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Effect of Age of Seedling and Phytohormones on Micropropagation of Indica Rice (*Oryza sativa* L.) from Meristem Culture.

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An efficient protocol for *in vitro* micropropagation of seven indica rice varieties was developed from meristem culture. Meristem (leaf base) was isolated from different age of seedlings and cultured on MS medium without hormones and supplemented with different concentrations of NAA and BAP. Regeneration of plantlets from meristem was observed within five days of culture. The meristem isolated from 4-day old seedlings gave highest regeneration on hormone free MS medium. Histological study of meristem (leaf base) from 4-day old seedlings confirmed the presence of meristematic cells. Regenerated plants were multiplied on MS medium supplemented with 0.05 mg/L NAA and 5 mg/L BAP. An average of five plants were obtained from single regenerated meristem. The plants regenerated from meristem showed morphological uniformity.

Keywords: *Oryza sativa* L., leaf base, meristem, plant regeneration, indica rice.

INTRODUCTION

In vitro tissue culture is an experimental approach for basic and applied research. Meristem culture has appeared as a distinct and independent technology in tissue culture for micropropagation of plant as well as disease elimination from many crops. In dicot plants, meristem present at the apex portion of growing shoot while in monocot, mostly in grasses, it lies among the organized tissues (Sautter *et al.*, 1995). Regeneration of plants from *in vitro* cultured cells is being applied for the improvement of economically important crops. Rice is one of the most important cereal and the staple food for more than two billion people, predominantly in developing countries (Anonymous, 1995). Cultivated rice is distinguished by two major subspecies, indica and japonica on the basis of morphophysiological variations and geographical distribution (Kato *et al.*, 1928; Glaszmann, 1987). The indica type accounts for 80% of the cultivated rice area in the world (Swaminathan, 1982). Indica rice showed poor response *in vitro* tissue culture as compare to japonica (Abe and Sasahara, 1982). Majority of work reported in the literature pertains to regeneration of indica rice from protoplast and callus through somatic embryogenesis (Lee *et al.*, 1989; Datta *et al.*, 1990; Jain *et al.*, 1996). Plant regeneration from protoplast and callus cultures is time consuming, genotype dependent (Abe and Futsuhara, 1986) and often associated with genetic and cytological variations (Abdullah *et al.*, 1989; Larkin and Scowcroft, 1981) which are not always desirable. The present investigation deals with *in vitro* micropropagation of seven indica rice varieties and evaluated the effect of seedling's age and phytohormones on meristem regeneration. This method of meristem culture holds oppertunities to regenerate plants in short duration and without somaclonal variations. The meristem (leaf base) can be used for genetic transformation of organized rice tissue with foreign genes using electroporation and biolistic gun techniques in order to get transgenic plants.

MATERIALS AND METHODS

Seeds of seven indica rice varieties Basmati 370, Super Basmati, Basmati Pak. IRRI6, Jhona, KS282 and BR11 were carefully dehusked and sterilized with 0.1% (w/v) $HgCl_2$ for 5 min followed by four washes with sterile distilled water. The seeds were germinated on MS (Murashige and Skoog, 1962)

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medium in dark at 25±2°C.

Isolation and Culturing of Meristem

Two leaf base pieces (2 mm) were isolated from 4-, 5- and 7-day old etiolated seedlings and cultured on MS full strength medium. The cultured leaf base pieces were kept at 25±2°C in 14 h light/10 h dark photoperiod. In order to investigate the effect of phytohormones, the leaf base pieces from 4-day old seedlings were cultured on MS medium supplemented with NAA and BAP (0.5-2.5 mg/L).

Meristem Regeneration and Shoot Multiplication

After four weeks the regenerated plantlets (20 cm) were shifted to MS medium supplemented with 0.05 mg/L NAA and 5 mg/L BAP for shoot multiplication. After two weeks the multiplied plants were carefully separated and washed with tap water to remove the agar from roots. The plants were kept into water for two days before shifting into soil. The seeds were harvested upon maturity and kept at 37°C for three days. The germination of seeds from regenerated plants was checked by growing them on MS medium.

Histological Procedures

The leaf base pieces (2 mm) isolated from 4- and 7-day old etiolated seedlings were fixed in formalin/acetic acid/alcohol for 24 h. After washing the material with different dilutions of alcohol it was dehydrated in tertiary butyl alcohol series and embeded in paraffin wax according to the procedure described by Johansen (1940). Serial sections were cut at 15 μm and mounted in Canada balsam.

RESULTS AND DISCUSSION

Attempts were made to regenerate multiple shoots from meristem of seven indica rice varieties commonly cultivated in South-East Asia. The effect of age of seedlings and phytohormones on meristem regeneration was evaluated. The meristem in rice lies at the base of growing leaf (Khanum *et al.*, 1996). In meristem culture experiment two pieces (2 mm) of leaf base were used and kept constant through out the study. After 4-days, initiation of root and shoot formation was observed in cultured leaf base pieces on MS medium without undergoing to callus formation. No visible difference was observed in the frequency of regeneration by keeping the explants in dark and night. The frequency of meristem regeneration was calculated by dividing the persentage of leaf base explant regenerated with total number of leaf base explants cultured. All varieties responded to regeneration at varying frequency. The highest regeneration in the range of 31-55% was observed from leaf bases isolated from 4-day old seedlings. The variety IRRI6 gave highest regeneration (55%) followed by Basmati 370 (51%). The frequency of meristem regeneration reduced with the increasing age of seedling (Table 1). It was almost impossible to isolate meristem from 3-day old seedlings and before. The average value of regeneration in all varieties was highest (42.5%) when meristem was isolated from 4-day old seedlings. The regeneration frequency decreased upto 38.4% and 18.4% when meristem was isolated from 5- and 7- day old seedlings respectively. No regeneration was observed when meristem was isolated from more than 8-day old seedlings. The decline in meristem regeneration was maybe due to the loss of meristematic activity with the increasing age of seedlings. After keeping the regenerated plantlets on MS medium supplemented with different combinations of NAA and BAP the effective concentration of phytohormones was selected. The best combination of NAA 0.05 mg/L and BAP 5 mg/L was observed which multiplied 4-5 plants/meristem (Fig. 1). It was observed that the concentration of NAA and BAP more than the effective

Table 1. Effect of age of seedling on meristem regeneration of indica rice (*Oryza sativa* L.)

Age (days) of seedlings	Variety						
	Bas. 370[a]	S[b]. Bas.	IRRI6	KS282	Jhona	BR11	Bas. Pak
4*	50±2.8	45±1.4	40±0.8	55±2.8	34±2.8	40±2.8	21±1.5
5	44±2.1	41±0.8	37±1.2	51±0.6	33±1.0	30±0.8	30±0.8
7	22±1.5	15±1.7	17±2.4	24±2.3	14±1.5	20±0.8	17±0.6

Data is the average of three experiments (%±SE)
*It was impossible to isolate meristem from 3-day old seedlings
a: Basmati
b: Super

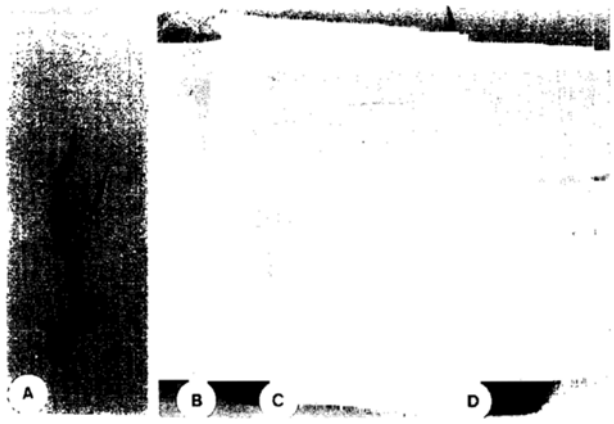

Fig. 1. Different stages of meristem culture of rice. (A) Plantlet formation from meristem after four days of cuture on MS medium. (B) Regenerated plant after two weeks on MS medium. (C) Regenerated plant from meristem was multiplied on MS medium supplemented with NAA 0.05 mg/L and BAP 5 mg/L. (D) Regenerated plant transferred into soil.

range could cause necrosis to the regenerated plants.

Histological study of leaf base isolated from 4-day old seedlings showed that cells were smaller in size, isodiametric in nature and less vacuolated, characteristic of meristematic cells (Fig. 2A). Leaf base from 7-day old seedlings showed less cytoplasmic, vacuolated cells and most of them underwent cellular cell death (Fig. 2B). Plant growth hormones (NAA and BAP) in culture medium significantly decreased the meristem regeneration efficiency. The hormone-free MS medium was found to be the best for meristem regeneration study. A clear decline in meristem regeneration was observed on MS medium containing NAA and BAP (Table 2). Highest percentage of meristem regeneration in hormone free medium is well documented by Anthony (1992) and Bobak *et al.* (1995). The regenerated plants after transferring into soil produced seeds showing 100% viability without any morphological variations. This procedure allows regeneration of rice plants from meristem tissue (without callus formation) within 6-8 weeks. The meristem of rice could be used for transformation by biolistic bombardment in order to introduce genes of agronomic importance. This tissue explant was proved to be good explant for transformation of reporter and marker genes by biolistic gun (Husnain *et al.*, 1995). High level of transient expression was studied in electroporated leaf bases (Dekeyser *et al.*, 1990). Meristem could be the best explant for bombardment by specially designed biolistc gun (Sautter, 1993).

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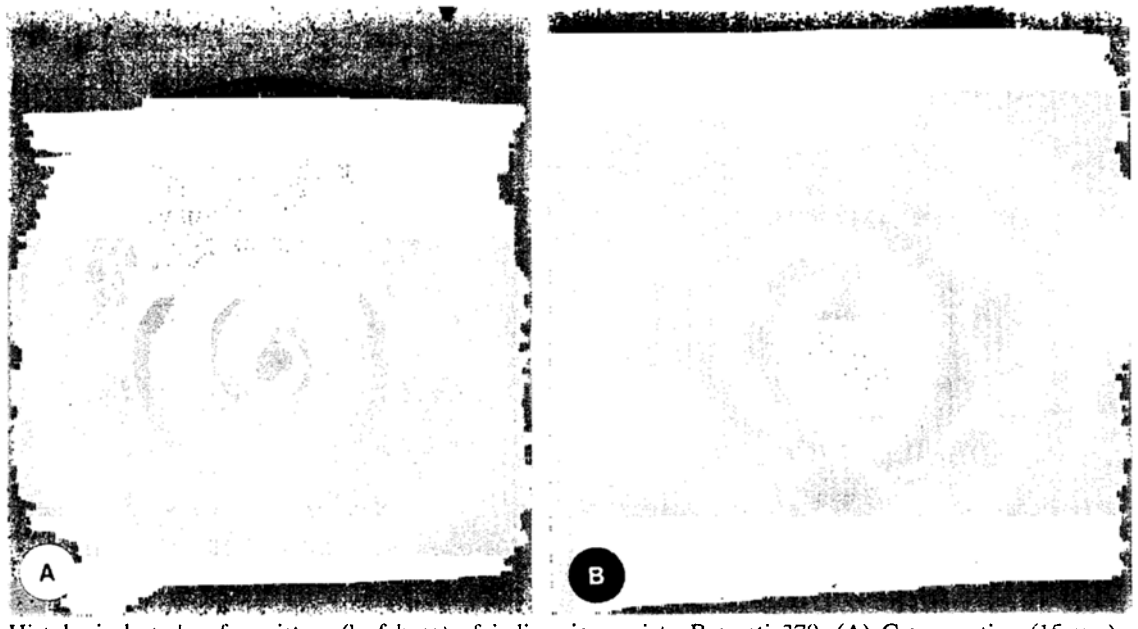

Fig. 2. Histological study of meristem (leaf base) of indica rice variety Basmati 370. (A) Cross section (15 μm) of meristem isolated from 4-day old seedling. (B) Cross section (15 μm) of meristem isolated from 7-day old seedling.

Table 2. Regeneration efficiency from meristem of different indica rice varieties on MS medium supplemented with different concentrations of NAA and BAP (mg/L)

NAA/BAP (mg/L)	Variety						
	Bas. 370[a]	S[b]. Bas.	IRRI6	KS282	Jhona	BR11	Bas. Pak
00/00	50±2.8	45±1.5	40±0.8	55±2.8	34±2.0	40±2.8	28±1.5
0.5/0.5	27±5.0	14±0.8	12±1.7	23±1.7	10±1.1	7±0.8	08±0.3
0.5/1.0	20±4.4	16±0.3	07±0.3	19±0.6	13±0.5	10±0.8	11±0.5
0.5/1.5	17±1.4	11±0.8	04±1.7	17±0.8	11±0.6	06±0.6	05±0.6
0.5/2.0	11±2.4	08±0.5	03±0.6	12±0.6	05±0.3	7±0.5	08±0.5
0.5/2.5	05±0.8	03±0.5	02±0.5	04±0.6	04±0.8	07±1.7	07±0.8
1.0/0.5	08±0.5	02±0.5	07±0.8	05±0.5	04±0.8	05±0.5	06±0.5
1.5/0.5	04±0.3	04±1.1	06±0.7	04±0.6	04±0.5	03±0.5	06±0.5
2.0/0.5	05±0.3	02±0.8	02±0.3	05±0.5	05±0.5	05±1.4	03±0.5
2.5/0.5	02±0.3	02±0.5	02±0.3	02±0.2	01±0.3	03±0.6	01±0.3

Data is the average of three experiments
a: Basmati
b: Super

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