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PROTOCOL OPTIMIZATION FOR EFFICIENT CALLUS INDUCTION AND REGENERATION IN THREE PAKISTANI RICE CULTIVARS

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Abstract

This study was undertaken to standardize an efficient and effective protocol for callus induction, subsequent growth and regeneration in three Pakistani rice (*Oryza sativa* L.) varieties viz., GNY-53, Basmati-370 and JP-5. MS and N6 media were used for callus induction. Overall, MS medium was found better for callus induction as compared to N6 medium. The growth regulator 2,4-D with varying concentrations (1-3mg/l) were tested for their callus induction and subsequent growth. GNY-53 (83%) and JP-5 (96.66%) showed maximum callus induction frequency on MS medium supplemented with 3mg/l 2,4-D while Basmati-370 (99%) showed higher callus induction frequency on the same medium supplemented with 1mg/l 2,4-D. The callus growth frequencies for varieties GNY-53 (60%) and Basmati-370 (94.73%) were best achieved on MS medium supplemented with 3mg/l 2,4D while JP-5 (73.68%) showed maximum callus growth frequency on the same medium with 1mg/l 2,4-D concentration. Mean square value for variety, treatment and their interactions were recorded for callus induction and growth on both MS and N6 media at probability level of $p \leq 0.05$. The regeneration efficiency of these varieties were tested alone on MS medium fortified with two different combinations of NAA and BAP (1mg/l NAA: 2mg/l BAP and 1mg/l NAA:4mg/l BAP). The two varieties GNY-53 (70.27%) and JP-5 (41.81%) showed maximum plantlets formation frequency on 1:2mg/l combination of NAA and BAP whereas Basmati-370 (43.33%) showed higher plantlets formation frequency on 1:4mg/l combination of NAA and BAP. Mean square value for variety, treatment and their interactions were also recorded for regeneration efficiency at probability level of $p \leq 0.05$ which showed that these entire factors significantly affect plantlets formation frequency.

Introduction

Rice (*Oryza sativa* L.) is one of the most versatile and important cereal crops of Poaceae family cultivated for more than 10,000 years (Sasaki, 2005). Currently this crop supports more than 50% of the world population (Christou, 1997). Rice consumers are increasing at the rate of 1.8% every year. But the rate of growth in rice production has slowed down. It is estimated that rice production has to be increased 50% by 2025 (Khush & Virk, 2000). A considerable improvement has already been made by exploiting the natural variation through conventional breeding. Albeit the success made in the last century, traditional breeding efforts alone can not meet the increasing demand of rice consumers in the 21st century. Therefore, at present various tissue culture techniques are being used for the genetic improvement of rice plant throughout the world (Raina, 1989).

Recent advancement in biotechnology, such as transformation and *In situ* and *In vitro* hybridization has enhanced the introgression of new genes from different sources to the cultivated species. However, the aforementioned methods are effective for rice when

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an efficient and reproducible regeneration system is available. Therefore, identification of useful genotypes for callus growth and successful *In vitro* plant regeneration is a prerequisite for such genetic improvement of rice (Hoque & Mansfield 2004). Callus induction as well as regeneration potential is affected not only by genotype and the type of explant but also by the composition of the culture medium including plant growth regulators and by the culture conditions (Rueb *et al*., 1994).

Materials and Methods

Three rice varieties viz., Basmati-370, JP-5 and GNY-53 were used in the present study for callus induction, proliferation and regeneration. The germplasm were kindly provided by Agriculture Research Centre, Swat and National Agricultural Research Centre (NARC), Islamabad, Pakistan.

Mature dehusked rice seeds were taken as source of explant. MS (Murashige & Skoog, 1962) and N6 medium (Chu *et al*., 1975) with varying concentrations of plant growth regulator 2,4-D (2,4-Dichlorophenoxyacetic acid) were used for callus induction whereas MS medium with varying ratios of NAA (Nephthalene acetic acid) and BAP (Benzyl aminopurine) in combination was used for plantlets regeneration These media were fortified with 3% sucrose (30g/l) as a source of carbon (organic matter), required concentration of growth regulators and 0.6% (6g/l) agar, the gelling agent. The sterilization step was carried out at 121°C for 20 minutes at 15 psi. Surface sterilization of seeds explant was done with chlorax (50%) for 20 minutes and then washed with sterilized distilled water 4-5 times to remove all the traces of chlorax.

Callus induction was carried out by transferring the surface sterilized dried seeds into the culture tubes containing the media and was kept under 16 hrs light and 8 hrs dark conditions at 25°C for three weeks (21-24 days). After the incubation period embryogenic calli were obtained and were maintained on proliferation medium. After 3-4 weeks of inoculation on proliferation medium, the calli were transferred to regeneration medium and were kept in growth room under 16 hrs light and 8 hrs dark photoperiod at 25 °C for about 3-4 weeks (27-30 days).

The response of 3 varieties of rice was determined in terms of callus induction, callus growth and regeneration frequencies. The data was subjected to ANOVA (Analysis of Variance) testing and the mean values were separated by least significance difference (LSD) by using standard statistical MSTAT-C software.

Results

Callus induction: Callus induction of dehusked rice seeds of three varieties viz., (GNY-53, JP-5 and Bas-370) was carried out on two types of media (MS and N6) fortified with three different concentrations of growth regulator 2,4-D (1mg/l, 2mg/l and 3mg/l). Calli were developed within 10 days of inoculation. Both types of media gave better response to callus induction ability of the three genotypes but MS media in general was found to be superior in its callus induction ability in all the three varieties. After 2-weeks of inoculation, the callus induction frequency was calculated as percentage of explants producing calli (Table 1).

Table 2. Mean square of callus induction for three varieties of *Oryza sativa* L. (GNY-53, JP-5 and Basmati-370) on MS and N-6 medium with three different concentrations of 2, 4-D (1mg/l, 2mg/l and 3mg/l).

	Callus induction on MS-medium	Callus induction on N-6 medium
Factor A	1210.47**	368.64**
Factor B	143.54^{ns}	1801.90**
Factor AB	295.74**	1061.34**
Error	**44.56**	**1.84**
CV (%)	7.80	1.383
Variety 1	72.39b	66.92c
Variety 2	89.98a	76.79b
Variety 3	94.24a	78.92a
LSD	**6.67**	**1.3**
Treatment 1	83.67	58.17c
Treatment 2	82.85	84.92a
Treatment 3	90.14	79.54b
LSD		**1.35**

*= Significant, **= Highly significant, ns= Non significant, CV= Coefficient of variation, Factor A= Variety, Factor B= Treatment, Factor AB= Interaction of variety and treatment on two growth medium (MS and N-6)
Values repeated with same letters are not significantly different at $\alpha \leq 0.05$.
Variety 1= GNY-53, Variety 2= JP-5, Variety 3= Basmati-370, LSD= Least significant differences
Treatment 1= 1mg/l 2, 4-D, Treatment 2= 2mg/l 2, 4-D, Treatment 3= 3mg/l 2, 4-D

Means square values of factor A (variety), factor B (treatment) and AB (interaction of A x B) on MS medium showed that factor A (variety) is highly significant (1210.47), factor B (treatment) showed non significant (143.54) results and the interaction of the two factors (variety × treatment) showed highly significant variation (295.74) at $\alpha \leq 0.05$ probability level. Mean square values of factor A (variety), factor B (treatment) and their interaction (AB) on N-6 medium supplemented with different concentrations of 2, 4-D (1mg/l, 2mg/l and 3mg/l) showed highly significant variations (368.64, 1801.90 and 1061.34) at probability level $\alpha \leq 0.05$ (Table 2).

Callus growth: After 3-4 weeks of inoculation, the calli showed maximum growth response (Table 3). Mean square values of callus growth for three different varieties of rice GNY-53, JP-5 and Basmati-370 showed highly significant variations for variety (2110.93), treatment (268.74) and their interaction (106.50) on MS-medium and variety (3729.93), treatment (1108.93) and their interaction (595.02) on N-6 medium supplemented with different concentrations of 2,4-D (1mg/l, 2mg/l and 3mg/l) at probability level $\alpha \leq 0.05$. Mean values of callus growth at probability level $\alpha \leq 0.05$ for the three varieties on MS and N6 media are given in (Table 4).

Table 4. Means square of callus growth on MS and N-6 medium for three different varieties of *Oryza sativa* L. (GNY-53, JP-5 and Basmati-370) using three different concentrations of 2, 4-D (1mg/l, 2mg/l and 3mg/l).

	Callus induction on MS-medium	Callus induction on N-6 medium
Factor A	2110.93**	3729.93**
Factor B	268.74**	1108.93**
Factor AB	106.50**	595.03**
Error	**4.36**	**4.50**
CV (%)	3.03	3.87
Variety 1	55.71c	34.97c
Variety 2	65.28b	53.69b
Variety 3	85.70a	75.62a
LSD	**2.08**	**2.12**
Treatment 1	70.46b	41.94b
Treatment 2	62.82c	60.97a
Treatment 3	73.41a	61.35a
LSD	**2.08**	**2.12**

*= Significant, **= Highly significant, ns= Non significant, CV= Coefficient of variation, Factor A= Variety, Factor B= Treatment, Factor AB= Interaction of variety and treatment on two growth medium (MS and N-6)

Values repeated with same letters are not significantly different at $\alpha \leq 0.05$.

Variety 1= GNY-53, Variety 2= JP-5, Variety 3= Basmati-370, LSD= Least significant differences

Treatment 1= 1mg/l 2, 4-D, Treatment 2= 2mg/l 2, 4-D, Treatment 3= 3mg/l 2, 4-D

Regeneration: Regeneration of the three rice varieties viz., GNY-53, JP-5 and Bas-370 was carried out on MS-medium fortified with two different combination of hormones NAA:BAP (1:2 and 1:4 mg/l). Plantlets formation frequency was calculated as percentage of green spots producing plantlets (Table 5). Green spots formation was higher was higher on 1:2 mg/l NAA:BAP combination as compared to 1:4 mg/l NAA:BAP in all the three varieties tested (JP-5=110, BAS-370=38 and GNY-53=37 green spots). Plantlets formation frequency for JP-5 (41.81%) and GNY-53 (70.27%) was higher on 1:2 mg/l NAA:BAP combination whereas BAS-370 (43.33%) produced its maximum value on 1:4 mg/l NAA:BAP combination.

Mean square values for regeneration efficiency of three different varieties of rice (*Oryza sativa* L.) on MS-medium supplemented with two different concentrations (1:2mg/l and 1:4mg/l) of BAP/NAA is given in Table 6. Results showed highly significant variation for variety (626.91), treatment (1002.18) and their interaction (913.89) at probability level $\alpha \leq 0.05$.

Discussion

Callus induction and growth: Mature dehusked rice seeds were used as an explants because calli initiated from scutellum of mature seeds of all rice varieties have high embryogenic potential (Ge *et al*., 2006, Khaleda & Al-Forkan, 2006) and was excellent material for transformation of rice by *Agrobacterium* (Rashid *et al*., 1996; Toki., 1997; Rashid *et al*., 2001, 2003; Cho *et al*., 2004 and Ge *et al*., 2006). Embryogenic calli obtained from mature seed explant have high regeneration capacity (Khalequzzaman *et al*., 2005).

Table 6. Means square of regeneration efficiency of three different varieties of rice (*Oryza sativa* L.) on MS-medium supplemented with two different concentrations (1:2mg/l and 1:4mg/l) of BAP/NAA (Benzyl aminopurine and Naphthalene acetic acid).

	Regeneration efficiency
Factor A	626.91**
Factor B	1002.18**
Factor AB	913.89**
Error	0.56
CV (%)	1.86
Variety 1	51.80a
Variety 2	37.45b
Variety 3	32.01c
LSD	0.968

*= Significant, **= Highly significant, ns= Non significant, CV= Coefficient of variation, Factor A= Variety, Factor B= Treatment, Factor AB= Interaction of variety and treatment on two growth medium (MS and N-6).
Values repeated with same letters are not significantly different at $\alpha \leq 0.05$.
Variety 1= GNY-53, Variety 2= JP-5, Variety 3= Basmati-370
LSD= Least significant differences

MS and N6 were the most commonly used basal media (Pandey *et al*., 1994, Rashid *et al*., 1996, Toki 1997). The results from our study revealed that all the three varieties gave better callus induction response on MS media (70%-100%) as compared to N6 media (40%-90%). These results are confirmatory to the finding of other researchers (Niroula *et al*., 2005, Islam *et al*., 2004, Azria & Bhalla, 2000). Our results are contradictory to earlier reports suggesting that callus induction on N6 medium is relatively more efficient (Gul *et al*., 2000; Naqvi *et al*., 2005).

To determine the optimum level of plant growth regulator, different concentrations of 2,4-D (1mg/l, 2mg/l and 3mg/l) were used in both MS and N6 media for callus induction and growth. Mostly 2,4-D has been used as the only growth regulator in callus induction media (Katiyar *et al*., 1999; Zhenyu *et al*., 1999). In the present study, the two varieties (GNY-53 and JP-5) showed better callus induction response on MS medium supplemented with 3mg/l 2,4-D. Other researchers (Khalequzzaman *et al*., 2005 and Islam *et al.,* 2005) found better callusing frequency at a concentration of 2.5 mg/l 2,4-D. Our results are contradictory to the findings of Naqvi *et a*l., (2005), Niroula *et al*., (2005) and Pandey *et al*., (1994). Whereas for Basmati-370, MS media supplemented with 1mg/l 2,4-D proved to be most beneficial for callus induction. On N6 media, the varieties GNY-53 and Basmati-370 showed higher callus induction frequency at 2mg/l 2,4-D concentrations whereas Basmati- 370 showed higher callus induction frequency on both 2mg/l and 3mg/l 2,4-D. These results are confirmatory to the findings of Ge *et al*., 2006, Rashid *et al*., (2001 and 2005). The response of the explants to different concentrations of 2,4-D in terms of callus induction was genotype dependent. These findings are in agreement with previous reports of other researchers (Khalequzzaman *et al*., 2005, Hoque & Mansfield 2004). The present investigation revealed that both genotype and media composition and their interaction largely affect on callus induction. This revelation is in agreement with the findings of Pandey *et al*., (1994) and Islam *et al*., (2005).

Regeneration: *In vitro* complete plant regeneration was investigated on MS medium supplemented with two combinations of growth regulators i.e., NAA and BAP (1:2mg/l and 1:4mg/l). Combinations of auxin and cytokinin along with the effect of basal salts played an important role for plant regeneration (Prodhan *et al*., 2001; Lee *et al*., 2002). It was observed that plant regeneration ability of plated calli depends on the genotypes and the callus induction media. After about a period of two weeks, the calli plated on regeneration medium produced green spots and at about the same time some of them became brown. This was supported by Hoque *et al*., (2004). Green spots were observed within 10 days of transfer of calli into regeneration medium (Khaleda & Al-Forkan, 2006). Highest regeneration frequency was produced by GNY-53, followed by Basmati-370 and then JP-5. It was supported by Hoque *et al*., (2004), who found that genotypic differences strongly influence on plant regeneration potential. The two varieties GNY-53 and JP-5 showed higher regeneration frequencies at 1:2mg/l combination of NAA and BAP. Best regeneration response on MS media with same hormonal combination (1mg/l NAA and 2mg/l BAP) was also found by Agarwal *et al.,* (2006) and Rachmawati & Anzai, 2006. Basmati-370 produces high regeneration frequency on hormonal combination of 1mg/l NAA and 4mg/l BAP. These results are contradictory to Rashid *et al*., (2003) who find high frequency of plant regeneration on MS media supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP. They used MS media with higher concentration of Sorbitol which may enhance the regeneration frequency (Higuchi & Maeda, 1991).

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