

## ANTHER CULTURE POTENTIAL OF INDICA RICE VARIETIES, *KURULU THUDA* AND BG 250

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Accepted: 7<sup>th</sup> October 2009

### ABSTRACT

The anther culture ability of two local indica rice varieties were evaluated by anther plating on N6 and SK-I callus induction media. The traditional rice variety, *Kurulu Thuda* displayed good anther culture potential with a callus induction frequency of 17.2%. Anthers most suitable for culture were those bearing bi-nucleate pollen. Anther response was relatively poor in the new improved variety, Bg 250 (1.4%). Callus induction was nearly three times higher on N6 medium than on SK-I medium in var. *Kurulu Thuda*. Shoot regeneration was achieved in var. *Kurulu Thuda* on SK-II medium, but only from callus induced on N6 medium. From a total of 115 calli transferred from N6 to regeneration medium shoot development occurred in 7 calli, thus limiting the overall anther culture efficiency. Of the calli with morphogenetic competence, 5 gave rise to albino shoots and 2 produced green shoots. After root development the green plantlets were potted in soil. Plant regeneration could not be observed from callus induced in var. Bg 250.

**Key words:** Anther culture, Callus induction, Indica rice

### INTRODUCTION

Anther culture is an innovative technique that can be utilized for the rapid development of homozygous plants. Pollen within the cultured anthers may be induced to form callus which will later regenerate haploid plants. Haploids upon chromosome doubling yield fully homozygous genotypes. The technique has been used successfully to produce homozygous breeding lines in japonica rice (Brar and Kush 2006). However, the potential for indica rice anther culture is yet to be fully exploited due to various constraints that include a recalcitrant genetic background in the indica varieties (He *et al.* 2006). Several attempts at anther culture of local indica rice germplasm have also met with limited success (Kumari *et al.* 2006; Silva and Achala 2008; Rathieka and Silva 2007).

The ability of pollen to redirect its development pathway from gametogenesis to embryogenesis depends on the stage of maturity of the pollen grains at the time of culture. For rice, the best stage has been described as uni-nucleate to early bi-nucleate stage (Jahne and Lorz 1995). Therefore, determining the development stage of pollen in the anthers is important to optimize the anther culture response of a given rice genotype (Silva 2010). Improved staining procedures have been used to identify rice pollen nuclei (Gupta and Borthakur 1987). A visual marker that shows good correlation with the pollen stage is used as a guide to identify the required stage of pollen during large scale culturing. Usually, the distance between the collar of the flag leaf and the ligule of the penultimate leaf of the tiller serves as a reliable guide to anther maturity

(Bishnoi *et al.* 2000). The objective of the experiment was to identify the anther culture potential of the rice varieties, *Kurulu Thuda* and Bg 250, facilitated by defining the appropriate stage of pollen development for successful anther culture.

### MATERIALS AND METHODS

#### Pollen staging using iron alum haematoxylin stain

The stage of pollen division was studied in anthers harvested at different maturity stages in *Kurulu Thuda* using iron-alum haematoxylin staining procedure (Gupta and Borthakur 1987). Panicle maturity was correlated with the distance between the flag leaf and the penultimate leaf collar. Panicles enclosed in leaf sheaths were harvested when this distance was 3, 4, 5, 6, 7 and 8cm. From each harvested panicle, spikelets were removed from the upper, mid, and lower portion that represented a maturity gradient of the florets. Three spikelets were sampled per region of the panicle, and from each spikelet a single anther was selected randomly for staining. The dissected out anther was dipped in 4% iron alum haematoxylin stain in a covered watch glass. The time required for differential staining of the nuclei was worked out for pollen grains of different maturity by trial and error. Accordingly, very young pollen collected from panicles when the distance between the flag leaf and the penultimate leaf auricle was 3cm were stained for up to 5min. and the duration of staining was increased up to 25, 60, 110, 130 and 140min. when this distance was 4, 5, 6, 7 and 8cm respectively.

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Stained anthers were squashed on a clean glass slide with a drop of haematoxylin stain, and observed under the light microscope for stages of pollen development.

### Anther Culture

Anthers were dissected out from panicles harvested at similar maturity stages as those used for pollen staging. The panicles enclosed in leaf sheaths were wiped with 70% alcohol, and wrapped in aluminum foil. Foil-wrapped and labeled panicles were placed in sealed plastic bags and subjected to cold pre-treatment at 10°C for 7d. Following cold-shock, panicles were surface sterilized in 20% Clorox for 20min. Anthers were dissected out in the laminar flow bench from separated spikelets. Each spikelet was snipped at the base while holding from the tip, to detach the anther lobes from the filaments. The released anthers were dropped onto agar solidified media contained in Petri dishes. Anthers were made to spread evenly on the agar surface by rotating the Petri dish during anther-plating. Anthers of the two varieties were cultured on N6 medium (Chu *et al.* 1975) while anther response of Kurulu Thuda was tested in addition on a second callus induction medium, SK-I (Raina and Zapata 1997), as a surplus of anthers were available from this variety during the culture phase. N6 and SK-I media were supplemented with 100mg L<sup>-1</sup> yeast extract and contained AgNO<sub>3</sub> (10mgL<sup>-1</sup>) as an ethylene inhibiting agent to delay anther senescence (Lentini *et al.* 1995). Two replicate plates were maintained for each representative stage of panicle maturity in each variety. A minimum of 318 and a maximum of 616 anthers were cultured per maturity stage depending on anther availability. Cultures were incubated in the dark at 25°C for callus induction.

Callus produced on anthers were allowed to develop to a size of about 3mm in diameter before transferring to regeneration medium. Shoot regeneration was tested on SKH II medium (Raina and Zapata 1997) contained in Petri dishes, with either agar or agarose as the solidifying agent. When agarose was used as the gelling agent, the initial transfer of callus was onto a hard medium containing 1% agarose for subjecting the callus to water stress. After two weeks, the stressed callus was moved on to a softer medium with 0.4% agarose and maintained until regeneration occurred. The growth regulators NAA, BAP, and kinetin were used, each at 1mgL<sup>-1</sup> in the regeneration medium. Two other growth regulator concentration regimes were also tested where either NAA or kinetin concentration was reduced to 0.5mgL<sup>-1</sup> while BAP concentration remained at 1mgL<sup>-1</sup>. Shoots that were regenerated on SKH II medium in Petri dishes were transferred onto fresh agar-solidified media in test tubes to allow shoot elongation. Shoots which produced roots were removed from the culture tube and the root

system was washed well in water. The plants with a weak root system were initially nurtured on Hoagland liquid medium and kept in the laboratory for 2 weeks, before moving into pots with soil and placed in the glass house.

## RESULTS

### Pollen staging

Anthers of young panicles of *Kurulu Thuda*, harvested when the distance between the flag leaf and penultimate leaf auricle was 3 and 4cm, contained only uni-nucleate pollen irrespective of the spikelet position within the panicle from which the anthers were obtained. When the above distance was 5 and 6cm, anthers within the panicles had both uni- and bi-nucleate pollen with a higher percentage of bi-nucleate cells in anthers of florets from the upper part of the panicle. Only bi-nucleate pollen was observed in anthers from any part of the panicle when harvested from boots that correlated to 7 and 8cm of the morphological marker distance.

### Callus induction from cultured anthers

Callus development was observed 4 and 8 weeks after anther plating in *Kurulu Thuda* and Bg 250, respectively. In *Kurulu Thuda*, mainly anthers from panicles corresponding to the marker distance of 7 and 8cm produced callus (Fig. 1a). In Bg250 callus induction was observed only from anthers collected from panicles when this distance was 6 and 7cm (Fig. 1b). This indicated that the best responsive pollen for *Kurulu Thuda* were those in the bi-nucleate stage. If the same correlation held for Bg250 then the most appropriate pollen would be of the late uni-nucleate or early bi-nucleate stages, but this has not been verified in this study. In *Kurulu Thuda*, frequency of callus induction was higher in anthers harvested from panicles at the 8cm distance (17.2%) compared to a much lower anther response (3.6%) relating to 7cm distance. In Bg250, callus induction frequency was only 1.4% in anthers from panicles of 6cm distance and even less (0.7%) for those from 7cm range (Table 1). Callus induction was better on N6 medium (3.6–17.2%) than on SK-I medium (0.6–3.3%) in *Kurulu Thuda* (Table 1). Callus produced in both varieties appeared creamy white in colour and friable in texture.

### Plant regeneration from induced callus

Further proliferation of callus occurred on the regeneration medium (Fig. 1c). Shoot regeneration was indicated by the appearance of yellow-green centers in the callus. Regeneration occurred in callus of *Kurulu Thuda* only, and this too from callus induced on N6 medium. A total of 115 calli were transferred from N6 callus induction medium to

**Table 1: Anthers producing callus from panicles harvested at different stages of maturity on N6 and SK-I media, and the number of regenerated green plants**

Rice variety	Callus induction medium	*Panicle maturity (cm)	No. of anthers cultured	No. of Callusing anthers	Frequency of callus induction	Number of green plants regenerated	
<i>Kurulu Thuda</i>	N6	6.0	365	00	0.0	12.0	
		7.0	616	22	3.6		
		8.0	542	93	17.2		
Bg 250	SK-I	6.0	322	02	0.6	-	
		7.0	546	18	3.3		
		8.0	379	09	2.4		
		N6	6.0	430	06		1.4
			7.0	594	04		0.7
		8.0	318	00	0.0		

\*Panicle maturity estimated by the distance between the auricles of flag leaf and penultimate leaf at panicle harvest

SKH II regeneration medium. However, the overall anther culture efficiency was poor and only 2 calli produced green shoots (Fig. 1d), while albino shoots were regenerated from 5 calli (Fig. 1e). It was not possible to assess the effect of growth regulator concentrations and media solidifying agent on regeneration potential due to the small number of calli showing morphogenetic competence. Clumps of green shoots developed roots when moved onto media in test tubes (Fig. 1f). Twelve fully regenerated green plants of *Kurulu Thuda* were transferred to pots in the glass house.

## DISCUSSION

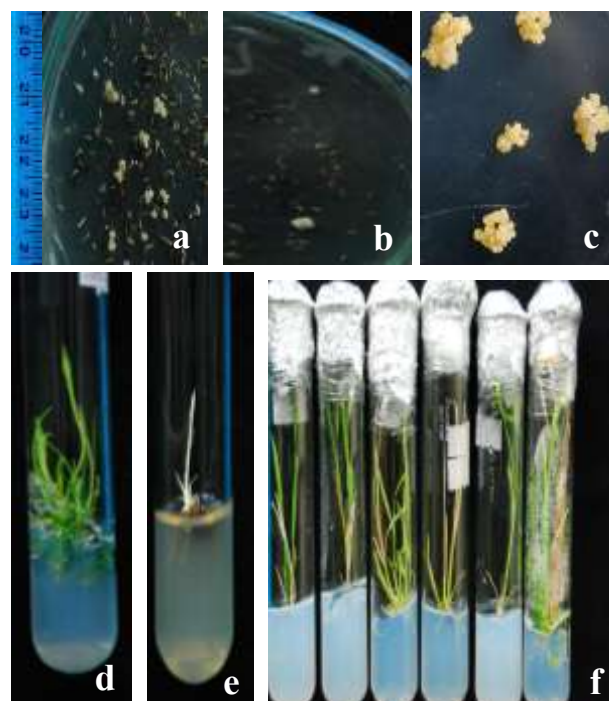
The distance from flag leaf to penultimate leaf auricle, a convenient morphological marker that has been previously used for estimating the maturity stage of pollen in different rice varieties (Bishnoi *et al.* 2000) could also be used as a visual guide in the indica variety, *Kurulu Thuda*.

A clear difference in the anther culture response was observed in the two varieties of rice. The highest anther response (17.2%) in *Kurulu Thuda* was produced from the most mature anthers examined (from panicles that correlated with the 8cm distance). This was over 12 times better than the best response (1.4%) observed for Bg250. Similar genotype specificity of anther response within the indica subspecies has been observed to be common (Lentini *et al.* 1995; He *et al.* 2006; Shahnewaz *et al.* 2003; Talebi *et al.* 2007).

N6 proved to be the better medium for callus induction in *Kurulu Thuda*. However, N6 medium which contains inorganic nitrogen in the form of nitrate and ammonium ions is generally preferred for japonica rice anther culture. Media in which nitrogen in the ammonium form is considerably low or absent, such as the SK-I, are considered better for indica varieties (Raina and Zapata 1997). However, in this experiment the callus induction frequency of indica variety *Kurulu Thuda* was distinctly better on N6 than on SK-I medium. This

suggests that while some generalization on media requirements for the two ecotypes is possible, requirements of a particular variety within a subspecies may be specific for that genotype. Callus induction frequency observed for *Kurulu Thuda* can be considered reasonably high by indica rice standards. However, manipulation of media components could yield further improvements.

Callus induction and plant regeneration are considered as two distinct phases in the anther culture process of rice. Also it has been reported that the rice cultivars that display high callusing ability show the best regeneration frequencies (Javed *et al.* 2007; Shahnewaz *et al.* 2003; Yan *et al.* 1996). The results obtained were consistent with this trend; callus induction was more successful in *Kurulu Thuda* and shoot regeneration also occurred from the same variety only. However, there are occasions in which genotypes that show high callus induction have displayed poor regeneration ability and vice versa (He *et al.* 1998; Talebi *et al.* 2007). Shoot regeneration in *Kurulu Thuda* occurred from callus that was induced on N6 medium after transfer to SK-II medium, but no regeneration occurred from callus transferred from SK-I induction medium. This indicates that the callus induction medium has an influence on the morphogenetic competence of the induced callus, determining its regeneration capability. Therefore, media manipulations should



**Figure 1: Callus induction and plant regeneration from cultured anthers of indica rice varieties.** a-anther callus of *Kurulu Thuda*; b-anther callus of Bg 250; c-proliferating callus of *Kurulu Thuda*; d-regenerated green shoots; e-regenerated albino shoot; f-rooted plantlets

target the production of embryogenic callus with good regeneration ability rather than simply inducing prolific callusing, from which regeneration would not be possible (Silva 2010).

Of the seven sub-cultured calli from which plant regeneration occurred, five gave rise to albino plants and only two produced green shoots. Albino plant regeneration is a problem that is commonly encountered in rice anther culture that diminishes the utility of the technique as a supplementary breeding tool for rice.

## CONCLUSIONS

Genotypic differences clearly influence the anther culture ability of rice, and local rice variety *Kurulu Thuda* has good anther culture potential. Pollen staging could contribute to the enhancement of anther culture efficiency by correctly identifying the maturity stage of anthers in each variety prior to culture. The N6 medium was found to be adequate for callus induction in *Kurulu Thuda*. Further improvements may be possible by manipulation of media components. However, anther culture efficiency measured in terms of the green plant regeneration ability was poor. Regeneration has to be addressed with detailed attention to media and culture conditions that would improve green plant production and lessen albino plant regeneration.

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