})				Regeneration media (mg l^{-1})			
	SK-I	N_6	DKN	Rev. N_6	SK-II	N_6	DKN	Rev. N_6
KNO_3	3,150	2,830	809	2,830	1,900	2,830	809	2,830
NH_4NO_3	–	–	–	–	1,650	–	–	–
$(NH_4)_2SO_4$	231.3	463	66	463	231.3	463	66	463
$NaH_2PO_4 \cdot H_2O$	–	–	312	–		–	312	–
KH_2PO_4	540	400	–	400	170	400	–	400
$MnSO_4 \cdot 4H_2O$	22.3	4.4	2.2	4.4	22.3	4.4	2.2	4.4
$ZnSO_4 \cdot 7H_2O$	1.5	1.5	2.2	1.5	8.6	1.5	2.2	1.5
H_3BO_3	6	1.6	2.9	1.6	6.2	1.6	2.9	1.6
KI	1	0.8	–	0.8	0.83	0.8	–	0.8
$CuSO_4 \cdot 5H_2O$	0.025	–	0.2	0.025	0.025	–	0.2	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	–	0.13	0.25	0.25	–	0.13	0.25
$CaCl_2 \cdot 2H_2O$	150	166	147	166	440	166	147	166
$MgSO_4 \cdot 7H_2O$	185	185	246	185	370	185	246	185
$FeSO_4 \cdot 7H_2O$	27.8	27.8	13.9	27.8	27.8	27.8	13.9	27.8
Na_2EDTA	37.3	37.3	18.7	37.3	37.3	37.3	18.7	37.3
Aspartic acid	–	–	666	–	–	–	666	–
Glutamin	–	–	731	–	–	–	731	–
Glycine	2	2	–	2	2	2	–	2
Thiamine HCl	2.5	1	10	2.5	1	1	10	1
Pyridoxine HCl	2.5	0.5	1	2.5	0.1	0.5	1	0.1
Nicotinic acid	2.5	0.5	1	2.5	0.5	0.5	1	0.5
Myo-inositol	100	–	100	100	100	–	100	100
Casein hydrolysate	500	2,000	2,000	2,000	–	2,000	2,000	2,000
2,4-D	0.5	2	0.5	0.5	–	–	–	–
NAA	2.5	–	–	2.5	1	0.1	–	1
IAA	–	–	–	–	–	–	0.2	–
BAP	–	–	–	–	1	–	0.5	1
Kinetin	0.5	–	–	0.5	0.5	0.5	–	0.5
Sucrose[1)]	40,000	60,000	30,000	50,000	30,000	30,000	30,000	50,000
Sorbitol	–	–	30,000	–	–	–	–	–
Agar	8,000	8,000	8,000	8,000	8,000	8,000	10,000	8,000

Modified contents of basal media are shown by underlines.

1) In Experiment 2, the same amount of maltose was used instead of sucrose, too.

uninucleate stage is good for callus induction (Afza et al. 2000; Kinoshita et al. 2000), panicles possessing spikelets with microspores at this stage were harvested. For cold pretreatment, panicles with flag leaf sheaths were wrapped in polythene bags and kept in the dark at 10°C (Reddy et al. 1985). Ten days later, appropriate spikelets were separated from the panicles, and sterilized with 70% ethyl alcohol and a 2% solution of NaClO after Misoo et al. (1991).

Anthers were inoculated on callus induction medium in Falcon petri dishes (3.5 cm in diameter) and cultured in the dark. Data on responding anthers and callus productivity were recorded on the 60th day. Nine androgenic calli (1–2 mm in size) were transferred to each regeneration medium in a petri dish (6 cm in diameter) and kept under light treatment (50 μmol $m^{-2}s^{-1}$, 14 h photoperiod). Then the numbers of regenerated green and albino shoots were recorded.

In this study, two experiments were carried out. The culture conditions of these experiments were as follows:

(*Experiment 1*) This experiment was conducted to examine the response of the two salt tolerant *indica* cultivars to anther culture, using N_6 (Chu et al. 1975), SK-I and SK-II (Raina and Zapata 1997), and DKN (Daigen et al. 2000) media with the modifications shown in Table 1. Two culture temperatures (Constant temperature: 25°C, 24 h; Alternating temperatures: 30°C/20°C, 14 h/10 h, respectively) were employed both at callus induction and regeneration stages. The calli produced were cultured under the respective experimental conditions for regeneration. More than 600 anthers were cultured for each treatment.

(*Experiment* 2) In this experiment, the effects of saccharides (sucrose and maltose) were studied at callus induction and regeneration stages using revised N_6 medium at alternating temperatures. The plant growth regulators and organic substances in SK media were used for revised N_6 medium together with some modifications of N_6 medium (Table 1). The calli produced in sucrose or maltose media were cultured on regeneration media containing sucrose or maltose. One thousand anthers were cultured in each treatment in this experiment.

Table 2 shows the effects of basal media and culture temperatures on callus induction and plant regeneration (Experiment 1). At the callus induction stage, Pokkali showed higher values than Nona Bokra for responding

Table 2. Effects of basal media and culture temperatures on callus induction and plant regeneration in anther culture of salt tolerant *indica* rice cultivars, Nona Bokra and Pokkali.

Cultivar	Culture temp.	CIM[1)]	No. of anthers cultured	Callus induction		PRM[4)]	No. of calli cultured	Plant regeneration		
				RA[2)]	CP[3)]			GS[5)]	AS[6)]	GSP[7)]
Nona Bokra	25 °C	SK-I	800	2.5	3.0	SK-II	24	0	0	0
		N_6	600	1.5	2.0	N_6	12	0	0	0
		DKN	600	1.0	1.5	DKN	9	0	0	0
	30/20 °C	SK-I	1600	3.0	3.9	SK-II	62	3.2	12.9	0.1
		N_6	600	2.3	3.0	N_6	18	0	0	0
		DKN	600	1.0	2.0	DKN	12	0	0	0
Pokkali	25 °C	SK-I	800	7.5	9.0	SK-II	72	5.6	8.3	0.5
		N_6	600	4.0	5.2	N_6	31	0	3.2	0
		DKN	600	1.0	3.5	DKN	21	0	0	0
	30/20 °C	SK-I	1200	12.8	24.3	SK-II	292	17.5	14.4	4.3
		N_6	600	4.8	7.2	N_6	43	0	0	0
		DKN	600	1.5	6.0	DKN	36	0	0	0

Abbreviations:
1) Callus induction media.
2) Rate of responding anther=(No. of anthers yielded calli/No. of anthers cultured)×100.
3) Callus productivity=(Total no. of calli yielded/No. of anthers cultured)×100.
4) Plant regeneration media.
5) Regeneration frequency of green shoot=(No. of green shoots/No. of calli cultured)×100.
6) Regeneration frequency of albino shoot=(No. of albino shoots/No. of calli cultured)×100.
7) Green shoot productivity=(No. of green shoots regenerated/No. of anthers cultured)×100.

anthers (RA) and callus productivity (CP). Regarding culture conditions, SK-I medium under alternating temperatures were more effective than other treatments. The highest values were obtained under these conditions both for Nona Bokra (RA=3.0; CP=3.9) and Pokkali (RA=12.8; CP=24.3). In this experiment, the lowest responses were observed in DKN medium. The interaction of genotype and medium has already been well documented (Khanna and Raina 1998; Bishoni et al. 2000). DKN medium, the new formula for anther culture of *cv*. Koshihikari (a leading cultivar in Japan), is an example of a genotype-specific culture medium (Daigen et al. 2000). The nitrogen content is reduced prominently in DKN medium (NO_3^- : 8 mM, NH_4^+: 1.75 mM) compared to other culture media (Table 1). Therefore, low nitrogen content could be one reason for poor response of salt tolerant cultivars to DKN medium. A low content of auxin in DKN callus induction medium may be another cause of low callus formation on this medium.

At the regeneration stage, green shoot regeneration was observed only in SK-II medium. At this stage, Pokkali also showed a higher response than Nona Bokra. In Pokkali, the regeneration frequency of green shoots (GS) and green shoot productivity (GSP) were 3.1 (17.5/5.6) and 8.6 (4.3/0.5) times higher under alternating temperature conditions than under constant temperature conditions. Culture temperature is an important factor in plant tissue culture. The effects of different day and night temperatures have been studied in *japonica* rice, which revealed that alternating temperatures (30°C/20°C, 12 h/12 h) remarkably enhanced anther culture efficiency (Okamoto et al. 2001). The present study also showed that alternating temperatures were effective for both salt tolerant *indica* cultivars. These observations suggest wide application of alternating temperatures in rice anther culture.

Since alternating temperatures showed remarkable effects on callus induction and plant regeneration in Experiment 1, Experiment 2 was carried out only under alternating temperature conditions. Table 3 gives the effects of saccharides in revised N_6 medium for callus induction and plant regeneration. At the callus induction stage, Pokkali showed a higher response than Nona Bokra, and maltose gave better results than sucrose. In particular, the maltose effect resulted in greater than 1.5 times higher values for CP of both cultivars, and Pokkali showed the highest CP value (42.1) among all the treatments employed in this study. At the plant regeneration stage, Pokkali exhibited high GS and GSP in every treatments. On the other hand, AS value was higher in Nona Bokra than Pokkali. In Nona Bokra, the maltose effects in callus induction media were still maintained for GS and GSP on regeneration medium with either sucrose or maltose, and the maltose effects in regeneration media were also observed for both treatments. The role of maltose in enhancing the anther culturability of cereals has been well documented (Jäne and Lörz 1995; Raina 1997). The effects of maltose in anther culture of rice have been reported also, but only at the callus induction stage (Lentini et al. 1995; Trejo-Tapia et al. 2002). In the present study, maltose clearly enhanced callus productivity in anther culture, and the highest values of GS and GSP were obtained when

Table 3. Response of salt tolerant *indica* rice cultivars, Nona Bokra and Pokkali, to revised N_6 media including sucrose and maltose at callus induction and plant regeneration stages.

Cultivar	Callus induction			Plant regeneration							
	Saccharide	RA	CP	Sucrose				Maltose			
				No. of calli	GS	AS	GSP	No. of calli	GS	AS	GSP
Nona Bokra	Sucrose	2.8	6.4	27	3.7	14.8	0.2	27	7.4	22.2	0.5
	Maltose	3.9	11.3	45	4.4	13.3	0.5	36	8.3	25.0	0.9
Pokkali	Sucrose	7.8	27.3	90	11.1	2.2	3.0	45	6.7	13.3	1.8
	Maltose	8.6	42.1	90	8.9	12.2	3.7	90	13.3	10.0	5.6

For explanation of abbreviations, see Table 2. One thousand anthers were cultured in each treatment.

maltose was used in media for both callus induction and regeneration. Maltose produce only glucose after degradation, but sucrose resolves into fructose and glucose in the medium. Fructose derived from sucrose may inhibit androgenesis as has already been reported (Last and Brettell 1990). The superiority of maltose might be associated with keeping a high proportion of swollen microspores and increasing their division rate (Xie et al. 1995). The calli produced in either sucrose or maltose medium showed maximum regenerability of green plants when transferred to a regeneration medium with maltose for Nona Bokra, while in Pokkali, the calli produced on sucrose and maltose media exhibited maximum regenerability of green plants on sucrose and maltose regeneration media, respectively. These results indicate differential requirements for saccharides at the callus induction or regeneration stages in anther culture of these varieties.

N_6 medium and SK medium, employed in this study, differ in several components such as inorganic salts, organic substances and plant growth regulators (Table 1). It was not clear which components in SK media affected callus induction and plant regeneration in these cultivars. Plant growth regulators, however, might have prominent effects because Nona Bokra and Pokkali showed high callus productivity and plant regeneration on revised N_6 medium, which included the same combination of plant growth regulators as SK-I and SK-II media. Revised N_6 medium contains the ingredients of SK medium that are lacking in N_6 medium. This formula was favorable to both salt tolerant cultivars, especially when maltose was used as carbon source. Therefore, it could be appropriate to use revised N_6 medium for all anther culture stages in these cultivars.

The profitable exploitation of somaclonal variation is of prime importance in plant breeding (Karp, 1995). Mandal et al. (1999) reported several somaclonal variations of agronomically important traits in Pokkali somaclones from mature seed-derived calli. Although anther culture is one of the most intensively investigated areas in rice tissue culture, most of the advances and achievements that have been made pertain to *japonica* rice, since anthers and pollen of *indica* cultivars show low response to *in vitro* culture. The present study is the first report to reveal that alternating temperatures and revised N_6 media including maltose could yield sufficient green shoots from anther culture to investigate the gametoclonal variation of agronomically important traits in Pokkali. It will also be possible to apply this anther culture system to the haploid method of breeding using this salt tolerant cultivar. Pokkali has the *Bph 9* gene, which shows resistance to three biotypes of BPH (Nemoto et al. 1989). Therefore, resistance against BPH could be integrated with the incorporation of salinity tolerance using the haploid method of breeding. Nona Bokra showed poor responses such as low callus formation and high albino shoot regeneration in the present study. We may need to develop other culture conditions for this cultivar to utilize it in an actual breeding program. Among the treatments employed in this study, the highest GSP values, 0.9 (Nona Bokra) and 5.6 (Pokkali), were observed under alternating temperature conditions with the combination of revised N_6 media and maltose. Even if there is an interaction between the genotype and the culture medium, the anther culture efficiency of recalcitrant *indica* rice cultivars could be improved even more by using maltose instead of sucrose and alternating temperatures instead of a constant temperature.

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