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Callus Induction and *in vitro* Plant Regeneration of Rice (*Oryza sativa* L.) Under Various Conditions

Muhammad Tariq, Gowher Ali, Fazal Hadi, Shakeel Ahmad, Nasir Ali and Aftab Ali Shah
Department of Biotechnology, University of Malakand, Chakdara, NWFP., Pakistan

Abstract: The objective of the present study was to develop an effective protocol for optimum callus induction and complete plant regeneration for four varieties of rice (*Oryza sativa* L.) i.e., Super Basmati, Basmati-370, Basmati-371 and Fakhre Malakand. Calli were induced from mature seed scutelum. The Murashige and Skoog (MS) and Chu's N6 media containing hormone 2, 4-D (2, 4-Dichlorophenoxy acetic acid) in different concentrations were used for callus induction. Fakhre Malakand produced maximum calli on N6 media containing 3 mg L^{-1} 2,4-D. while other three varieties showed maximum callus induction on N6 media containing 2.5 mg L^{-1} 2,4-D. N6 media was found better than MS media for callus induction. For complete plant regeneration the calli of two varieties i.e., Basmati-370 and Basmati-371 were plated on N6 media containing different concentrations of NAA (1-Naphthalene acetic acid) and BAP (6-benzyl aminopurine). The maximum regeneration frequency (%) was observed on N6 media containing NAA 1 mg L^{-1} and BAP 2.5 mg L^{-1} . It took 27-30 days for the callus to regenerate into a complete plant. Basmati-370 produced 4-7 plantlets per callus whereas Basmati-371 produced 4-8 plantlets per callus with regeneration frequencies of 61 and 69%, respectively.

Key words: *Oryza sativa*, mature seed scutelum, callus induction, plant regeneration, 6-benzyl aminopurine, 1-naphthalene acetic acid

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important food crop in the world and feeds over half of the global population (Sasaki, 2005). In Asia where it covers half of the arable land used for agriculture in many countries (Cantrell and Hettel, 2004). About 84% of rice production growth has been attributed to the use of modern technologies (Maclean *et al.*, 2002). Rice production needs increase to meet the predicted demands of increasing population. One option is to increase the area under rice cultivation, which is getting harder as more farm areas are being converted to residential areas in the developing world. The most viable option, therefore, is to increase productivity by advances in biotechnology. Further, being the staple food for most of the developing world, the nutritional improvement of rice can also help to reduce malnutrition (Bajaj and Mohanty, 2005). During the last decade, tremendous progress has been made in the area of plant biotechnology (cell, tissue and organ culture). *In vitro* techniques constitute an important component of biotechnology and have the potential not only to improve the existing cultivars, but also for the synthesis of novel plants and early release of high-yielding plants resistant

to various diseases, pests, stresses and temperature. The successful application of plant tissue culture techniques for crop improvement requires suitable plant regeneration methods. The ability of plant regeneration from seed-derived callus of rice is influenced not only by physiological factors but also by genotypes. Among these factors, the genotype of plants is a strong determinant of the regeneration ability from seed callus and this character is under genetic control (Henry *et al.*, 1994). Several genetic studies have been performed to improve the regeneration ability from seed derived calli in rice. However, the use of tissue culture in rice improvement is limited, since the regeneration can be obtained only in limited number of genotypes (Taguchi-Shiobara *et al.*, 1997). Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies. Studies related to the *in vitro* culture of plants require a basic protocol for callus induction and subsequent plant regeneration. Tissue

culture of dicots is simple as compared to monocots (Reinert and Bajaj, 1976). The regeneration of whole plant is possible today from cereal species; such as bread wheat (Redway *et al.*, 1990; Vasil *et al.*, 1990), maize (Duncan *et al.*, 1985), rice (Yamada *et al.*, 1986) and barley (Luhers and Lorz, 1987). In rice, which is one of the most important food crop in the world (Sasaki, 2005). However, the use of tissue culture in rice improvement is limited, since the regeneration can be obtained only in limited number of genotypes (Taguchi-Shiobara *et al.*, 1997). Objectives of the present study was to determine the most suitable concentrations and combination of growth regulators for improvement in calli induction and *in vitro* complete plant regeneration efficiency in different varieties of rice which is essential for gene transformation in Rice varieties.

MATERIALS AND METHODS

Experimental studies were conducted on four varieties of rice (*Oryza sativa* L.), Super Basmati, Basmati-370, Basmati-371 and Fakhre Malakand taken from National Agriculture Research Centre (NARC) Islamabad. This research work was carried out in department of biotechnology university of Malakand in 2006. All of these varieties were used for callus induction study. Two varieties, Basmati-370 and Basmati-371 were subjected to regeneration experiments. The dehusked seeds were surface sterilized using 70% (v/v) ethanol for 1 min and 0.1% (w/v) mercuric chloride (HgCl₂) for 4 min. The seeds were then washed three times with sterilized distilled water (Ramesh and Gupta, 2005). The calli were induced from mature seed scutellum on agar solidified MS (Murashige and Skoog, 1962) and Chu's N6 (Chu *et al.*, 1975) media containing basal salts, 3% sucrose (30 g L⁻¹) as a source of carbon, 0.6% (6 g L⁻¹) agar (Difco) and different concentrations (2.0, 2.5 and 3.0 mg L⁻¹) of 2,4-D (2,4-Dichlorophenoxy acetic acid). The pH of the media was adjusted to 5.8 with 1N NaOH and 1N HCl using electronic pH indicator. The media was autoclaved at a temperature of 121°C and pressure of 15 lbs psi for 20 min. The medium-filled tubes were kept in laminar airflow cabinet and were subjected to UV light for 20 min. All tissue culture work was carried out in a sterilized environment in a laminar airflow cabinet. The seeds were inoculated in Lamina flow cabinet and then transferred to growth room. The cultures were kept in controlled environment at 23±2°C and 16-8 h's light/dark regime under fluorescent light. Due to the best calli formed in Basmati-370 and Basmati-371 in about 3 weeks (21-24 days) on Chu's N6 media, they were regenerated on Chu's N6 (Chu *et al.*, 1975) medium modified with different concentrations of NAA: BAP (1:2, 1:2.5 and 1:3 mg L⁻¹) The combination of

these hormones produced the shoots and roots on the same media thereby saving time as well as labor. The frequencies of the callus induction and regeneration were determined as the percentage of explants producing callus and the percentage of calli producing fully regenerated plants, respectively.

$$\text{CIF (\%)} = \frac{\text{No. of explants producing callus}}{\text{No. of explants plated}} \times 100$$

$$\text{PRF (\%)} = \frac{\text{No. of calli regenerated plantlets}}{\text{No. of calli plated for regeneration}} \times 100$$

Where:

CIF = Callus Induction Frequency

PRF = Plant Regeneration Frequency

RESULTS

Experiments for callus induction and regeneration in rice (*Oryza Sativa* L.) was conducted in four varieties, Super basmati, Basmati-370, Basmati-371 and Fakhre Malakand. Callus was invariably developed from the scutellar region of the seeds and was visible within 7-10 days. MS (Murashige and Skoog, 1962) media and N6 (Chu *et al.*, 1975) media were used for callus induction in mature seeds. Three different concentrations (2, 2.5 and 3 mg L⁻¹) of 2,4-D (2,4-Dichlorophenoxy acetic acid) were used to induce callus in the mature seeds. The response of the explants to different concentrations of 2,4-D in terms of callus induction and the percentage of explants producing calli were calculated for all varieties and is shown Table 1. The overall frequency (%) of callus induction on Chu's N6 media was found better than that on MS media, showing the superiority of N6 media over the MS media for the induction of callus as shown in Table 1. The callus induction frequency was maximum for Basmati-370 (75%), followed by Basmati-371 (73%), Super Basmati (60%) and lowest for Fakhre Malakand (40%). The calli were also compared for their mean weights on MS and N6 media containing different concentrations of 2,4-D as shown in Table 1. Three varieties Super Basmati, Basmati-370 and Basmati-371 showed the mean weights (0.22, 0.27 and 0.25 g, respectively) on media containing 2.5 mg L⁻¹ 2, 4-D whereas Fakhre Malakand gave its highest mean weight (0.26 g) on 3 mg L⁻¹. The two varieties i.e., Basmati-370 and Basmati-371 were further studied for shoots and roots regeneration due to their excellent callus forming ability on N6 media. Single concentration (1 mg L⁻¹) of hormone NAA (1-Naphthalene acetic acid) and variable concentrations (2, 2.5 and 3 mg L⁻¹) of BAP (6-benzyl

Table 1: The frequency of callus induction, mean weight of callus, regeneration frequency of different varieties of rice (*Oryza sativa* L.) on MS and N6 media containing different hormonal concentrations

Varieties	Frequency of callus induction (%)		Mean weight of callus (g) on given concentrations (mg L ⁻¹) of 2, 4-D			Regeneration frequency (%) on given conc. (mg L ⁻¹) of NAA: BAP		
	On MS media	On N6 media	2.0	2.5	3.0	1:2	1:2.5	1:3
			(mg L ⁻¹)			(%)		
Super Basmati	33.00	60.00	0.19	0.22	0.20	-	-	-
Fakhre Malakand	20.00	40.00	0.17	0.21	0.26	-	-	-
Basmati-370	23.00	75.00	0.20	0.27	0.21	5	61	15
Basmati-371	27.00	73.00	0.19	0.25	0.20	10	69	30

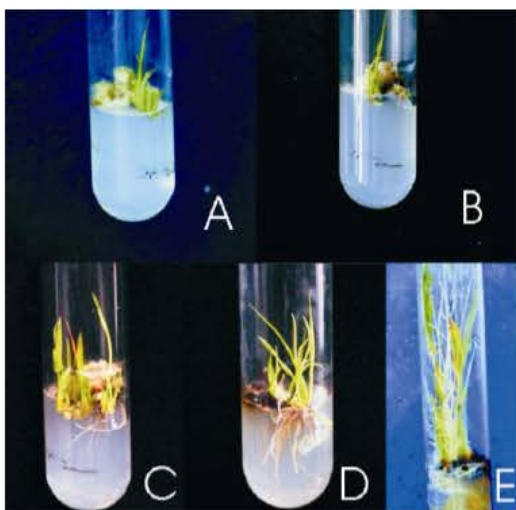


Fig. 1: (A) 18 days shoots of Basmati-370, (B) 18 days shoots of Basmati-371, (C) 27 days rooted shoots of Basmati-370, (D) 27 days rooted shoots of Basmati-371 and (E) Fully regenerated shoots with well developed roots

aminopurine) were used in combination for regeneration on N6 media. The data obtained from regeneration study is given in Table 1. The maximum regeneration frequency was found on media containing 1 mg L⁻¹ NAA and 2.5 mg L⁻¹ BAP but slight difference was found between the regeneration frequencies of the two varieties (61% for Basmati-370 and 69% for Basmati-371) (Fig. 1A and B). The number of plantlets produced per callus was also determined. Basmati-370 produced 4-7 plantlets per callus (Fig. 1C). Whereas Basmati-371 produced 4-8 plantlets per callus (Fig. 1D).

DISCUSSION

In present study the efficiency of MS (Murashige and Skoog, 1962) and N6 (Chu *et al.*, 1975) media containing different concentrations of 2,4-D (2,4-Dichlorophenoxy acetic acid) was tested for callus induction in four varieties of *Oryza sativa* L. Super Basmati, Basmati-370,

Basmati-371 and Fakhre Malakand. *In vitro* complete plant regeneration was investigated on N6 media containing different concentration of NAA (Naphthalene acetic acid) and BAP (Benzyl aminopurine) in two varieties, Basmati-370 and Basmati-371. The results showed that the incidence of contamination was very low (2-3%) during whole experiment. All the varieties were inoculated for their callus induction ability. The callus was induced successfully in all varieties and it was according Rashid *et al.* (2004) and Rashid *et al.* (2001) reporting that callus can be induced and grown on MS and N6 media. Our results showed that N6 media was better than MS media with callus induction frequency in all the four varieties of rice, Super Basmati showed 33%, Fakhre Malakand 20%, Basmati-370 showed 23% and Basmati-371 27% callus induction frequency on MS media as compared to 60, 40, 75 and 73%, respectively on N6 media. It is, perhaps due to the reason that N6 media contained more nitrogen than the MS media (Rashid *et al.*, 2004). These results are contradictory to the findings of Khatoon *et al.* (2003) who reported that MS was better than N6 media; however, they modified their media with 2 mg L⁻¹ 2, 4-D, 1 mg L⁻¹ NAA and 3 mg L⁻¹ casamino acid. Here, we used only 2, 4-D for callus induction. The difference in the composition of culture medium can result in variation in callus induction (Torbet *et al.*, 1998). Furthermore, genotype variation also plays its role (Khanna and Raina, 1998). Present results are similar to the findings of Rashid *et al.* (2004, 2001) they used Super basmati. Mandal and Gupta (1995) reported that MS media is better than N6; however, they used anthers as explants whereas in the present study callus was induced invariably from mature seed scutelum. The concentrations of hormones also affect the callus induction ability of plant cultivars. Previous studies showed that callus can be induced in rice using 2, 4-D singly (Katiyar *et al.*, 1999; Zhenyu *et al.*, 1999). Therefore, in the present study this growth regulator was used singly in different concentrations in MS as well as in N6 media, response of the four genotypes was different at different concentrations of 2, 4-D. Three varieties Super Basmati, Basmati-370 and Basmati-371 gave their highest mean weights (0.22, 0.27 and 0.25 g, respectively) on N6 media containing 2.5 mg L⁻¹ 2, 4-D whereas Fakhre Malakand gave its highest mean (0.26 g) on N6 media containing

3 mg L⁻¹ 2, 4-D. The mean weights of the first three varieties decreased on increasing the concentration of 2, 4-D whereas Fakhre Malakand showed an increase in its mean weight with increasing concentration of 2, 4-D i.e., 0.17 g on 2.0 mg L⁻¹, 0.21 g on 2.5 mg L⁻¹, 0.26 g on 3.0 mg L⁻¹. Therefore, Fakhre Malakand needs to be further tested on increased concentrations of 2, 4-D to find out the optimum concentration of the hormone for maximum callus induction. On transferring the calli to regeneration media, green spots becomes visible on the calli within 3-4 days. After 27-30 days fully regenerated rooted shoots were observed. Basmati-370 produced 4-7 plantlets per callus whereas Basmati-371 produced 4-8 plantlets per callus. Saharan *et al.* (2004) reported 7-10 plantlets per callus; however, they used 48 h desiccated calli. The highest regeneration frequency for both the varieties (61% for Basmati-370 and 69% for Basmati-371) was obtained on N6 medium containing NAA (1-Naphthalene acetic acid) 1 mg L⁻¹ and BAP (6-Benzyl aminopurine) 2.5 mg L⁻¹. Present results are contradictory to those of Rashid *et al.* (2004) who reported higher frequencies of regeneration for Basmati varieties (81.60%) with NAA (1-Naphthalene acetic acid) 1 mg L⁻¹ and BAP (6-Benzyl aminopurine) 5 mg L⁻¹. They used MS media containing high concentrations of sorbitol (the osmoticum) which enhances the regeneration frequency (Higuchi and Maeda, 1991). From the present study, it could be concluded that callus induction frequency was found better on N6 media for all varieties while Fakhre Malakand variety showed increase in callus mean weight with increase in 2, 4-D concentration, therefore further investigation should be carried out to find out the optimum concentration of the 2,4-D for maximum callus induction in Fakhre Malakand. Further, Basmati-370 and Basmati-371 were tested for their ability to regenerate in to complete plant. Regeneration rate for both varieties was found maximum on N6 media containing ratio of NAA 1 mg L⁻¹: BAP 2.5 mg L⁻¹. It took 27-30 days for the callus to regenerate into a complete plant.

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