*Indian J. Genet.*, 68(1): 77-80 (2008)

*Short Communication*

# Establishment of an *in vitro* haploid production system for aromatic rice cultivars of Assam

**T. V. Murali and Akshay Talukdar[1]**

Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat 785 013

(Received: December 2007; Revised: January 2008; Accepted: February 2008)



Production of haploid plants from immature pollen grains offers a good technique for rapid development of homozygous lines and early release of new crop varieties. For induction of haploids, the most promising and successful system is microspore androgenesis (culture) within or in isolation from the anther. For successful haploid production, it requires establishment of an efficient and reproducible regeneration system which is lacking for the local cultivars of Assam. In the present investigation, we could optimize the *in vitro* haploid production system technique suitable for aromatic rice cultivars of Assam.

Two popular aromatic rice cultivars *viz*., Kolajoha and Joha, and one non-aromatic cultivars of Assam-Ranjit were taken as plant materials. Boots from the tillers were collected between 7.00-8.00 a.m. on sunny days from healthy plants prior to emergence from the flag leaf sheath when the distance of the flag leaf auricle to that of the next lower leaf was 5-8 cm. Panicles were wrapped in moistened polyethylene bags and kept in the dark in an incubator for 7 days at 8-10°C for providing cold pretreatment. Approximately 100 anthers were inoculated in each culture petriplates containing 25 ml of the media.

Initially, three basal media *viz.* $N_6$ [1], modified $N_6$ [2] and $B_5$ media [3] each supplemented with 1 $mgl^{-1}$ 2,4-D, 1 $mgl^{-1}$ Kinetin, 3 $mgl^{-1}$ NAA and 5% sucrose were tested for callus induction efficiency. Based on callus induction performance, $B_5$ supplemented with different concentrations of 2, 4-D (*viz*., 0.0, 1.0, 2.0, 3.0 and 4.0 $mgl^{-1}$) along with fixed concentration of Kinetin (1 $mgl^{-1}$) and NAA (3 $mgl^{-1}$) was used. For regeneration purpose, different concentrations of Kinetin (*viz*., 0.5, 1.0, 1.5 and 2.0 $mgl^{-1}$) and fixed concentration of NAA (1 $mgl^{-1}$) were used. After 10 days of initiation, the anther-derived primary calli were sub-cultured on $B_5$ + 1.00 $mgl^{-1}$ 2,4-D + 1.00 $mgl^{-1}$ Kinetin + 3.00 $mgl^{-1}$ NAA + 5% sucrose medium for production and proliferation of sufficient callus. The embryogenic calli after 10 days in sub-culturing medium were transferred to modified MS (MS inorganic + $B_5$ organic salts) regeneration medium. Multiplication of plantlets were obtained in MS medium supplemented with 0.5 $mgl^{-1}$ 2, 4-D whereas for rooting MS + 2% sucrose was used. All the cultures were incubated in dark at 25 ± 1°C for 4 to 5 weeks. After induction, calli were transferred to sub-culture media and kept under light (700 lux) until transfer to regeneration medium. For regeneration, the cultures were kept at 25 ± 1°C temperature under light intensity of 4000-5000 lux for 16 hrs a day. For ploidy level testing, propiono orcein squash technique [4] was used.

In this study, anthers with the pollen which were at the uninucleate or early binucleate stage (Fig. 1) were used. Cultivars with high percentage of viable uninucleate pollen (51.51% and 46.27% for Kolajoha and Joha, respectively) responded more favorably to callus induction (Fig. 2).

Callus induction of all the 3 cultivars occured in $B_5$ media with fairly good frequencies (Table 1). Success of the $B_5$ might lies on its lower N content. A low N concentration is beneficial for callus induction while high concentration is inhibitory [1, 5].

Genotypic variation to callus induction was

[1]Present address: Division of Genetics, Indian Agricultural Research Institute, New Delhi 110 012

**Table1**. Effect of different media on callus induction of rice cultivars

| Cultivars | Anthers cultured (No.) | | | Callus induction (%) | | |
|---|---|---|---|---|---|---|
| | $B_5$ | $N_6$ | Modified $N_6$ | $B_5$ | $N_6$ | Modified $N_6$ |
| Kolajoha | 300 | 300 | 300 | 8.66 | 2.30 | 1.33 |
| Joha | 300 | 300 | 300 | 10.00 | 1.33 | 3.33 |
| Ranjit | 300 | 300 | 300 | 1.67 | 0.00 | 0.00 |

prominently observed in all the three media tested. Non-aromatic *indica* cultivar Ranjit showed callus induction only in one media i.e. $B_5$ while the two aromatic cultivars Kolajoha and Joha showed callus induction in all the 3 media.

The ratio of auxin: cytokinin in the *in vitro* culture medium is most critical for obtaining good callusing and regeneration response. Lower doses of 2, 4-D ($1mgl^{-1}$ for Kolajoha and $2mgl^{-1}$ for Joha and Ranjit) with $1mgl^{-1}$ Kinetin and $3mgl^{-1}$ NAA gave better callusing than its higher doses (Table 2). Also, NAA ($3mgl^{-1}$) in combination with 2, 4-D showed better callusing responses than NAA alone. It thus indicated that at lower concentration, auxin might have some synergistic effect favoring better induction of callus. The improved efficiency of 2,4-D was reported to be due to the conversion of 2,4-D to relatively inactive glucosyl esters or active amino acid conjugates which might have helped in DNA synthesis or mitosis [6,7].

Kinetin concentration influences regeneration of plantlets from callus. We transferred the calli to the MS media supplemented with different concentrations of Kinetin (*viz*., 0.5, 1.0, 1.5 and 2.0 $mgl^{-1}$) with fixed concentration of NAA ($1mgl^{-1}$). Of the 3 cultivars, Ranjit did not regenerate in any of the treatments (Table 3). In case of Kolajoha, highest regeneration (55%) was obtained from the media containing $1mgl^{-1}$ Kinetin (Fig. 3). However, frequencies of albino plant regeneration were also high (Table 4). On the other hand, when the Kinetin concentration was 0.5 $mgl^{-1}$, maximum green plant regeneration (27.5%) took place. Similarly, Kinetin at a concentration of $1.5mgl^{-1}$ gave 92.5% regeneration in Joha, but all the regenerated plants were albino. It thus indicated that excess concentration of Kinetin is not beneficial.

Occurrence of albino plant is a nuisance in anther or pollen culture of rice [8]. Here, appearance of green plants along with albino plants was observed in Kolajoha while in Joha only albino plants (100%) were regenerated. Occurrence of such albino plants may primarily be due to pDNA deletions in the pollen grains during development or during culture. Kawata *et al*. [9] reported a positive correlation between the stage of

**Table 2**. Callusing response of different cultivars on $B_5$ medium with different concentrations of growth regulators

| Cultivars | Treatments* | | | | |
|---|---|---|---|---|---|
| | $CI_1$ | $CI_2$ | $CI_3$ | $CI_4$ | $CI_5$ |
| Kolajoha | 3.67 | 10.33 | 7.67 | 6.00 | 3.33 |
| Joha | 3.33 | 11.00 | 14.67 | 7.67 | 5.00 |
| Ranjit | 1.67 | 3.00 | 7.33 | 3.67 | 1.67 |

*Concentration of 2, 4-D in treatments $CI_1$, $CI_2$, $CI_3$, $CI_4$ and $CI_5$ is 0.0, 1.0, 2.0, 3.0 and $4.0mgl^{-1}$, respectively. All the treatments also contained 5% sucrose, $1mgl^{-1}$ Kinetin and $3mgl^{-1}$ NAA.

**Table 3**. Callus induction and plant regeneration potential of the cultivars

| Cultivars | Anthers inoculated (no.) | Calli obtained (no.) | Green plants $100^{-1}$ calli | Green plants $100^{-1}$ anthers | Albino plants $100^{-1}$ calli | Albino plants $100^{-1}$ anthers |
|---|---|---|---|---|---|---|
| Kolajoha | 1800 | 91 | 18.68 | 0.94 | 57.14 | 2.89 |
| Joha | 1800 | 125 | 0.00 | 0.00 | 90.40 | 6.28 |
| Ranjit | 1800 | 52 | 0.00 | 0.00 | 0.00 | 0.00 |

**Table 4**. Effect of different concentrations of NAA and Kinetin on plant regeneration in *Kolajoha*

| Treatments* | Green plants(%) | Albino plants(%) | Total Regeneration(%) |
|---|---|---|---|
| $R_1$ | 27.50 | 20.00 | 47.50 |
| $R_2$ | 12.50 | 42.50 | 55.00 |
| $R_3$ | 0.00 | 30.00 | 30.00 |
| $R_4$ | 5.00 | 40.00 | 45.00 |

*Concentration of Kinetin in the treatments $R_1$, $R_2$, $R_3$, and $R_4$ is 0.5, 1.0, 1.5 and 2.0 $mgl^{-1}$, respectively. Also, each treatment contained 3% sucrose and $1mgl^{-1}$ NAA.

**Fig. 1. Uninucleate pollen used for culture**



**Fig. 2. Pollen at different stages of callusing**



**Fig. 3. Embryonic callus in regeneration media**




**Fig. 4. Regeneration of green and albino shoots from single callus**



**Fig. 5. Putative haploid plant in shooting media**



**Fig. 6. Putative haploid plants with well developed roots and shoots**



**Fig. 7. Haploid plant established in pot**

pollen grains and pDNA deletions in rice. Harda *et al*. [10, 11] reported that albino rice plants derived from microspores usually contains plastid genome that had suffered large-scale deletions. It was observed, in a few cases, that both albino and green plants regenerated from single callus in Kolajoha. It might be that the callus was chimeric and differentiation of green and albino plants took place from different sectors (Fig. 4). Sinha [12] also reported about differentiation of green and albino plants from different chimeric sectors of callus. The green plantlets were finally separated from the regeneration media and sub-cultured for shoot growth and rooting (Figs. 5 and 6). Here, 40% of the regenerated plants established successfully in the pot (Fig. 7). Following the propiono orcein squash technique [8], the putative haploid plants were found to contain only one set of chromosome (n = 12). Morpho-physiologically also, the plants were inferior to the normal diploids (2n = 24). Further, the haploid plants found to have smaller stomata (<0.001mm) located more closely as against those in diploids (0.002mm). All these evidences showed that the regenerated and established plants were true haploids. The study thus established an effective *in-vitro* system suitable for successful culturing and regeneration of anther derived haploid plants from aromatic rice cultivar of Assam.



## References

1. **Chu C. C., Wang C. C., Sun C. S., Hsu C., Yiu K. C., Chu C. Y. and Bi F. Y**. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen sources. *Scientia Sinica*., **18**: 659-668.
2. **Murashige M. and Skoog F**. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant., **15**: 473-479.
3. **Gamborg O. L., Miller R. A. and Ojima K**. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res., **50**: 151-158.
4. **Mukherji P. and Mukherji D. K.** 1979. A simple propiono orcein squash technique forthe karyomorphological studies on rice (*Oryza sativa* L.). Curr. Sci., **48**: 496-497.
5. **Zapata F. J. and Cho M. S.** 1988. Callus formation and plant regeneration in isolated pollen culture of rice (*O. sativa* L., cv. Taipei 309). Plant Sci., **58**: 239-244
6. **Huang C. R., Wu Y. H. and Chen C. C**. 1985. Effects of plant growth substances on callus formation and plant regeneration in anther culture of rice. *In*: Proc. Int. Rice Genet.Symp. Khush, G.S. (ed.), IRRI, Los Banos, pp. 763-771.
7. **Lenka N. and Reddy G. M**. 1994. Role of media, plant growth regulators in callusing and plant regeneration from anthers of *indica* rice. Proc. Indian Natl. Sci., Acad., **60**: 87-92.
8. **Islam M. A., Asaduzzaman M., Bari M. A., Rahman M. H., Khatun N. and Rahman M**. 2003. *In vitro* plant regeneration through anther culture of five rice varieties. On Line J. Biol. Sci., **3**: 167-171.
9. **Kawata M., Ohmiya A., Shimamoto Y., Oono K. and Takaiwa F**. 1995. Structural changes in the plastid DNA of rice (*Oryza sativa* L.) during tissue culture. Theor. Appl. Genet., **90**: 364-371.
10. **Harda T., Sato T., Asaka D. and Matsukawa I**. 1991. Large scale deletions of rice plastid DNA in anther culture. Theor. Appl. Genet., **81**: 157-161.
11. **Harda T., Ishikawa R., Niizeki M. and Saito K**. 1992. Pollen derived rice calli that have large deletions in plastid DNA do not require protein synthesis in plastids for growth. Mol. Gen. Genet., **233**: 145-150.
12. **Sinha S. K., Mandal N. and Gupta S**. 1998. Anther culture of *Oryza rufipogon* Griff. Oryza, **35**: 232-236.