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Efficient plant regeneration from long-term callus cultures of rice by spermidine

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Abstract. A significant reduction in regeneration potential with increasing age (upto 12 months) in rice (*Oryza sativa* L. cv.TN-1) embryogenic callus cultures was observed. Spermidine, while having an inhibitory effect on plant regeneration in fresh callus cultures, promoted morphogenesis in long-term callus cultures. A massive accumulation of polyamines, particularly putrescine (5-fold) was observed in 12 month old cultures resulting in a change of putrescine/spermidine ratio, which seems to be important for maintaining the morphogenetic response. Application of exogenous spermidine to 12 month old cultures showed increased levels of polyamines and restored the putrescine/spermidine ratio comparable to that found in freshly induced cultures, concomitantly, promoting the plant regeneration via somatic embryogenesis in long-term rice callus cultures.

Key words : Morphogenesis - *Oryza sativa* L. - Polyamines - Spermidine

Abbreviations : PA, Polyamines; PCA, Perchloric acid; PUT, Putrescine; SPD, Spermidine; SPM, Spermine.

Introduction

Efficient plant regeneration from rice callus and cell cultures has been reported and reviewed extensively (Abe and Futsuhara 1991, Croughan and Chu 1991). A rapid decrease in morphogenetic capacity with age in culture still remains a serious limitation for regeneration in rice (Kavikishor and Reddy 1986) as well as in other cereals (Pius *et al.* 1993, Vasil *et al.* 1984). In rice, high frequency regeneration has been obtained from long-term cultures by changing the osmolarity of culture media (Kavikishor and Reddy 1986, Kavikishor 1987), increase in subculture interval, modification of growth and regeneration media (Ozawa and Komamine 1989), and by salt pretreatment (Binh and Heszky 1990; Binh *et al.* 1992).

The diamine putrescine (PUT) and polyamines (PA) spermidine (SPD) and spermine (SPM) participate in a variety of plant developmental events such as growth, differentiation, aging, and stress responses (Evans and Malmberg 1989, Galston and Kaur-Sawhney 1990, Rajam 1989, 1993, Rajam *et al.* 1985, Slocum *et al.* 1984). Exogenous application of PA, particularly SPD, leads to retardation of symptoms of senescense (Kaur-Sawhney *et al.* 1982). Moreover, the levels of PA increase in rejuvenating cereal cultures (Tiburcio *et al.* 1991). PA, especially SPD have been implicated in somatic embryogenesis (Altman *et al.* 1990, Feirer *et al.* 1985, Mengoli *et al.* 1989, Robie and Minocha 1989), flower bud differentiation (Kaur-Sawhney *et al.* 1990) and morphogenesis (Sanchez-Gras and Segura 1988). In the present study, the influence of exogenous SPD on morphogenesis in fresh and long-term callus cultures of rice is reported.

Materials and Methods

Mature rice seeds (*Oryza sativa* L. cv. TN-1) were kindly supplied by Dr. M.P. Pandey, Department of Plant Breeding, G.B. Pant Agricultural University, Pant Nagar, India. They were dehusked, surface-sterilized with 0.1% mercuric chloride for 20 min, and rinsed thrice with sterile distilled water. Callus cultures were initiated from mature embryos of the seeds by anchoring the endosperm into MS (Murashige and Skoog 1962) medium (pH 5.8), supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L sucrose and 8 g/L agar-agar (Himedia, Bombay, India). Following 3 weeks of culture in the dark at 26±1°C, the callus contained both embryogenic (white to light yellow in colour, compact, nodular and

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friable) as well as non-embryogenic sectors (yellow in colour, mucilagenous and smooth) (Heyser *et al.* 1983). The embryogenic sectors were excised and subcultured on the same medium for 2 weeks, whereas non-embryogenic sectors were discarded. The same procedure was followed at each subculture. For plant regeneration through somatic embryogenesis, embryogenic sectors of the callus were cultured on MS medium supplemented with 0.5 mg/L benzyladenine (instead of 2,4-D)(Raghava Ram and Nabors 1985) at 26±1°C with 16/8 h photoperiod with light intensity of 40-60 μmol s^{-1} m^{-2}. Spermidine, where used, was filter-sterilized (0.22μm filters - Millipore Corporation, Bedford, MA, USA) and added to autoclaved medium cooled to 46-48°C to get final concentrations of 0.01, 0.1, 1 and 5 mM. The data were scored after 4 weeks in culture. The percent regeneration frequency was based on 15 replicates and the average number of plants were calculated per embryogenic callus culture.

Free and bound PA were analysed in fresh and 12 month old callus treated with or without SPD according to the procedures of Flores and Galston (1982) and Tiburcio *et al.* (1985), respectively. Briefly, callus pieces (app. 100 mg) were grown for 5 days in culture (just before the onset of differentiation on plant regeneration medium), extracted with 1 ml of 10% perchloric acid (PCA), and centrifuged at 16,000 g for 20 min at 4°C. After centrifugation, the supernatant and the pellet were separated. The supernatant was the source of free and PCA soluble conjugated (with phenolic acids and other low molecular weight compounds) PA, whereas the pellet contained PCA insoluble bound conjugated PA (with macromolecules). The pellet was resuspended in 1 ml of 1N NaOH by vortexing and without heating (Tiburcio *et al.* 1985). Aliquots from each of pellet suspension and the original supernatant were hydrolyzed with 12N HCl. The non-hydrolyzed PCA supernatant containing free PA, the hydrolyzed PCA supernatant and the hydrolyzed pellet suspension containing PA liberated from conjugates were dansylated, extracted in benzene, and loaded on precoated high resolution TLC plates (Silica Gel on 20x20 cm glass, particle size 5-17μm and layer 250 μm - Sigma Chemical Co., St. Louis, USA), and developed in cyclohexane:ethyl acetate (5:4 v/v). Dansylated PA bands marked under UV lamp were scraped off, eluted in ethyl acetate and quantified using Shimadzu RF540 spectrofluorophotometer with excitation wavelength of 350 nm and emission wavelength of 495 nm. All the experiments were conducted at least twice with consistent results. The data presented are representative of a single experiment.

Results and Discussion

Fresh callus cultures (3 weeks in the initial culture and subcultured once), and 3, 6 and 12 month old calli were tested for plant regeneration via somatic embryogenesis. In fresh cultures, callus response to plant regeneration was 76%, and regeneration frequency decreased with increasing age of the callus (Fig. 1). After 3 months of subculture, regeneration frequency dropped to 61% and subsequently to 53% and 28% in 6 and 12 month of subculture, respectively. These observations are consistent with earlier reports on regeneration in rice cultures (Abe and Futsuhara 1991, Croughan and Chu 1991, Binh *et al.* 1992). Kavikishor and Reddy (1986) reported failure of rice callus to initiate shoots when subcultured for 100 days. In the present study, exogenous SPD, while having an inhibitory effect on plant regeneration frequency in fresh cultures, promoted the regeneration response in 3 and 6 month old cultures, but it had no effect on regeneration frequency in 12 month old cultures (Fig.1).

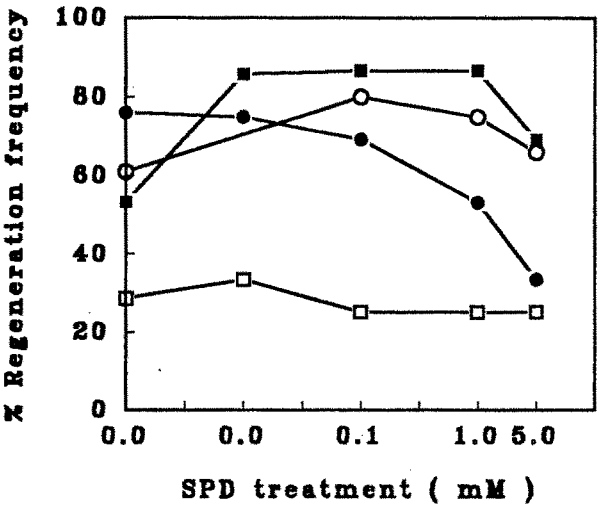

Fig.1. Effect of exogenous spermidine on regeneration frequency in fresh (●), 3 month (○), 6 month (■) and 12 month old (□) callus cultures of rice. Data are based on 15 replicates and scored after 4 weeks of culture.

Table 1 shows statistical analysis of the effect of SPD on plant regeneration in fresh and long-term callus cultures. In fresh callus (app. 100 mg) cultures, an average of 10 plants/culture could be obtained. However, as the callus aged, a significant reduction in number of plants was recorded. The exogenous SPD caused a decrease in plant number in fresh callus cultures. By contrast, SPD significantly promoted the number of plants in long-term cultures. The amount of SPD required for the induction of maximum number of plants was 1 mM for 3 and 6 month old cultures and 5 mM for 12 month old cultures. Fresh and 12 month old calli treated with or without SPD (5 mM) were utilized for analysis of cellular PA levels (Table 2). PUT constituted the major PA followed by SPD and SPM. The free fraction was the most abundant form followed by conjugated and bound fractions. Similar observations have previously been reported in rice leaf (Chatterjee *et al.* 1988) and coleoptile (Reggiani *et al.*

1989). A massive accumulation of PA, particularly PUT (5-fold) was observed in 12 month old cultures as compared to fresh callus cultures. The increase in cellular SPD levels was much less (2.5-fold). This could be due to reduced conversion of PUT to SPD (Galston and Kaur-Sawhney 1990). Such alterations in the cellular PA pools resulted in an increase of PUT/SPD ratio, especially soluble and conjugated fractions in 12 month old callus cultures over fresh callus cultures. However, an increase in SPD levels with the addition of exogenous SPD (5 mM) in 12 month old cultures, resulted in a change of PUT/SPD ratio, which is closer to the ratio found in fresh callus cultures (Table 2). It has been shown earlier that exogenously supplied PA are preferentially compartmentalized inside the vacuole and cytoplasm (DiTomaso *et al.* 1992).

Table 1. Effect of exogenous spermidine on plant regeneration in fresh and long-term callus cultures of rice.

SPD (mM)	No. of plants/embryogenic callus			
	Callus age (months)			
	0	3	6	12
0	9.75±1.69^{a}	3.00±0.36^{b}	4.12±0.81^{bc}	2.75±1.5^{b}
0.01	8.44±1.69^{ab}	2.60±0.88^{b}	6.08±0.58^{ac}	5.50±0.86^{abd}
0.1	7.11 ±0.75^{a}	5.70±0.49^{a}	8.07±1.03^{ad}	6.33±0.79^{abd}
1.0	5.71±0.68^{a}	13.0±0.73^{c}	9.60±1.03^{d}	7.66±0.82^{ad}
5.0	2.33±0.66^{b}	6.40±1.73^{abd}	5.55±0.78^{abc}	9.33±1.76^{d}

Each value is the mean ± SEM, based on 15 replicates and the data scored after 4 weeks of culture.

Values followed by different letters within rows and columns are significantly different at $P<0.05$ using Fischer's LSD test.

The decrease in plant regeneration in long-term cultures may be because of accumulation of PUT and high PUT/SPD ratio. These changes could be due to aging of the cultures (Shen and Galston 1985) and may be responsible for the blockage of conversion of immature embryos to mature embryos. Bradley *et al.* (1984) have shown that when carrot cells were transferred to an embryogenic medium containing 40 mg/l arginine, they reached the globular embryo stage but failed to develop further but synchronous development of the arrested embryos resumed when arginine was removed from the embryogenic medium. This observation indicates that precise regulation of endogenous PA levels is required during critical stages in plant development (Flores *et al.*

Table 2. Polyamine levels in freshly induced and 12 month old callus treated with or without spermidine in rice

	PCA fraction	PUT	SPD	SPM	Total PA levels	PUT/SPD ratio
		nmol/g fresh wt.				
A	S	534±84	166 ±17	65±20	766	3.19
	SH	150±70	66±26	72±37	288	2.77
	PH	96±28	47±5	36±8	178	2.04
	Total	780	279	173	1232	2.79
B	S	2997±52**	405±27*	30±7	3431	7.40
	SH	1208±33**	253±21*	30±7	1492	4.77
	PH	198±22	30±5	8±3	237	6.60
	Total	4403	688	68	5159	6.39
C	S	4702±62**	1352±38**	61±7	6116	3.47
	SH	2485±54*	779±33	35±6	3299	3.18
	PH	560±41	48±10	21±4	629	11.66
	Total	7747	2179	117	10044	3.55

A = Fresh callus, B = 12 month old callus, C = 12 month old callus with 5 mM SPD.

S = Soluble fraction, SH = Soluble conjugated fraction, PH = Bound fraction, PCA = Perchloric acid.

Each value is the mean ± SEM, based on three replicates.

*and ** denote significant differences from fresh cultures and 12 month old cultures (for 12 month old cultures treated with 5 mM SPD) at 5 and 1% level, respectively.

1989). Faure *et al.* (1991) have suggested that the abnormal growth behaviour of somatic embryos and their low rate of development into plantlets in *Vitis vinifera* could be due to excessively high free PA content and/or to an inadequate PUT/SPD ratio. Further, it has been shown that treatments that decrease PA levels, like the addition of PUT synthesis inhibitors (Mengoli *et al.*1989) or treatments that modify the PUT/SPD ratio, such as the addition of SPD (Altman *et al.*1990), increased the conversion of somatic embryos into plantlets in carrot and celery, respectively. In our study, the increase in both percent regeneration frequency and number of plants/culture in long-term cultures following SPD treatment may be due to its enrichment effect on plant regenaration, and the restoration of PUT/SPD ratio in such cultures. However, there was no effect on the percent regeneration frequency (but more number

of plants/culture were recorded) in SPD-amended 12 month old cultures.

In conclusion, it appears that the plant regeneration potential can be promoted in long-term callus cultures of rice by maintaining an optimal PUT/SPD ratio, using exogenous SPD.

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