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EFFICIENT EMBRYOGENIC SYSTEM FROM TISSUE CULTURE OF MATURE EMBRYOS FOR SOME COARSE VARIETIES OF RICE (*ORYZA SATIVA* L.)

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Abstract

Five coarse cultivars of rice (*Oryza sativa* L.) viz., Pakhal, Swat-1, JP-5, KS-282 and IR-6 were evaluated for their ability to undergo somatic embryogenesis. Results indicated that N6 basal medium supplemented with 2 mg/L of 2, 4-D without BAP was the most appropriate for callus growth but inferior in its regeneration potential. Among all the genotype tested JP-5 showed highest 84 %) whereas IR-6 showed the lowest (63 %) callus induction frequency. (Regeneration rate was highest (82 %) on N6 basal media supplemented with 5 mg/L of BAP and 0.5 mg/L of IAA. All of the varieties showed a good regeneration potential on culturing, the highest for KS-282 and Pakhal while JP-5 showing the lowest regeneration compared with other genotypes studies. It was observed that sucrose supported regeneration better than sorbitol and mannitol. Calli on sucrose containing media showed higher regeneration and produced longer shoots whereas mannitol could not support the growth.

Introduction

The early success in the production of transgenic rice plants was reported in 1988 for Japonica varieties (Zhang & Wu, 1988) and in 1990 for an Indica variety (Peng *et al.*, 1990). However, with advances in rice genetics and recognition of rice as a model crop plant in addition to its importance as food, rice transformation is becoming a more routine exercise in laboratories involved in agricultural biotechnology.

The major obstacles to the genetic manipulation of many plant species are still due to a lack of an effective tissue culture system for the regeneration of whole plants from tissue parts after transformation. Most of the methods of biotechnology, presently used or envisaged, require the culture of cells and subsequent regeneration into a plant. Therefore, tissue culture must be considered a gateway which all forms of genetic engineering must pass through. Success in genetic engineering, therefore, relies heavily on the availability of efficient tissue culture protocols (Quraishi, 2001).

Gautheret (1934) laid the foundation of "tissue culture" studies by raising an indefinitely growing callus from cambium of trees. The callus is a rapidly proliferating and undifferentiated mass of cells, which can be obtained by culturing explants on a nutrient medium containing specific growth hormone. Callus is an abnormal growth with a potential to develop into normal roots, shoots or embryoid that can develop into plants. Efficient plant regeneration depends upon several factors including the composition of the culture media and especially the genotype of the plant. Some other factors that affect plant regeneration frequency from rice callus are concentration of gelling agents, osmoticum and specific combination of plant growth regulators (Tsukahara & Hirosawa, 1992).

The purpose of the present study was to compare the callus induction and regeneration capacity of different coarse cultivars of rice under different concentrations of various hormones (2-4D, BAP and IAA). Effect of different carbon sources (sucrose, sorbitol and mannitol) on regeneration was also studied. The study will make genetic engineering efforts possible for improving local rice cultivars and will help to develop a system for functional genomics. Through this system functions of cloned DNA sequences and putative regulatory elements may be assayed by over/under expression.

Materials and Methods

Seeds of 5 rice cultivars viz., IR-6, JP-5, KS-282, Pakhal and Swat-1 were provided by Rice Program, NARC, Islamabad in 2002 and were used as an explant source. Hulls were removed manually from mature and healthy seeds followed by surface sterilization with a liquid detergent (Lemon Max), rinsing off thrice under tap water and finally with distilled water. Seeds were treated with absolute ethanol for 1 minute under aseptic conditions in the laminar flow and dipped in commercial bleach for 15 minutes with continuous shaking. Three quick washes were given with autoclaved distilled water. Finally seeds were washed with distilled water for 5 minutes and placed on an autoclaved filter paper.

The N6 basal medium (Chu, 1978) supplemented with sucrose (30g/L), 2,4-dichloro-phenoxy acetic acid (2,4-D) @ 0, 1, 2 and 3mg/L along with benzylaminopurine (BAP) @ 0, 0.1, 0.2 and 0.3 mg/L were used for callus induction. The pH of the medium was adjusted to 5.80 and solidified with 2 g/L of Phytagel™ (Sigma®). The medium was then poured into test tubes and plugged with cotton, and autoclaved at 20 psi pressure for 15 minutes. One surface sterilized seed per test tube was cultured under aseptic conditions in a laminar flow cabinet. Cultures were incubated at 32 ± 2ºC with a light of approximately 2000 lux provided by general electric florescent tubes. Twelve to fourteen days old, healthy and well growing calli were cleared off any seed part/non-growing/brown part and used for regeneration.

N6 basal medium with different BAP/IAA compositions (either 2/0.2 mg/L or 5/0.5 mg/L) was used for regeneration. Sucrose (30g/L) was added and media were prepared as described previously for callus induction. Cultures were kept under similar environmental conditions as were used for callus induction. After three weeks of inoculation data regarding regeneration (number of green spots and number of shoots per combination) were recorded. After three weeks on regeneration media, parts of calli without any signs of regeneration were separated from regenerated parts, and were transferred to their respective medium combination. Different C-sources such as sucrose, sorbitol or mannitol @ 30 g/L were also added in the regeneration medium to monitor their effect on regeneration. All experiments were conducted at least twice and average of the results was used for data analysis.

Results and Discussion

Effect of genotype on callus induction: Five different rice genotypes were used for this study. Callus growth and callus induction frequencies apparently varied for all of the five rice genotypes tested (Fig. 1). IR6 showed the lowest growth potential, while JP-5, KS-282 and Pakhal were similar in their growth response. Statistical analysis however revealed that the observed differences in callus induction frequency were non-significant except for IR-6.

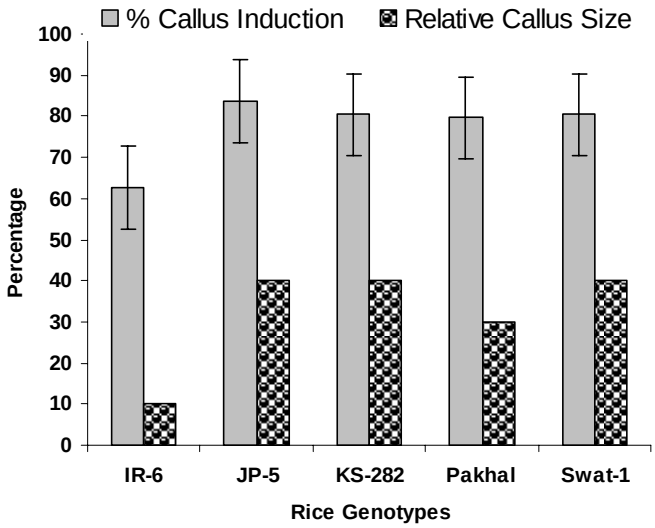

Fig. 1. Genotypic responses to callus induction and growth. The bars show mean ± LSD. Only IR-6 showed significant difference from other varieties

Callus induction frequency was the highest (81.5%) in KS-282 and significantly lower (65.9%) in IR-6. Apparently better callus induction was observed in medium containing 2 mg/L of 2,4-D without BAP but the differences were statistically insignificant (Fig. 1). A differential response of rice cultivar has also previously been reported for somatic tissue and anthers from different rice genotypes (Abbasi *et al.*, 2000; Karim *et al.*, 1991; Naqvi *et al.*, 1989).

Callus induction frequency was studied with four different combinations of plant growth regulators. Callusing was better with only 2,4-D alone. Inclusion of BAP alongwith 2,4-D in callus induction medium resulted in a decrease in callus growth but the difference was not significant (data not shown).

Regeneration: Regeneration through a tissue culture procedure means development of a whole plant in *In vitro* conditions (Burgess, 1989). There is a variety of growth regulators like Benzyl aminopurine, Kinetin, Zeatin, IAA and NAA etc., used for regeneration of a plant. IAA, a naturally occurring auxin, causes cell elongation, induction of cell division and maintenance of apical dominance, also plays a vital role in the plantlet development. BAP, a synthetic cytokinin, on the other hand is responsible for shoot development, shoot tip proliferation and cell division (Burgess, 1989). Frequency of *In vitro* morphogenesis has been reported to be influenced by genotypes and concentration of different phytohormones (Bell, 2003).

Two combination of regeneration medium were used in this study. Green spot (primordia) formation started after three days following the transfer of cultures to regeneration medium and the plantlet formation started after one week. After three weeks of subculturing the shoot tips started browning (Fig. 3). Shoot primordia appeared in the form of green spots on the calli indicating their regeneration potential.

Table 1. Table of Analysis of Variance showing the effect of genotype, callusing media, regeneration media and their interactions.

Source	Degree of freedom	Sum of squares	Mean square	F-value	Probability
Varieties	4	4985.627	1246.407	3.7725	0.0073
Callusing media	3	3369.323	1123.108	3.3993	0.0217
Regeneration media	1	13.879	13.879	0.042	
Interactions					
Variety: Callusing medium	12	8037.253	669.771	2.0272	0.0321
Variety: Regeneration medium	4	3240.097	810.024	2.4517	0.0526
Callusing: Regeneration medium	3	2214.262	738.087	2.2339	0.0906

Effect of genotype on regeneration potential: All the 5 genotypes were compared for their regeneration potential. Although the differences in callus induction frequency were not much evident except in IR6, the differences in regeneration were significant (Table 1). Among the different genotype studied, Pakhal and KS282 showed the highest regeneration efficiency, while JP5 and Swat-I were the lowest with the IR6 exhibiting an intermediate response. Interestingly the two varieties, the Pakhal and KS282 which showed a high regeneration potential have also performed better in callus induction.

Effect of callusing medium on regeneration potential of calli: The 2,4-D, a synthetic auxin, is not efficiently metabolized by plant tissues. (Ashton & Crafts, 1981; Naqvi *et al*., 1989). However, it is diluted due to increase in callus mass, but 2,4-D, once taken up by a callus may still have a carry over effect on subsequent morphogenesis. Khanna & Raina (1998) reported that regeneration percentage as well as the shoot bud induction frequency was influenced by rice genotype, callus induction medium, regeneration medium, interaction between genotype and two different compositions of regeneration medium as well as the interaction between the callus induction medium and regeneration medium. In order to test this hypothesis we have studied the effect of different callus media on regeneration potential in different genotypes of rice. Calli induced on four media with different concentrations of 2,4-D (with and without BAP) were subjected to two different regeneration media. The calli induced with 1mg/L of 2,4-D and 0.1 mg/L of BAP showed significantly higher regeneration (92.3%); while those induced on 3mg/L of 2,4-D and 0.3 mg/L of BAP showed lower regeneration. Interestingly, omitting BAP from callusing medium further decreased the subsequent regeneration potential. The interaction between the callus induction medium and the genotype was also significant. On comparison, both combinations of regeneration medium revealed no significant differences. The interactions either between varieties and regeneration medium or between callusing medium and regeneration medium were non-significant (Table 1).

Effect of sub-culturing of calli on regeneration: Calli were kept on the regeneration medium for about a month. In order to fully explore the regeneration potential, parts of these calli, apparently non embryogenic, were separated and subcultured on a similar fresh regeneration medium for another one month. Highest combined regeneration rate on all compositions was observed in IR-6 (89.3 %) followed by KS-282 (75 %), Pakhal (46.5 %), and much lower in Swat-1 (16.9 %) and JP-5 (13 %) as shown in Fig. 2. Valdez *et al*., (1997) reported that the ability to regenerate could be directly linked to genetic differences among Indica cultivars.

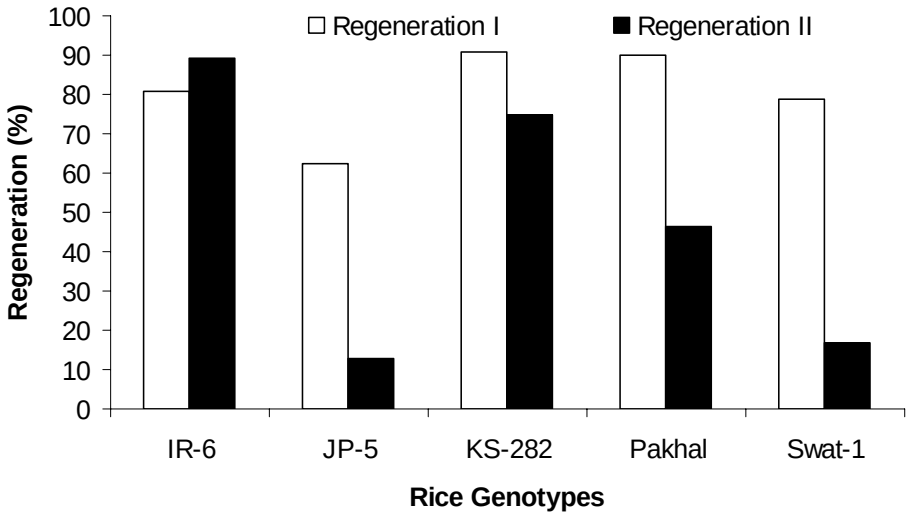

Fig. 2. Genotypic response during morphogenses in all media. Rengenerarion I: Regeneration from calli on first exposure to regeneration medium. Regeneration II: Undifferentiated calli separated from first exposure and cultured on similar fresh regeneration medium.

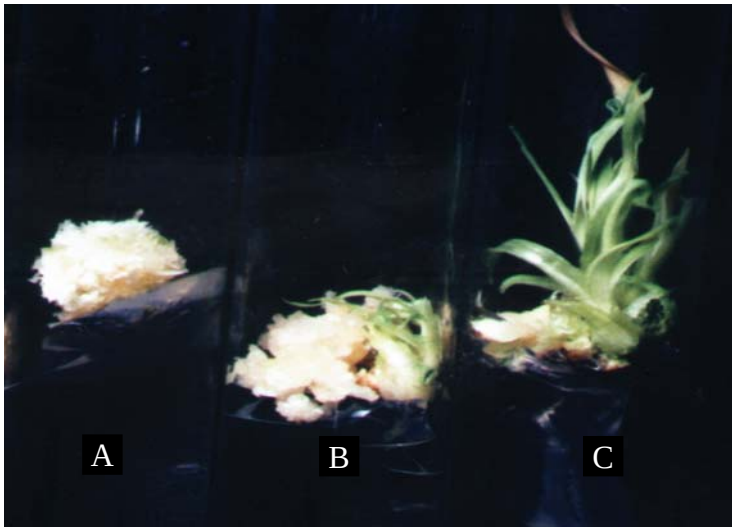

Fig. 3. Different stages of regeneration in KS-282 on N6 media with BAP (5 mg/L) and IAA (0.5 mg/L).

A=Three weeks old callus showing shoot primordial,
B= Shoot elongation from six weeks old callus,
C= Mature shoot from eight weeks old callus

Table 2. Regeneration responses to different carbon sources.
The values are average of three observations.

C-source	Rice genotypes	Regeneration-I				Regeneration-II			
		Response /genotype (%)	Response/ C-source (%)	Callus response (%age)		Response/ genotype (%)	Response/ C-source (%)	Callus response (%age)	
				Green spots	Shoots			Green spots	Shoots
Sucrose	KS- 282	86.2	80.6	24.0	76.0	80.0	56	0.0	100.0
	JP-5	75.0		41.66	58.33	40.0		66.66	33.33
Sorbitol	KS-282	57.1	54.4	83.33	16.66	33.33	50	100.0	0.0
	JP-5	51.7		86.66	13.33	62.0		87.5	12.5
Mannitol	KS-282	0.0	0.0	0.0	0.0	0.0	0	0.0	0.0
	JP-5	0.0		0.0	0.0	0.0		0.0	0.0

Effect of carbon source on regeneration: It is well known that carbohydrates do not function only as a carbon source in metabolism, but also play an important role in the regulation of osmotic potential. Klenovska (1973) reported that osmotic stress affects the callus growth, shoot regeneration, somatic embryogenesis and metabolism of specific compounds.

In this study the calli were induced on N6 media containing 30 g/L of sucrose. Fourteen days old healthy calli were transferred to the regeneration media containing 2 mg/L of BAP, 0.2 mg/L of IAA, and 30g/L of sucrose, sorbitol or mannitol. When sucrose was used @30g/L in medium, the highest combined regeneration response (80.6 %) was obtained (Table 1) with KS-282 (86.2 %) and JP-5 (75 %). Calli were healthy, whitish green or slightly pale and proliferative showing a high regeneration potential with green fleshy shoots. When these calli were sub-cultured on a fresh medium with sucrose as a carbon source for the second time, the highest regeneration (56 %) was again observed (Table 2) with KS-282 (80 %) better than JP-5 (40 %).

In sorbitol containing medium better regeneration was observed in KS-282 (57.1%), followed by JP-5 (51.7 %). Calli were healthy but smaller in size and less proliferative. Regeneration potential was observed in the form of green primordia but few calli developed the shoots. When the remaining calli were sub-cultured on a similar medium for second time, the results were nearly same with KS-282 showing less regeneration (33.3%) than JP-5 (62 %). Alkhayri *et al.,* (1996) also studied the effect of sucrose and sorbitol on callus induction and regeneration, and reported that medium containing sucrose supported the callus proliferation and addition of either sucrose or sorbitol improved regeneration potential.

No regeneration was observed in the medium containing mannitol. This may be due to the fact that it is very weakly metabolized, hence, not available as an energy source. Mannitol also causes osmotic stress. Huang *et al*., (1999) studied callus induction in Indica rice using sorbitol and mannitol. Their results showed that with 2 % and 4 % of sorbitol explants survived for more than 25 days, and regeneration rates of green plantlets were 52.6 % and 90 % respectively. With 2% or 4% of mannitol, the explants turned pale, gradually shrunk and dried out and lost their regenerative ability. In mannitol regeneration media callus size reduced and turned blackish with very loose and watery texture. No signs of regeneration were observed.

From these studies it may be concluded that either sorbitol or mannitol alone is not superior to sucrose for regeneration, however, one might study their effect in combination with sucrose.

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