International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **3(4),** 73-79, April **(2014)** *Int. Res. J. Biological Sci.*

Androgenic Callus Induction of the Indica Rice Hybrid of Chakhao Amubi and Basmati 370

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Available online at: www.isca.in, www.isca.me
Received 19th December 2013, revised 28th January 2014, accepted 25th February 2014

Abstract

Among the different varieties of rice available worldwide some of the specialty rice viz., the fragrant Basmati of India and Pakistan, medicinal purplish-black Jiengo rice of China known as Laos and Cambodia a glutinous sticky rice of Thailand are the most desirable rice fetching upto 10 times more than the common rice in international markets. In the last few decades, Basmati rice has become the most favored rice due to its soft fluffy texture with fine slender grains and high content of acetyl-1-pyrroline (0.09 ppm). Dark-purple glutinous aromatic rice known as "Chakhao Amubi" of Manipur, India, was found to have its own aesthetic value and high nutraceutical properties. Although the dark sticky glutinous rice is also one of the most preferred rice, the grain quality of this aromatic rice is still poorly understood and neglected because of its low yield, non-responsiveness to fertilizers and high susceptibility to rice blast. Thus in the present study we tried to develop a novel rice hybrid of "Chakhao Amubi and Basmati 370" to improve its yield and provide a better grain quality rice having high nutraceutical properties using anther culture development techniques of homozygous breeding lines of double haploid. We believed our present study would provide desired homozygous and improve genotype for sustainable development for rice variety.

Keywords: Androgenous callus, Basmati, Chakhao Amubi, homozygous, genotype.

Introduction

Rice is an important food component for Asians in general and Indians. Asia countries cultivate around 137 million hectare of paddy and Indian contribution to 45 million hectare. Rice plays an important role in the growth of annual GDP of India by 15% and provides essential nutrient with 43% calorie to more than 70% of the total population[1]. Rice is a vital part of many cultures. It is the basis of livelihood for most of the Asian farmers and staple food for seventeen countries in Asia -Pacific, eight African countries, seven Latin America and Caribbean. Unmilled rice comes in different shapes and colors including brown, red, purple and black. These types of rice are very popular among the rustic population for their health properties. Although, specialty rice varieties may not be grown on larger acreage, there is an increasing demand in the market for rice having novel cooking, flavor, processing and appearance characteristics. Therefore, investigation is needed to develop cultivars that meet various niches in marketing so that the local rice producers can compete in international market arenas. In this respect, development of a "novel rice" variety, which has the characteristics of specialty rice with better grain quality, nutraceutical properties, will certainly result in specialty rice. This can be achieved by combining the useful traits through crosses involving the elite rice genotypes possessing the desired characters. However, it takes 7 to 8 years to develop a new cultivar using conventional methods. Few more years are spent in field trials making upto10 to 11 years to get a new variety. Therefore, techniques that reduce the costs in terms of time and labor to release new cultivars are always welcome. In this context, double haploid breeding through anther or pollen culture reveals itself as a useful tool. The advantage of anther culture is that it provides production of homozygous lines in a short period as compared to several generations required using conventional whole plant techniques [2-4]. Unlike conventional breeding programs, in which numerous cycles of planting and harvesting, segregating populations are necessary, regeneration of fertile plants from gametophytic cells, without fertilization, allows rapid development of pure lines. This technique shortens the time necessary to attain homozygous from seven to one generation by production of double haploids, and selection becomes more simple and efficient. Therefore, the production of new rice varieties with high yielding potential, aromatic, better grain quality and nutraceutical properties in a very short time through biotechnological interventions is the specific need of the moment. There has been a substantial improvement in anther culture technique but a through detail study on the factors involving the culture response under *in vitro* condition of indica rice is very limited. The generation of green plants from androgenic calli is very low irrespective of its varieties. The low anther culture response, high percentage of albino plantlet generation and abundance of haploids are the main constraints in establishing a successful anther culture in rice. The development of plantlets directly from microspores provides ample scope for developing homozygous doubled haploids (DH) without heterozygosity interference. Thus, using anther

culture provides an easy way to handle *in vitro* selection for genetic improvement of characters for superior growth and yield[5]. Androclonal variation has immense prospect though it was quantified in a very few cases in rice[6]. Studies have proved that doubled haploids lines produced from microspore culture and anther culture were highly potential for genetic improvement by broadening the genetic diversity through production of homozygous lines within a short time[3,7], in contrast to conventionally practiced. However, *indica* cultivars respond poorly to *in vitro* techniques[8-9], 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 involves two steps; induction of embryogenic calli from microspores and regeneration of green plants from calli. In the case of *indica* rice, major problems include early anther necrosis, poor callus proliferation and albino plant regeneration[10]. The important role of another culture in breeding program is to provide plants with special agronomic characters like development of earliness, increased grain weight, superior grain quality, disease resistance [11,12], dwarf plant type and abiotic stress tolerances[13]. The application of biotechnology is very essential for the successful improvement of crops using cell culture and tissue. The success of this approach depends upon reliable callus culture and plant regeneration procedures. Callus induction frequencies and plant regeneration tissue culture are influenced by many factors like culture medium composition, explants source, genotype and environmental condition[14]. Genotype and nutrient composition are regarded as the major sources of variation *in vitro* culture[15]. The shoot regeneration problem also encountered during plant regeneration experiments[16]. The success rate can be enhanced by improving the composition of the tissue culture medium and manipulating plant growth regulators[17], osmotic pressure[18] and partial desiccation[19].

A number of studies have been carried out for regeneration of rice plant such as mature embryos [20-23] or scutellum-derived callus[24-26] immature embryos[22,27,28]. In present, biotechnological interventions such as the technique of anther culture for development of homozygous breeding lines of double haploid have been successfully employed. Such study will be a boon for sustainable progress of nutraceutical varieties and improved rice based production.

Material and Methods

Plant material: i. Selected F5 plants of the cross between local aromatic dark purple rice "*Chakhao-amubi*" of Manipur and high yielding Basmati rice derivatives "*Basmati 370*". ii. Selected F5 plants of the cross between dark purple non-aromatic rice "*Hei Bao*" and high yielding aromatic rice line "*Pusa1302-3-3-1-10".*

Method I: Conventional Breeding Technique: Experiments were carried out under green house conditions for advancement generation i.e. to obtain two generations of crosses in a year. The F1 plant populations resulted from the cross between selected F5 plants of local aromatic dark purple rice "*Chakhao-amubi*" x high yielding Basmati derivative "*Basmati 370*" and selected F5 plants of dark purple non-aromatic rice "*Hei Bao*" x aromatic high yielding "*Pusa 1302-3-3-1-10*" were bulked for growing F2 plant population using single panicle descent Method. Using the same single panicle descent method F2, F3 and F4 plant population was grown under standard cultural practices for individual plant selection and seeds of selected individual plants was collected separately as F6 seeds.

Method II: Non-Conventional Breeding Technique (Biotechnological interventions) using anther/pollen culture: During the first and second year the protocols were developed for *in vitro* production of the androgenetic double haploid lines through anther and pollen culture of the selected F1, populations resulted from the cross between selected F5 plants of local aromatic dark purple rice "*Chakhao-amubi*" x high yielding "*Basmati 370*" and selected F5 plants of dark purple non-aromatic "*Hei Bao*" x "*Pusa 1302-3-3-1-10*". Three to four panicles were collected from the plants early morning. Anthers from the central spikelets with middle to late uninucleate stage were wrapped in moist polythene bags for 10 days at 10-12 ^{0}C before culture. Panicles were rinsed with 70% alcohol for 1 min and spikelets were removed. Then they are surface sterilized with 0.1% aqueous $HgCl_2$ for 5 mins and rinsed thoroughly with sterilized distilled water. Base of the spikelet was cut with the sterilized scissor and cultured on both liquid and semi solid N6 medium supplemented with various concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% maltose. Then, the culture was maintained in the dark condition at 25±2^{0}C. Culture was examined and the frequency of calli formation was recorded every after six to seven weeks. The yield calli were then cultured in Murashige and Skoogs medium (MS) supplemented with different concentration and combination of auxin and cytokinins 3% sucrose for regeneration experiment. Percentage of calli regeneration data was recorded after 7 weeks of total incubation. For each experiment, 20 replicate were taken and repeated thrice. The collected data were analyzed using ANOVA and mean separation was carried out using Tukey's comparison test. This test of statistical significance was performed at 5% level using SPSS program (Version 17). The healthy regenerated plants were later transferred in a large tub and grown in net house under control condition.

Results and Discussion

Crosses of 50 specific (*Chakhao-amubi x Basmati 370*) and (*Hei Bao x Pusa 1302-3-3-1-10*) were made in the year 2009, 2010 and 2011 during the crop season to obtain F_1 hybrids and F_2 plants. In the subsequent year (2011), *in vitro* culture experiment was carried out to evaluate the anther culture response of the parental indica and Basmati rice varieties, and their heterotic F1 hybrids. Callus induction and plant regeneration were carried out using N6 medium[29-34], which was first designed for anther culture of rice by Chu et al.[35].

Subsequently, intense efforts have been undertaken to improve the recipe of N6 medium by adjusting its growth regulator combinations[29,32, 33,36]. In the present study, both liquid and semi solid N6 medium supplemented with different concentration of 2,4-D (0.5-4 mg/L) was tested for callus induction. Callus was invariably developed from anther under different concentration within 2-3 weeks. The response of the explants with different concentration of 2,4-D and percentage frequency of callus induction is shown in table 1. Callus were induced and grown in both liquid and semi solid N6 medium[37] and the difference of composition of culture medium can result in variation in callus induction[14]. However, semi solid medium does not show mark response in callus induction (table -1). The highest frequency of callus induction (60%) was observed in liquid N6 medium supplemented with 1 mg/L (table-1) (figure-1). Among the different modification made, significant ones are the used of liquid N6 medium in all the culture tested. In the present study, it can be inferred that 2,4-D alone can be used for callus induction which is in conformity with Katiyar et al.,[38]. The frequency of the anthers forming Calli varied between 0 to 60% depending upon the culture medium tested (table-1). Callus induction frequency as determined by number of anthers forming calli, was 24% to 60% in liquid medium and 9.33% to 26.67% in semi solid medium. Notably, anthers responded quicker and more responsive in liquid medium forming visible colonies within 2-3 weeks while increasing concentration of 2,4-D shown reduction in the calli initiation (table-1). The N6 medium[39] which are widely applied for Japonica rice anther culture, was found suitable for the F1 hybrid of (*Chakhao-amubi x Basmati 370*) and (*Hei Bao x Pusa 1302-3-3-1-10*). Since, sugars in a culture medium function as a source of carbon and osmotic regulator, both are critical for embryoid or callus formation[40]. The maltose role is to improve the anther culture ability of cereals has been documented well[41,42]. In the present study, 3% maltose was utilized as a source of carbon and shows enhanced anther culture efficiency, which is in conformity[43,44]. The induced calli from the above experiment were transferred into semi-solid MS medium supplemented with various concentration and combination of α-napthalene acetic acid and Kinetin (Kn), 6-benzyl-amino-purine (BAP) + 3% sucrose, for shoot regeneration (table-2). Regeneration of plant started after 15 days of transfer and the generation of shoot and green spots were visible within 1 week and after 15 days of fully regenerated roots and shoots were observed. Highest green shoot regeneration frequencies (70%) were found in N6 medium supplemented with 1 mg/L NAA and 2mg/L of BAP (table- 2) (figure- 2). Production of albino plants has been the major problem anther rice culture especially in indica rice varieties and hybrids involving indica rice parent[11-45,46]. However, sufficient number of green plants could be obtained in all the culture tested, but some culture does not show mark response in percentage shoot regeneration. Plantlet regeneration is dramatically decreased when the concentration of BAP in medium increases. Fully-grown regenerated plantlets were transplanted into plastic tub and maintained in shade house with 90% survival rate. The established plants were then shifted outside the shade house and grown into maturity (figure-3).

Table-1
Relative proliferation rate of embryonic calli on N6 media with varying 2,4-D concentrations

Plant growth regulator conc. (mg/L) 2,4-D	Type of media	Number of anther inoculated	% of anthers forming callus
0	Semi solid	150	0±.00
	Liquid	150	0±.00
0.5	Semi solid	150	17.5±.02^{b}
	Liquid	150	60.00±.04^{h}
1	Semi solid	150	20.24±.07^{b}
	Liquid	150	42.25±.10^{g}
2.0	Semi solid	150	18.00±.05^{b}
	Liquid	150	40.00±.06^{f}
3.0	Semi solid	150	22.45±.22^{c}
	Liquid	150	27.15±.06^{e}
4.0	Semi solid	150	9.33±.07^{a}
	Liquid	150	24.00±.08^{d}

Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

Table-2
Effect of different plant growth regulators in shoot regeneration from calli of (Chakhao-amubi x Basmati370) and (Hei Bao x Pusa 1302-3-3-1-10) hybrid

Sl. No.	Plant growth regulator conc. (mg/L)		Shoot regeneration frequencies
	NAA	BAP	
1.	0	0	0±.00
2.	0	1	0±.00
3.	0	2	44.55±.09^{j}
4.	0	4	42.5±.06^{i}
5.	1	1	0±.00
6.	1	2	70.00±.12^{k}
7.	1	4	45.35±.15^{j}

Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

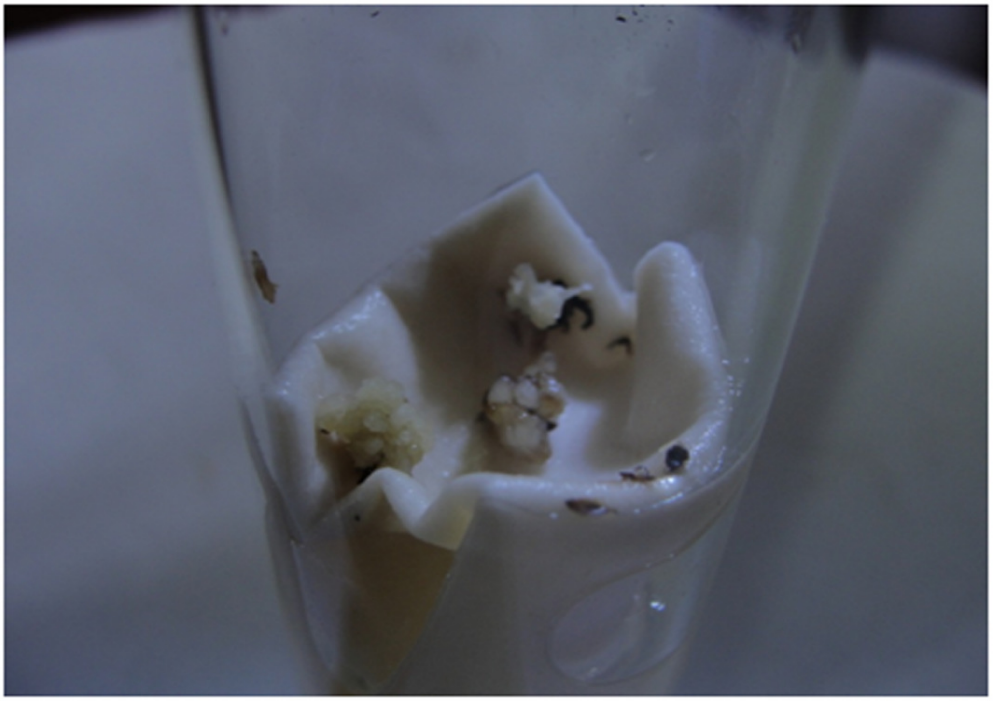

Figure-1
***In vitro* callus induction in liquid N6 medium supplemented with 0.5 mg/L 2-4D**

Figure-2
***In vitro* shoot regeneration in semi solid MS supplemented with 1 mg/L NAA + 2 mg/L BAP**

Figure-3
Established *in vitro* hybrid plant in plastic tub

Conclusion

In the present study the anther culture technique was successfully employed and it showed the potential of tissue culture technique for mass propagation of (*Chakhao-amubi x Basmati 370*) and (*Hei Bao x Pusa 1302-3-3-1-10*) hybrid which will help to improve the variety into a nutraceutical rice. Manipur state which are blessed with rich genetic resources and a variety of customary rice-based products. Being a land of assorted food habits, it has the advantage to develop culturally and ethnically acceptable preparations of rice for commercial use. This will eventually give the abundant needed support to the farmers to cultivate the specialty rice. This technique also shortens the time necessary to attain homozygous from seven to one generation by production of double haploids, and selection becomes more simple and efficient. In this context, there is a growing interest in synthesis of better nutraceutical rice for food and medicinal purposes. Underutilized dark purple rice of Manipur "*Chakhao-Amubi*" can be sustainable developed by breeding with aromatic "*Basmati 370*". The present study successfully employed the technique of anther culture for production of haploid plants from the anthers of the F1 hybrid. This will help in selection of the desired homozygous genotype.

Acknowledgement

We thank all members of the lab for their valuable suggestions and Director IBSD, Department of Life Sciences Manipur University and Central Agricultural University Imphal for providing the infrastructure for the study. The authors would like to thanks to Department of Science and Technology, New Delhi, India for the financial assistance.

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