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IN VITRO REGENERATION OF AROMATIC RICE (*ORYZA SATIVA* L. VAR. DOAIRGURA)

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Abstract

Rice (*Oryza sativa* L.) is the world's most important cereal crop. The Doairgura variety (*O. sativa* L.) is a low-yielding, high-quality aromatic rice-producing variety in Bangladesh. This study was undertaken to develop an *in vitro* regeneration protocol through in vitro callus induction. MS media supplemented with different concentrations of 2, 4- D (0.5, 1.0, 2.0 and 3.0 mg /l) were used for callus induction from the mature dehusked rice seeds. The best results for callus formation was obtained on MS media supplemented with 2.0 mg/l 2, 4-D. Shoot regeneration from in vitro cultured callus was tested with different combinations (BAP, NAA, Kn mg/l) of hormone containing MS media. The highest regeneration frequencies (100%) as well as the highest average shoot per culture (15.3) were recorded on the MS media supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA. For root induction, hormone free MS media showed better results. After hardening, the plantlets were transferred to soil. This protocol is relatively simple and reproducible.

Introduction

Rice (*O. sativa* L.) belongs to the family Poaceae and feeds over half of the global population (Saskai *et al.* 2005). In Asia, it covers half of the arable land used for agriculture in many countries (Cantrell and Hettel 2004). Almost 114 countries are growing rice with an annual production of 100,000 tons or more (http://www.irri.org/stastistics). Bangladesh is an agro-economy-based middle-income country. Rice is the staple food of Bangladesh and the main agricultural activity of this country is involved in producing rice. It provides nearly 48% of rural employment, approximately 75% of the calories in the average daily diet (Bhuiyan *et al.* 2002) and ensures the country's political stability. The Rice sector contributes 60% of GDP to agriculture and accounts for 69% of the value added in crop production. Rice accounts for about 77% of the total cropped area **(**http://www.irri.org/statistic**s).**

Rice has 24 species, of which 22 are wild and two, viz., *Oryza sativa* and *Oryza glabrrima* are cultivated (Ray *et al*. 1985). Aromatic rice constitutes a small, special group of rice, which is considered the best in quality. Aroma is caused by extremely small amounts of volatile compounds, which are contained as a complex mixture. More than 100 compounds contributing to the aroma of rice have been identified (Tsugita *et al*. 1983). The biochemical basis of aroma was identified as 2-acetyl-1-pyrroline (Widjaja *et al.* 1996, Weber *et al.* 2000). Aromatic rice has long been popular in the Orient and is now becoming more popular in the Middle East, Europe and the United States of America. Most of the trade in aromatic rice is from India, Pakistan and Thailand in the world market (Singh *et al.* 2005). In Bangladesh, aromatic rice is also very popular. With the attainment of self-sufficiency in rice, the demand for quality aromatic rice in the domestic and international market is on the increase. The price of fine aromatic rice is higher than that of coarse rice. Most of the scented rice varieties in Bangladesh are of the traditional type, photoperiod

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sensitive and cultivated during the Aman season. The majority of these indigenous aromatic rice cultivars are low yielding, but their higher price and low cost of cultivation generate higher profit margins compared to other varieties.

Most of the rice varieties have been developed traditionally by selection, hybridization and back crossing with locally adapted high-yielding lines. The conventional methods of plant selection for aroma are not easy because of the large effects of the environment and the low narrow-sense heritability of aroma. The aromatic variety can be improved (biotic and abiotic stress tolerance variety) through genetic transformation followed by tissue culture techniques.

Furthermore tissue culture of aromatic rice may help to get somaclone and their performance can be observed in the field. Organs treated with chemical and physical mutagenic agents produce mutant callus, which aids in the production of somaclones, disease, pest, or insect resistant, stress, or salt tolerant mutant lines of aromatic rice. In vitro regeneration of aromatic rice can be a crucial technique for the improvement of the crop. There are many aromatic rice varieties in Bangladesh. Some varieties are now in endangered condition and are not cultivated due to low yield. *O. sativa* var. Doairgura is a low-yielding high-quality aromatic rice variety in Bangladesh. Therefore, the experiment was undertaken with the aim of finding out the potential of the aromatic rice (*O. sativa* L.) variety Doiargura for callus induction and plant regeneration from mature dehusked seeds as well as conservation of the variety. This protocol will also help in genetic transformation studies for this variety.

Material and Methods

O. sativa var. Doairgura, was used as plant material for this investigation. Seeds were collected from the Bangladesh Rice Research Institute (BRRI), Gazipur, Dhaka. The present experiment was conducted by the Bangladesh Council of Scientific and Industrial Research (BCSIR). Mature and viable seeds of an aromatic rice variety were dehusked manually and used as explants for callus induction. Approximately 2.0 mm diameter calli derived from these mature seeds were used for plant regeneration. Rice seeds were surface sterilized initially with mild detergent and washed with tap water for 3-5 min. After that, explants were surface sterilized with 70% (v/v) ethanol for 1 min, followed by 0.1% $HgCl_2$ by gentle shaking for 8 min and rinsed four times with autoclaved distilled water. Then, the explants were soaked with sterilized filter paper. Finally, they are ready for inoculation on an appropriate nutrient medium. Explants were cultured on MS containing BAP, NAA, IAA, IBA, 2, 4-D and Kn (mg/l) singly or in combinations for regeneration. All the culture were maintained in growth room in which 16 hrs photoperiod and 25^0C temperature were maintained. In vitro regenerated shoots were sub-cultured to fresh medium every 12-15 days for further multiplication. Fully elongated shoots were treated with hormone-free MS medium and also with MS medium supplemented with IBA for root formation. Following sufficient development of roots, plantlets were taken out and washed thoroughly with water to remove the culture medium from the roots. Then transplanted into small plastic pots. All the experiment carried out by three replicate to minimize error. For data analysis Microsoft Excel was used.

Results and Discussion

The present study has been carried out in order to establish an efficient regeneration protocol for the Doairgura aromatic rice variety of Bangladesh. The overall experiments were carried out in two phases. In the first phase of experiments, callus induction for the selected aromatic rice variety (Doairgura) was developed. The responses of MS media with various hormones towards callus induction were recorded. In the second phase, *in vitro* plant regeneration was carried out from the

callus of the aromatic rice variety Doairgura. The effect of MS media supplemented with various hormones towards *in vitro* plant regeneration was tested.

To reduce the level of surface organisms, the explants (dehusked embryos) were sterilized with a 0.1% $HgCl_2$ solution for 8 minutes. Then the explants were washed five times with sterilized distilled water. Mercuric chloride solution at a concentration of 0.1% was also effectively used to sterilize the surface of seeds of *Brassica* (Goswami *et al.* 2020).

In this investigation, *in vitro* plant regeneration was tried with the intervention of callus. The induction and proliferation of callus was carried out in MS medium supplemented with various concentrations of 2, 4-D. It was observed that maximum callus induction was obtained with the increased amount of 2, 4 -D. Callus initiation was found to start after 7-8 days of transferring the dehusked embryo or caryopsis to culture medium containing the hormonal supplements**.** The color of all callus masses was light yellow and friable in texture (Fig 1a). It was noticed that MS media with 2.0 mg/l 2, 4-D as well as 3.0 mg/l 2,4-D produced 100% callus formation (Table 1). The MS medium with 0.5 mg/l 2, 4-D failed to produce callus (Table 1). Callus culture is significantly important in plant biotechnological studies, particularly in the case of rice. *In vitro* responses of different explants have been reported by Ullah *et al.* 2007, Khaleda and Forkan 2006, Carsono and Yoshida 2006. The results obtained from the study demonstrated that MS medium with different concentrations and combinations of 2, 4-D was effective in producing callus. This result is in agreement with that of the findings of Sikder *et al.* 2006, Bano *et al.* 2005.

Table 1. **The effect of different concentrations of 2, 4-D on callus induction in Doairgura.**

2,4-D (mg/l)	Number of explants (seeds) inoculated	Number of explants responded to callus induction	% of mean number of callus forming explants	Days to callus formation
0.5	60	-	-	-
1.0	60	43	69.99 ± 1.36	10-12
2.0	60	60	100 ± 1	7- 8
3.0	60	60	100 ±1.52	7- 8

The MS medium supplemented with different concentrations and combinations of BAP, Kn and NAA was employed to examine their effect on shoot development from the promising callus of Doairgura. The results of these observations are presented in Table 2. The best regeneration response was observed on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA in Doairgura. The colour and diameter of the callus are presented in Table 3. More or less, 2-5 days were required to induce regeneration through callus formation (Fig. 1b). Numerous shoot buds were initiated from the induced calli within 4-5 days of culture (Fig. 1c). The best mean number of shoots in this case (15.3 shoots/callus) was found on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA (Table 2). Moreover, shoot multiplication, elongation and proper development were observed in the same medium (Fig. 1d, e, f and g). Regenerated shoots were sub cultured on the same medium for their multiplication and elongation at regular intervals of 14-15 days.

The efficiency of regeneration of shoots from calli was mainly dependent on the callus size transferred to the regeneration media. In the present study, optimum efficiency was obtained when calli were 3.0 mm in diameter. The necessity of a minimal size for producing shoots may be related to the generation of hormonal gradients in the callus body from the auxins and cytokinins provided in the medium. Martinez-Trujillo *et al.* (2004) achieved good regeneration efficiency

using 3-4 mm callus. The highest average number of shoots (15.3) per callus was obtained from the size of 3.0 mm calli in Doairgura variety. Marassi *et al.* (1996) found the best shoot responses on MS media supplemented with 0.1 or 1.0 mg/l NAA and 1.0 or 1.5 mg/l BA. Sharma *et al.* (2008) observed better shoot bud regeneration on MS medium supplemented with 100 mg/l adenine sulfate, 2.0 mg/l BAP and 1.0 mg/l NAA. Rashid (2006) has reported higher frequencies of regeneration for Basmati varieties with 1.0 mg/l NAA and 5.0 mg/l BAP.

Table 2. Effects of different combinations of BAP and NAA in MS medium on regeneration of multiple shoots from the callus of Doairgura.

Hormonal combinations			No. of callus inoculated	No.of responsive callus	Days to shoot initiation from callus	Mean no. of shoots/callus after 30 days of inoculation
BAP	NAA	Kn				
0.5	0.5	0.5	60	24	4-5	6.7
1.0	1.0	-	60	38	4-5	4.6
1.5	1.0	0.5	60	52	3-5	5.5
0.5	-	0.5	60	29	4-5	2.7
1.0	1.0	-	60	56	2-3	5.8
1.0	1.0	1.0	60	51	3-5	3.2
2.0	**1.0**	**-**	**60**	**60**	**2-4**	**15.3**
2.0		1.0	60	50	3-5	10.2

Table 3. Callus colour and diameter of Doairgura variety.

Hormonal combinations			Callus diameter	Callus colour
BAP	NAA	Kn		
0.5	0.5	0.5	2-3 mm	White creamy colour
1.0	1.0	-	1-3 mm	White colour
1.5	1.0	0.5	1-2 mm	Light yellow colour
0.5	-	0.5	1-1.5 mm	Light yellow colour
1.0	1.0	-	1.5-2.5 mm	Light yellow colour
1.0	1.0	1.0	2.5-3 mm	White creamy colour
2.0	1.0	-	2-3 mm	Light yellow colour
2.0		1.0	1.5-2.5 mm	White colour

Induced root growth is an essential part of the successful development of plantlets after successful regeneration of shoots spontenuous root formation was also observed (Fig. 1f and g) in regeneration media (MS with 2.0mg/l BAP and 1mg/l NAA). For root induction, regenerated shoots were cultured on hormone-free MS medium (Fig. 1h and i). Many researchers (Goswami *et al.* 2020, Banu *et al*. 2017) also report similar findings for root induction in *Brassica* and *Gynura* respectively. After sufficient development of roots, the plantlets obtained from the Doairgura variety of aromatic rice were successfully transplanted into small plastic pots (Fig. 1j and k).

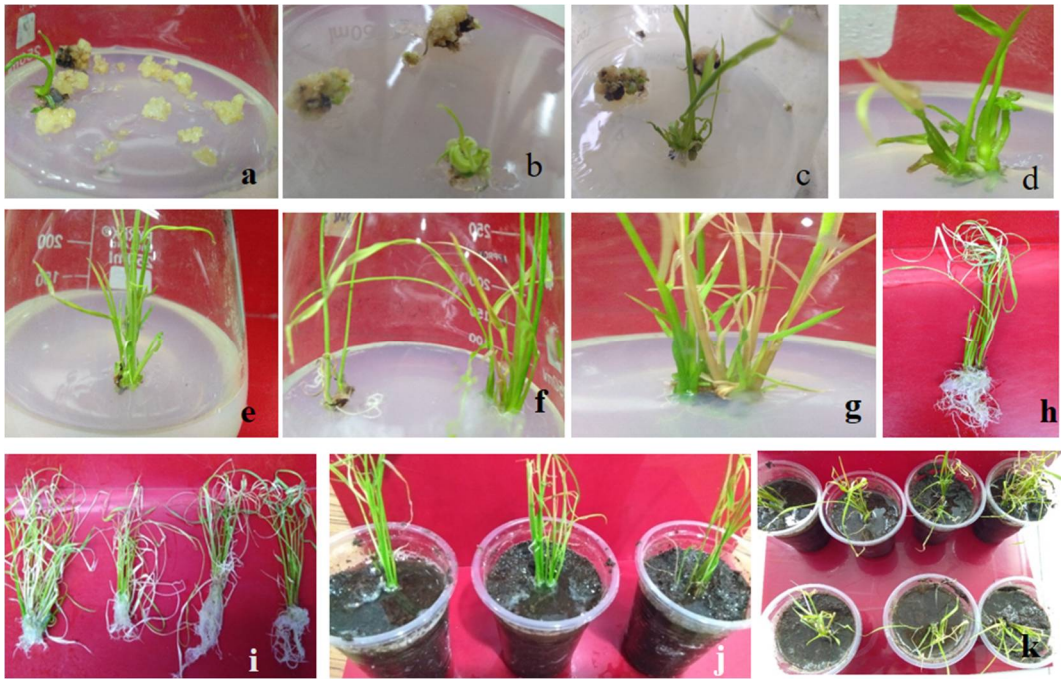

Fig. 1 (a-k): Different stages of *in vitro* regeneration of *O. sativa* var. Doairgura. a) Formation of callus from dehusked seed explants of Doairgura on MS medium supplemented with 2.0 mg/l 2,4-D; b) Initiation of shoots from callus of Doairgura on MS medium supplemented with 2.0 mg/l BAP and 1 mg/l NAA, c) formation of Multiple shoots from callus in Doairgura on MS medium supplemented with 2.0 mg/l BAP and 1mg/l NAA, d) Multiple shoots of Doairgura on the same media as mentioned fig. 1c; e) Elongated shoots of Doairgura variety on the above mentioned media, f&g) Spontaneous root formation on shoot regeneration medium (2.0 mg/l BAP and 1.0 mg/l NAA) in Doairgura variety, h&i. Root formation of *in vitro* regenerated shoots of Doairgura variety on hormone free MS medium, j&k. Hardening of *in vitro* regenerated plantlets in plastic pots.

It may be concluded here that the *in vitro* regeneration protocol developed for the aromatic rice variety of Bangladesh through callus culture is reproducible. This protocol is relatively simple and can be exploited in future biotechnological investigations, particularly for genetic transformation studies. This protocol will also help for the conservation of the aromatic rice variety Doairgura in Bangladesh.

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