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Callus Induction and Regeneration from *In Vitro* anther Culture of Rice (*Oryza sativa* L.)

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Abstract

The standardization of anther culture media for callus induction, and regeneration from anther derived callus of Azucena and Budda rice varieties. Anthers from panicles in which the distance between flag leaf and subtending leaf was 11cm in Azucena and 8 cm in Budda were used for anther culture.At this stage of development, anthers contained uni-nucleate pollen grains. Panicles were subjected to cold pretreatment of 5°C for 8 days. Callus induction frequency in different media combination ranged from 0.66% to 6.66% was observed in N6 medium supplemented with 1.0 mg^{-l},2, 4-Dichlorophenoxy acetic acid, 2.0 mg^{-l} α-Naphthalene acetic acid and 0.5 mg^{-l} Kinetin. In Azucena variety, highest callus induction (6.66%) while no callusing was found in Budda variety. Highest shoot regeneration (0.33%) from callus was observed in MS medium supplemented with 0.5 mg^{-l} 6- Benzyl amino purine, 0.5 mg^{-l} Kinetin and 80 mg^{-l}adenine sulphate.

Highlights

- Azucena and Budda rice varieties were used for anther culture.
- Azucena was showing response for anther culture but no response in Budda.
- All Regenerated plantlets were albino

Keywords: Anther culture, Panicle**,** Callus, Regeneration

Rice (*Oryza sativa* L., 2n=24), a member of the family Poaceae is one of the most important cereal food crops of the world and feeds over half of the global population(Sasaki, 2005).In direct proportion to the predicted rise in the world's human population, rice consumption and demand will increase over the next several decades(Kathuria *et al.,* 2007).

Haploid plants have the genotypic number of chromosomes that is a single set of chromosomes in sporophyte. Haploidy may be induced by different techniques, the most promising and successful one being microspore androgenesis.

Many new rice cultivars have been developed through biotechnological techniques like anther culture, embryo rescue and somaclonal variation(Brown and Thorpe, 1995; Zapata *et al*., 2004).Anther culture is a tissue culture technique which can be applied in plant breeding to accelerate the process of obtaining pure lines with numerous advantages: shortening breeding cycle by immediate fixation of homozygosity in one generation, increased selection efficiency and allowing early expression of recessive genes.

In addition, the screening of haploid cells against cold tolerance, salinity, pathotoxins and other biotic and abiotic factors before plant regeneration also becomes possible. Haploids are also valuable to detect and fix desirable recessive traits introduced through mutation(Chen *et al*., 2001) or hybridization (He *et al*., 2006).

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 (He *et al.,* 2006) and it is difficult to obtain a large pollen plantlet population in the indica varieties (Manonmani and Khan, 2004).

The production of haploid rice plants by anther culture was first reported by Niizeki and Oono (1968). Subsequent studies have shown that the development of microspores into fertile plants depends on many factors, any of which can be limiting. These include plant genotype (Shen *et al.*,1982), the developmental stage of the microspore (Chen 1976), cold pretreatment of the anthers (Chaleff and Stolarz 1981; Chen *et al.*,1982; Zapata *et al.,* 1982), growth conditions of the donor plants e.g. photoperiod and light intensity (Lee *et al.*,1988), the orientation of the plated anthers (Mercy and Zapata, 1987), and the nitrogen source of the callus-induction medium (Chen *et al.,* 1982; Tsay *et al.,* 1982).

The objective of this research was to standardize culture media for callus induction and regeneration from callus.

Materials and Methods

The present experiment was conducted in 2011-12 at the Plant Tissue Culture Laboratory, Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India.

Plant material

Rice varieties Azucena (Aromatic with high Fe and Zn content) and Budda (Drought tolerance) were used as the source of explants. These varieties were grown in the field till the time of flowering. Recommended fertilizers and plant protection measures were adopted to raise healthy plants.

Stage of panicle harvest

Panicles were harvested at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Panicles were collected between 8.00 and 9.00 am and washed with water and sprayed with 70 % (v/v) ethanol. Panicles with a maximum distance between the subtending leaf and the flag leaf, of 11cm for Azucena and 8 cm for Budda were selected (Fig 1a).These panicles were kept in refrigerator for cold pretreatment at 5°C for 8 days. In order to identify the stage of pollen development, anthers of Azucena and Budda were stained with 2% (v/v) acetocarmine and observed under light microscope (Fig 1b). The panicles with uni-nucleate, pollen were used for anther culture.

Sterilization, preparation of explants for callus induction

The panicles were surface sterilized by immersion in 70 % (v/v) ethanol for 20 seconds followed by 0.2% $HgCl_2$ for 10 minutes. The treated panicles were washed 3-4 times with sterile distilled water.Later the anthers were isolated from spikelet avoiding any mechanical damage, followed by inoculation in 60 mm x 15 mm petri dishes(Fig 1c), each containing 10 ml of solidified induction solid N6medium containing 5% sucrose and 0.8% agar callus induction.The medium was supplemented with different concentrations of phytohormones (2,4-D at 0.5,1.0, 1.5 and 2.0 mg^{-1}; NAA at 1.0 and 2.0 mg^{-1} and Kinetin at 0.5 and 1.0 mg^{-1}). The cultures were sealed with parafilm and kept in dark at 23±2°C. The treatments were replicated thrice. Observation on frequency of callus induction was recorded 7-8 weeks after inoculation.

Regeneration

The anther derived calli were transferred to 25 mm x150 mm culture tubes (Borosil) containing 15 ml of solidified regeneration Murashige and Skoog medium consisting with 3% sucrose and 0.8% agar (Fig 1f). The growth regulators BAP and Kinetin, each at 0.5, 1.0, 2.0 mg^{-1} in combination with NAA at 0.0 and 0.5 mg^{-1} was added to the media. The treatments were replicated thrice. The pH of the both media for callus induction and regeneration was adjusted to 5.8 with 1 N HCl or 1 N NaOH before adding agar and autoclaving.The plated calli were incubated in culture room at 23±2°C with 16-h of light, at light intensity of about 3000 Lux. Observations on response of callus were recorded after 15 days of culture.

Results and Discussion

Callus induction

Cultured anthers turned black or brown in both varieties after two weeks. First indication of callusing was swelling

of the anther wall. Later callus appeared from the cut ends after eight weeks (Fig 1d).This observation is in accordance with the report of Gupta and Borthakar (1987).

The callusing ability of anthers were influenced by the growth regulator treatment.Percent callus induction due to growth regulators ranged from 0.66%to 6.66% in Azucena. The treatments failed to induce callusing in Budda.

Callus induction frequencies were variable and ranged from 0.66% to 6.66% (Table 1). Highest frequency of callus induction(6.66%) was obtained on N6 media with 1.0 mg^{-l} 2,4-D, 2.0 mg^{-l} NAA, and 0.5 mg^{-l} Kinetin in Azucena variety (Fig 1e). These results are consistent with the findings of Gueye and Ndoye (2010) who observed 4.24% callus induction in IKP (*Japonica*) variety on N6 medium supplemented with 3 mg^{-l} 2, 4-D and 1 mg^{-l} NAA and 1 mg^{-l} Kinetin.

Anthers of Budda (*Indica*) variety did not produce callus. Similar results were found by Ramkrishnan *et al.,* (2005) in ADS16 (*Indica*); Silva and Ratnayake (2009) in KuruluThuda (*Indica*) and Shahnewaz and Bari (2004) in BRRI Dhan 29 (*Indica*). Similar results were reported on N6 medium supplemented with 2, 4-D, NAA and Kinetin. Miah *et al*., (1985) reported that anther culture response varied from 41 % for a *japonica* cultivar to 0 % for an *indica* cultivar.

Genotypic variation in androgenic response as observed in the present study in *japonica* rice and *indica* rice has been demonstrated earlier by many authors (Mandal and Bonyopadhyay, 1997; Raina and Zapata, 1997). In rice, it has been demonstrated that *japonica* genotypes produce more androgenic callus and plants than *indica* genotypes (Zapata *et al*., 1990; Yamagishi *et al*., 1998).

Regeneration

Many factors such as culture medium, growth regulators, culture environment, explant and genotype of donor are known to influence regeneration of plants. In regeneration medium, most of the calli did not respond (Table 2). After 15 days of culture, the callus turned green in colour (Fig. 1g) in media containing 0.5 mg^{-l} BAP, 0.5 mg^{-l} Kinetin and 80 mg^{-l} adenine sulphate and after 2 months of culture it

Table1: Callus induction from Azucena rice anthers culture on N6 medium with different concentration of growth regulators

Treatment No.	2,4-D (mg^{-l})	NAA (mg^{-l})	Kinetin (mg^{-l})	Mean no. of anthers inoculated	Mean no. of anthers response	% Callus* induction
T_0	0.0	0.0	0.0	50	-	0.0
T_1	0.5	0.0	0.0	50	-	0.0
T_2	0.5	1.0	0.5	50	-	0.0
T_3	0.5	1.0	1.0	33.3	2	4.0
T_4	0.5	2.0	0.5	50	0.33	0.66
T_5	0.5	2.0	1.0	33.3	0.33	0.66
T_6	1.0	0.0	0.0	33.3	-	0.0
T_7	1.0	1.0	0.5	33.3	-	0.0
T_8	1.0	1.0	1.0	33.3	-	0.0
T_9	1.0	2.0	0.5	50	3.33	6.66
T_{10}	1.0	2.0	1.0	50	0.33	0.66
T_{11}	1.5	0.0	0.0	16.6	-	0.0
T_{12}	1.5	1.0	0.5	50	1.33	2.66
T_{13}	1.5	1.0	1.0	50	-	0.0
T_{14}	1.5	2.0	0.5	50	-	0.0
T_{15}	1.5	2.0	1.0	50	-	0.0
T_{16}	2.0	0.0	0.0	33.3	-	0.0
T_{17}	2.0	1.0	0.5	33.3	0.33	0.66
T_{18}	2.0	1.0	1.0	33.3	-	0.0
T_{19}	2.0	2.0	0.5	16.6	0.66	0.66
T_{20}	2.0	2.0	1.0	33.3	-	0.0

Total number of anthers = 50 in each treatment per replication
*= number of anthers produced callus divided by number of anthers cultured
Significant at P = 0.05

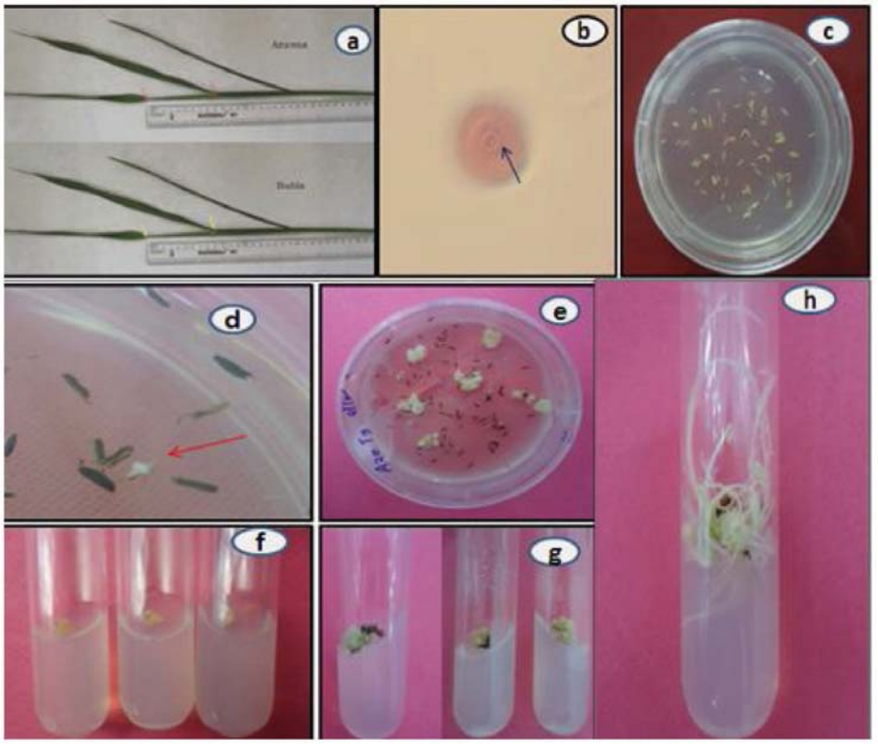

Fig. 1: Anther culture in rice variety Azucena. (a) panicle harvest stage(b) pollen development stage observed under light microscope at 400x (mid uninucleate) (c) anthers inoculated on N6 medium (d) callus appeared from cut end of swelled anther wall (8 weeks after culture) (e) highest frequency of callus induction after 10 weeks of culture (f) callus transferred to regeneration medium (g) callus turned in green colour after 15 days of culture (h) shoot and root formation after 2 months of culture.

showed shoot and root formation (Fig. 1h). Highest regeneration frequency of (0.33%) was observed in regeneration media containing 0.5 mg^{-l} BAP, 0.5 mg^{-l} Kinetin and 80 mg^{-l} adenine sulphate in Azucena variety. These results are consistent with the findings of Gueye and Ndoye (2010) who observed 5.75% regeneration frequency from callus in IKP (*japonica*) variety on MS medium supplemented with 0.5 mg^{-l} NAA and 1.0 mg^{-l} BAP. The mean frequency of shoot regeneration was 1.84%. All the shoots induced from anther derived calli were albinos in Azucena. Statistical analysis,randomized completely designwas done, but all the treatments were not significant, because most of the treatments were not responding for callus induction and regeneration. The anther culture is influence by many factors with modification of these we can increase the dedifferentiation and differentiation of the tissue.

Table 2: Regeneration from anther derived callus in rice variety Azucena

Treatment No.	NAA (mg^{-l})	BAP (mg^{-l})	Kinetin (mg^{-l})	Response
T_0	0.0	0.0	0.0	Root
T_1	0.0	0.0	0.5	-
T_2	0.0	0.0	1.0	-
T_3	0.0	0.0	2.0	Shoot
T_4	0.0	0.5	0.0	-
T_5	0.0	1.0	0.0	Shoot
T_6	0.0	2.0	0.0	Shoot
T_7	0.0	0.5	0.5	Shoot and Root
T_8	0.0	0.5	1.0	-
T_9	0.0	0.5	2.0	-
T_{10}	0.0	1.0	0.5	-
T_{11}	0.0	1.0	1.0	-
T_{12}	0.0	1.0	2.0	-
T_{13}	0.0	2.0	0.5	-

Contd.

TreatmentNo.	NAA (mg^{-l})	BAP (mg^{-1})	Kinetin ($mg^{-1)}$	Response
T_{14}	0.0	2.0	1.0	-
T_{15}	0.0	2.0	2.0	-
T_{16}	0.5	0.5	0.5	-
T_{17}	0.5	0.5	1.0	-
T_{18}	0.5	0.5	2.0	-
T_{19}	0.5	1.0	0.5	-
T_{20}	0.5	1.0	1.0	-
T_{21}	0.5	1.0	2.0	-
T_{22}	0.5	2.0	0.5	-
T_{23}	0.5	2.0	1.0	-
T_{24}	0.5	2.0	2.0	-

Legend: T = Treatment, - = No response

Albinos

Albino plant production is one of the main problems in rice anther culture(Bishnoi *et al*., 2000).In the present study all the shoots regenerated from callus were albinos. Many factors have been found to affect the degree of albinism, such as the genotype and physiological state of the donor plants(Knudsen *et al*., 1989). Sun *et al.*(1979) reported that the basic cause of albinism in rice is impairment of DNA either in nuclear genome or cytoplasmic genome. This might be due to presence of chemicals such as high level of auxin and cytokinin added to the medium.The Azucena variety will be used in future for anther culture studies.

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References

Bishnoi, U., Jain R., Rohilla, J., Chowdhury, V., Gupta, K., and Chowdhury, J. 2000.Anther culture of recalcitrant indica x Basmati rice hybrids.*Euphytica* **114:** 93–101.

Brar, D. and Kush, G. 2006. Cytogenetic manipulation and germplasm enhancement of rice (*Oryza sativa*L). In: Genetic Resources, Chromosome Engineering and Crop Improvement Cereals **2:**115-158.

Brown, D.C.W. and Thorpe, T.A.1995.Crop improvement through tissue culture.*World Journal of Microbiology and Biotechnology* **11:** 409-415.

Chaleff, R. and Stolarz, A.1981.Factors influencing the frequency of callus formation among cultured rice (*Oryza sativa*) anthers.*PhysiologiaPlantarum* **51:** 201-206.

Chen, C. C.1976. Studies on the anther culture of rice: pollen stage and low-temperature treatment. *National Science Council Mon Taipei* **4:** 2187-2190.

Chen, Q.F., Wang, C.L., Lu, Y.M., Shen, M., Afza, A., Duren, M.V., Brunner, H. 2001.Anther culture in connection with induced mutations for rice improvement. *Euphytica* **120:**401–408.

Chen, C., Lai, P., Liao, C. H. andTsay, H. S.1982. Medium evaluation for rice anther culture. Plant tissue culture(ed., Fujiwara Maruzen,) Tokyo pp 551-552.

Gueye, T. andNdoyr, K. 2010.In vitro production of doubled haploid lants from two rice species (*Oryza sativa* L. and *Oryza glaberrima*Steudt.) for the haploid development of new breeding material.*ScientificResearchand Essays* **5**(7): 709-713.

Gupta, H.S. and Borthakar, D.N. 1987.Improved rate of callus induction from rice anther culture following microscopic staging of microspores in iron-alum-haeraatoxylin.*Theoretical Applied Genetic*s **74:** 95-99.

He, T., Yang, Y., Tu, S.B., Yu, M.Q. andLi, X.F. 2006.Selection of interspecific hybrids for anther culture of indica rice.*Plant Cell Tissue Organ Culture* **13:** 484-489.

Kathuria, H., Giri, J., Tyagi, H., Tyagi, A. 2007.Advances in transgenic rice biotechnology.*Critical* Reviews in *Plant Sciences* **26:** 65-103.

Knudsen, S.I., Due, K. and Andersen S. B. 1989.Components of response in barley anther culture.*Plant Breeding* **103:** 241-246.

Lee, S. Y., Lee, Y. T. and Lee, M. S. 1988. Studies on the anther culture of *Oryza sativa*L..Growing environment of donor plant in anther culture, effects of photoperiod and light intensity.*Research Report to Rural Development Administration.* Suwon, Korea **30:** 7-12.

Mandal, A. B. and Bonyopadhyay, A.K. 1997.In vitro anther culture response in indica rice hybrids. *Cereal Research Communications* **25:** 891-896.

Manonmani, M.S. and Khan, F.A.K. 2004. Effect of growth regulation on androgenesis and regeneration in rice (*Orize sativa*). *Indian Journal of Genetics* **64**(1):17-20.

Mercy, S. T., Zapata, F. J. 1987.Position of anthers at plating and its influence on anther callusing in rice.*Plant Cell Reports* **6:**318-319.

Miah, M.A.A., Earle, E.D. and Khush, G.S. 1985. Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa*L. *Theoretical and Applied Genetics* **70:** 113-116.

Niizeki, H. and Oono, K.1968. Induction of haploid rice plant from anther culture. *Proceedings of the Japan Academy* **44:** 554-557.

Raina, S. K. and Zapata, F. J. 1997.Enhanced anther culture efficiency of indica rice (*Oryza sativa*L.) through modification of the culture media. *Plant Breeding* **116:** 305-315.

Ramkrishnan, S. H., Saravanan, S., Kumar, A. C.R. andKannanbapu, J. R. 2005.In vitro Androgenesis in Rice (*Oryza sativa*).*Asian Journal of Plant Science* **4:** 600-602.

Sasaki, T. 2005. The maped base sequence of the rice genome. *Nature*, **436:**793-800.

Shahnewaz, S. and Bari, M.A. 2004.Effect of concentration of sucrose on the frequency of callus induction and plant regeneration in anther culture of rice (*Oryza sativa* L.).*Plant Tissue Culture* **14(1):** 37–43.

Shen, J. H., Li, M. F., Chen, Y. Q. and Zhang, Z. H.1982.Breeding by anther culture in rice varieties improvement.*Scientia Agricola* **2:**15-19.

Silva, T.D. andRatnayake, W.J.2009.Anther Culture Potential of Indica Rice Varieties, KuruluThudaAndBg 250. *Tropical Agricultural Research & Extension* **12**(2).

Sun, C. S., Wu, S. C., Wang, C. C. and Chu, C. C. 1979.The deficiency of soluble proteins and plastid robosomal RNA in the albino pollen plantlets of rice.*Theoretical.Applied Genetics* **55**(5): 193-197.

Tsay, H.S., Chen, L.J., Tseng, T.H. and Lai, P.C. 1982. The culture of rice anthers of japonica X indica crosses. Plant tissue culture. (ed., Fujiwaraand Maruzen) Tokyo, 561-562.

Yamagishi, M., Otani, M., Higashi, M., Fukuta, Y., Fukui, K., Yano, M. and Shimada, T. 1998. Rice chromosomal region controlling anther culturability (*Oryza sativa*). *Euphytica* **103:** 227-234.

Zapata, A. F.J., Torrizo, L.B. and Ando, A. 2004.Current developments in plant biotechnology for genetic improvement: the case of rice (*Oryzasativa*L.). *World Journal of Microbiology and Biotechnology*, **11:** 393-399.

Zapata, F.J., Torrim, L.B., Ramero, R.O. and Alejar, M.S. 1982.Androgenesis in Oryza sativa.. In 'Plant tissue culture' (ed., Fujiwaraand Maruzen) Tokyo.

Zapata, F.J., Aldemita, R.R., Ella, E.S. and Cho, M.S. 1990. Isolated microspore culture of rice at the International Rice research Institute. In: Rice genetics II Proc. Second Intl. Rice Res. Genet. Symp., IRRI, Manila.

