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# Improved method for anther culture induction and regeneration in indica rice hybrids

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## ABSTRACT

Anthers of three rice hybrids (SK2096, SK2096-1 and SK2060) having late uninucleate microspores were investigated for induction of haploid rice plants. Anthers were cultured on RZM medium and RZM medium supplemented with three concentrations of yeast extract, 0.25 gm $L^{-1}$, 0.500 gm $L^{-1}$, 0.75 gm $L^{-1}$. The best callusing from cultured anther obtained for SK2096-1 hybrid in RZM medium supplemented with 0.25 gm $L^{-1}$ yeast extract. It gave 58% callus induction. The other hybrids performed well on RZM media with the other two concentrations of yeast extract. However, the callus induction percentages obtained were less than that for SK2096-1. For regeneration haploid plantlets anther derived calli were transferred to MS and MSR1 media. The regeneration was very poor on MS medium compared with MSR1 medium. SK2096-1 gave the highest regeneration percentages, extended from 13 % to 36.6 %. Percentage of haploid rice plants obtained was defined. The investigation showed the importance of yeast extracts in enhancing callus initiation of the indica genotypes.



## KEYWORDS

Rice;
Anther culture;
*Oryza indica*;
Yeast extracts;
Casamino acid.

## INTRODUCTION

"At present, anther culture is a powerful tool for plant breeding, offering the possibility to rapidly obtain the genetically diverse haploids or homozygous doubled haploids from F1 hybrids"[1-4]. However, the use of anther culture as a routine tech-nique for breeding of indica rice is extremely limited by the poor induction of androgenic calli and subsequent plant regeneration. The average frequency of green plant regeneration from cultured anthers of indica rice was only 1 % in comparison with the 10% of japonica rice[5-8].

Extensive works have been made on various factors influencing the anther culture efficiency of indica rice[9-12]. It was reported that indica rice anther culture can be enhanced by improving the composition of tissue culture medium especially by manipulating plant growth regulators[10,13-17], osmotic pressure adjustment[18-20] and nutrients[21-23]. However, the frequency of whole plantlet induction through androgenesis is still low in indica rice.

The purpose of this study was to improve callus induction and subsequent plant regeneration of some cultivars of indica rice in Egypt (SK2096, SK2096-1, SK2090) by deriving calli from microspores and using RZM medium fortified with 0.25, 0.50 or 0.75 g $L^{-1}$

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yeast for callus induction, and MSR and MSR1 media for plant regeneration.

## MATERIALS AND METHODS

Three indica rice hybrids namely SK2096, SK2096-1 and SK2060 were grown in the experimental garden of Rice Research and Training Center, Sakha, Kafr El Sheikh, Egypt, during 2006/2007 growing season.

Closed flower buds (Boots) of rice hybrids having the late uninucleate microspores suitable for induction of androgenesis were collected for conducting investigation. Identification of late uninucleate stage was made as described by Sharmin et al.[24]. Panicles thus collected were wrapped in a moist muslin cloth, sealed within polyethylene bags and then subjected to shock at 6°C for 10 days in the dark.

Cold treated panicles were cleaned. Individual spikelets from the middle of the panicles were taken out and put in clean and sterilized Petri-dishes. The spikeletes were sterilized by dipping into NaOCl solution (1.2% V/V for 10 minutes). Then they were thoroughly rinsed with sterilized distilled water. Anthers were picked up from the central spikeletes by sterilized forceps[25,26] and then placed horizontally on the culture medium RZM and culture medium RZM supplemented with 0.25, 0.50 or 0.75 g $L^{-1}$ yeast. An average of 2000 anthers of SK2096, SK2096-1 and SK2060 cultivars were planted. The conversion of embryogenic structures in plants was performed in the MS[27] and its modified medium MSR1, supplemented with 1.0 mg $L^{-1}$ BAP and NAA 1.0 mg $L^{-1}$ hormones. The plants of two regeneration media transferred into MS medium free hormones to make strength of plants[28-32]. The well regenerated plants were transferred into semi synthetic soil (Peat moss/Soil, 1:1) till they become strong enough to be transferred to greenhouse till harvest.

Rice root tips of regenerated plants were prepared for determination of haploidy chromosomes as described by Sharmin et al.[24]. Chromosome numbers were determined by chromosome counting of root tips.

## RESULTS AND DISCUSSION

When about 2000 anthers of each indica hybrids SK2096, SK2096-1 and SK2060 were cultured on basal RZM medium, only 400 to 723 anthers represented 20 to 36.16% produced the calli from microspores (TABLE 1). The frequency of induction of callus accounted to 36.16% in SK2096, 20 % in SK2096-1, 28% in SK2090. This data showed that SK2096 was more responsive to callus induction than the other two tested hybrids on RZM medium. The percent of callus induction in this study (20 % - 36.16 %) was more than four fold (0.0–8.23 %) higher than that reported by Chen and Lin[33], Hakim et al.[34], Sripichitt et al.[2], Shahnewaz et al.[15] and Ramakrishnan et al.[10] who found poor callusability in indica rice variety. The wide variation in callus induction between our data and the data of other workers was attributed to many known and unknown factors, such as donor plants, genotypic variation, media composition, and handling of cultures[35]. These factors may have a greater influence on the response of androgenesis. It can also be concluded that RZM medium used in our study was more efficient in callus induction of indica rice than chu and N6N media used by Sripichitt et al.[2] which gave callus induction 1 % -1.4 %. The efficient of RZM medium could be due to the presence of maltose in its gradients which was found to enhance embryo induction and plant regeneration dramatically in cereals[35] or/and the presence of exogenous auxins which has proved to be the most efficient treatment to induce somatic embryogenesis. Although the process of embryo induction from cells in culture is not fully understood, it is now generally believed that, in the continued presence of auxin, a differential change in gene expression (probably associated with increased demethylation of DNA) in PEMs occurs[36].

When anthers cultured on basel RZM medium supplemented with o.25, 0.500, 0.75 gm $L^{-1}$ yeast extract, SK2096-1 hybrid showed a higher frequency (58 %) on medium supplemented with 0.25 gm $L^{-1}$ yeast extract. The other two hybrids, SK2090 and K2096, showed a higher frequency (43.33, 47.25%) on a medium supplemented with 0.500, 0.75 gm $L^{-1}$ yeast respectively TABLE 1. This data indicated that the rate of induction increased with the addition of yeast extract, which may have some role in enhancing cell division and development of somatic embryos of indica hybrids because it contains Vitamin B group, fatty acids and Casamino acid[6,37-43]. Our data also indicated that rate of anther callus induction varied with the geno-

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type. This variation was consistent with the previous works of Reddy et al.[9], Bishnoi et al.[44] and Sripichitt et al.[2]. The variation in the rate of anther callus induction between genotypes can be attributed to the variation in the levels of endogenous hormones in the tissue. Furthermore, the variation between genotypes in the rate of anther callus induction might be influenced by the specific pathway combining with spontaneous doubling of chromosomes. Microspore derived calli and embryoids often show aneuploids, dihaploids and polyploids[35]. On the other hand, the media used for callus induction had a varied effect for the different genotypes of the same species[21,2,10]. A given concentration of yeast extract gave relatively different effects in the percentage anther callus induction of SK2096, SK2096-1 and SK2060 hybrids. For example, RZM supplemented with 0.75 gm $L^{-1}$ was more effective for SK2096, whereas RZM supplemented with 0.50 gm $L^{-1}$ was more effective for SK2090. Two ways analysis of variance indicated that anther callus induction is dependent on

**TABLE 1 : Callus induction from microspores on RZM medium and RZM medium supplemented with 0.25, 0.50, 0.75 mg$L^{-1}$ yeast extract (YE)**

| Hybrids | Media | No. of anthers cultured | No. of anthers forming callus | % of anthers forming callus |
|---|---|---|---|---|
| SK2096 | RZM | 2000 | 723 | 36.16 |
| | RZM + 0.25 mg$L^{-1}$ YE | 2000 | 720 | 36 |
| | RZM + 0.50 mg$L^{-1}$ YE | 2000 | 790 | 39.5 |
| | RZM + 0.75 mg$L^{-1}$ YE | 2000 | 945 | 47.25 |
| SK2096-1 | RZM | 2000 | 400 | 20 |
| | RZM + 0.25 mg$L^{-1}$ YE | 2000 | 1160 | 58 |
| | RZM + 0.50 mg$L^{-1}$ YE | 2000 | 620 | 31 |
| | RZM + 0.75 mg$L^{-1}$ YE | 2000 | 230 | 11.5 |
| SK2090 | RZM | 2000 | 560 | 28 |
| | RZM + 0.25 mg$L^{-1}$ YE | 2000 | 480 | 24 |
| | RZM + 0.50 mg$L^{-1}$ YE | 2000 | 867 | 43.33 |
| | RZM + 0.75 mg$L^{-1}$ YE | 2000 | 333 | 16.66 |
| **Analysis of Variance for induction percentages** | | | | |
| Medium | | | 351.9462** | |
| Hybrid | | | 413.0173** | |
| Medium X Hybrid | | | 564.9970** | |

**** Significance at 0.05%**

**TABLE 2 : Plant regeneration of anther calli (induced on RZM medium supplemented with 0.25, 0.50, 0.75 mg$L^{-1}$ yeast extract) on MS medium**

| Hybrids | Callus induction media | No. of calli transferred to regeneration medium | No of plants regenerated (%) | | | | No of diploid rice plants obtained (%) |
|---|---|---|---|---|---|---|---|
| | | | Green | Albino | Green | Albino | |
| SK2096 | RZM | 74 | 0.00 | 0.00 | 1 | 0.00 | 0.00 |
| | RZM + 0.25 mg$L^{-1}$ YE | 31 | 20 (64.5) | 11 (35.5) | 0.00 | 31 | 17 (85) |
| | RZM + 0.50 mg$L^{-1}$ YE | 18 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | RZM + 0.75 mg$L^{-1}$ YE | 98 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| SK2096-1 | RZM | 43 | 0.00 | 0.00 | 1 | 0.00 | 0.00 |
| | RZM + 0.25 mg$L^{-1}$ YE | 93 | 8 (8.6) | 9 (9.7) | 6 (6.5) | 32 | 5 (62.5) |
| | RZM + 0.50 mg$L^{-1}$ YE | 28 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | RZM + 0.75 mg$L^{-1}$ YE | 12 | 0.00 | 1 (8.3) | 1 (8.3) | 2 | 0.00 |
| SK2090 | RZM | 32 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | RZM + 0.25 mg$L^{-1}$ YE | 19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | RZM + 0.50 mg$L^{-1}$ YE | 22 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | RZM + 0.75 mg$L^{-1}$ YE | 13 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

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the media, genotypes and the interaction between them TABLE 2.

The anther calli formed from microspores cultured on basal RZM medium and basal RZM supplemented with 0.25, 0.500, 0.75 gm $L^{-1}$). yeast were transferred to the regeneration media MS and MSR1 (Table Generally, the percentage of regeneration of the three hybrids was extremely high on MSR1 medium in comparison with MS medium TABLE 1. The regeneration of the three hybrids on MS medium was very poor, except for the calli formed from microspores of SK2096 cultured on RZM supplemented with 0.25 $gmL^{-1}$ yeast, gave 74.19 % regenerated plants.

In Sk2096-1, between 13%-36.5% of the anther calli induced on RZM medium and RZM media supplemented with 0.25, 0.500, 0.75 gm $L^{-1}$ yeast regenerated plants. In SK2096, between 8%-10% of the anther calli induced on RZM medium regenerated plants. On the other hand, the regeneration frequency was less than 4% in Sk2096. The percentage of albino plants ranged between 5.5% and 39%. Datta[35] reported that the extent of albinism is mainly attributed to genetic background of the donor plants. However, our data indicated that the anther calli, derived from the same hybrid SK2096-1, gave a different percentage of albinism TABLE 3. Interestingly, it was noticed that anther calli developed on RZM supplemented with 0.25 gm $L^{-1}$ yeast extract gave a higher percentage of albinism on MSR1 medium and this percentage decrease with increasing the concentration of yeast extract (TABLE 3). Therefore, we can infer that albinism may be affected by the nutrient of the media which not only provides nutrition to the microspores but also directs the pathways of embryo development. The frequency of the undifferentiated dead brown calli was higher in SK2090 and Sk2096 than SK2096-1. This might be due to the deleterious effect of endogenous hormones hormone in SK2096-1 and exogenous hormones in the regeneration medium.

The green plantlets regenerated from the anther calli were examined for the ploidy level. The percentage of diploid plants ranged between 62.6% to 85% on MS medium TABLE 2 and between 66.6% and 100 % on MSR1 medium TABLE 3.The frequency of diploid plants was very high in anther callus induced on RZM medium supplemented with 0.25 gm $L^{-1}$ yeast extract. SK2096-1 gave a good performance on MSR1 medium compared with the other two hybrids.

The success rate of anther culture in indica rice is still limited due the low frequency of from microspores and subsequent regeneration of green plants. Further studies are required.

**TABLE 3 : Plant regeneration of anther calli (induced on RZM medium supplemented with 0.25, 0.50, 0.75 $mgL^{-1}$ yeast extract) on MSR1 medium**

| Hybrids | Callus Induction media | No. of calli transferred to MSR1 medium | No of plants regenerated (%) | | | | No of diploid rice plants obtained (%) |
|---|---|---|---|---|---|---|---|
| | | | Green | Albino | Roots | Total | |
| SK2096 | RZM | 143 | 7 (4.8) | 8 (5.6) | 3 (0.04) | 18 | 5 (71.4) |
| | RZM + 0.25 $mgL^{-1}$ YE | 77 | 4 (5.1) | 4 (5.2) | 2 (2.6) | 10 | 4 (100) |
| | RZM + 0.50 $mgL^{-1}$ YE | 79 | 3(3.8) | 1 (1.3) | 3 (3.8) | 7 | 2 (66.6) |
| | RZM + 0.75 $mgL^{-1}$ YE | 91 | 5 (5.5) | 0.00 | 6 (6.6) | 11 | 4 (80) |
| SK2096-1 | RZM | 37 | 8 (21.6) | 2 (5.4) | 2 (5.4) | 12 | 5 (100) |
| | RZM + 0.25 $mgL^{-1}$ YE | 23 | 3 (13.0) | 9 (39.1) | 0.00 (0.00) | 12 | 2 (66.6) |
| | RZM + 0.50 $mgL^{-1}$ YE | 34 | 11 (32.4) | 2 (1.2) | 2 (5.9) | 15 | 8 (81.8) |
| | RZM + 0.75 $mgL^{-1}$ YE | 11 | 4 (36.4) | 1 (9.1) | 0.00 (0.00) | 5 | 3 (75) |
| SK2090 | RZM | 84 | 0.00 | 2 (2.4) | 0.00 (0.00) | 2 | 0.00 (0.00) |
| | RZM + 0.25 $mgL^{-1}$ YE | 72 | 1 (1.4) | 11 (15.3) | 0.00 (0.00) | 12 | 0.00 (0.00) |
| | RZM + 0.50 $mgL^{-1}$ YE | 130 | 1 (0.77) | 8 (6.2) | 1 (0.77) | 10 | 0.00 (0.00) |
| | RZM + 0.75 $mgL^{-1}$ YE | 37 | 0.00 | 2 (5.4) | 0.00 (0.00) | 2 | 0.00 (0.00) |

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