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PLANT REGENERATION FROM PROTOPLASTS OF RICE : RELATIVE EFFICIENCY OF DIFFERENT SOURCES OF PROTOPLASTS

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ABSTRACT

Plant regeneration from protoplasts of rice has been reported for several varieties using different explant as source of protoplast. However, no general conclusion can be drawn from these experiments as to which source is better. It seems; use of one explant or the other was determined by the ease with which one explant could be manipulated in vitro. We report here a general protocol for plant regeneration from protoplast including development of cell suspension from three different explants of rice. The protocol can be used for both Japonica and Indica rice. Plating efficiency was as high as 4.85%. Among the different explant sources, immature embryos were the best and gave highest plating efficiency. Relative efficiency of different sources and beneficial effect of nursing of protoplasts have been described in detail.

INTRODUCTION

Plant regeneration from protoplast has been reported in more than 20 varieties of rice including japonica and indica lines (Lee et.al., 1989; Bhattacharjee et.al., 1998). Rice protoplasts have been used in production of somatic hybrids (Terada et.al., 1987; Hayashi et.al., 1988) and in transfer of cytoplasmic male sterility (Bhattacharjee et.al., 1999) establishing its potential in genetic manipulations. Although Agrobacterium can now be used very efficiently for rice transformation, protoplasts are still considered important for chloroplast transformation. Thus, efficient plant regeneration from protoplasts is still a key requirement for various genetic manipulations of rice.

Different explants like seed, immature embryo, anther-derived calli, leaf base cells, microspores etc. were used to obtain protoplasts. However, no general conclusion can be drawn from these experiments as to which source is better. It seems; use of one explant or the other was determined by the ease with which one explant could be manipulated in vitro. Thus, in Japonica genotypes, where in vitro response was higher, all types of explants were used for initiation of cell suspension whereas in Indica genotypes explant source was restricted to seeds and immature embryos. Various culture media and protocols were used for protoplast culture and plant regeneration depending on genotype and protoplast source (Abdullah et.al., 1986; Kyozuka et.al., 1987; Gupta and Pattanayak, 1993; Bhattacharjee et.al., 1998). Here we report a common protocol for plant regeneration from rice protoplasts obtained from cell suspensions of various explants.

MATERIAL AND METHODS

Experiments were carried out with 7 genotypes viz., NEH Megha Rice 1, NEH Megha Rice 2, RCPL 1-2C, RCPL1-3C(all japonica), IR64, IR72 and IR74 (all Indica). Calli were induced from mature and immature embryos in LS medium (Linsmaier and Skoog, 1965) supplemented with 4.0 mg/l 2,4D,

500 mg/l casein hydrobysate, 500 mg/l L-proline, 3.0% sucrose and solidified with 0.8% agar (pH 5.8) following the procedure described by Gupta et. al. (1989). Callus induction from anthers was carried out following the protocol of Pattanayak and Gupta (2000). Both embryo and anther-derived calli were proliferated and maintained on N6 medium (Chu et.al., 1975) supplemented with 4 mg/l 2,4-D, 3% mannitol and 2% sucrose. Calli were sub-cultured every 15 days and at each sub-culture nodular whitish embryogenic calli were selected.

Friable calli obtained after 3-4 subcultures were used for initiation of cell suspension. Calli (0.5 - 1.0) were inoculated into 25 ml of Amino Acid medium (Miller and Grafe, 1978) supplemented with 4 mg/l 2,4-D(AA4 medium) in 250 ml flasks. Cultures were maintained on a shaker (120 rpm) at 27+ 2oC in the dark. Initially sub-culturing was done every alternate day but after a month sub-culturing was done every fourth day. Fine cell suspension was developed by periodically sieving the suspension through a 500 μm nylon sieve.

Embryogenic cell suspensions (Ecs), obtained after about 2 months of subculture were used for protoplast isolation. Cell clumps of > 250 μm size were digested in an enzyme mixture containing 1% cellulose, 0.1% Pectolyase, 5 mM MES in CPW 13M (Frearson et.al. 1973) medium at 50 rpm in the dark (25±1oC) for 3 hours and then kept stationary for 1 hour. After digestion the mixture of enzyme and cells was passed through a stack of pre-wetted 64 μm, 45 μm and 30 μm nylon sieves, washed twice by centrifugation (80xg) in CPW13M, heat shocked t 45oC for 8 min followed by 20 sec. on ice and then purified by floating over 1M sucrose at 60 x g. Purified protoplasts were finally washed in protoplast culture medium and cultured in one of the following 3 ways :

i) in liquid N6 medium supplemented with 1.5 mg/l 2,4-D, 0.2 mg/l zeatin, 500 mg/l casein hydrolysate (N6PCMZ) and 0.7 M glucose.

ii) Suspended in liquid N6PCMZ with 0.7M glucose and cultured inside donut surrounded by feeder cells and

iii) Suspended in N6PCMZ with 0.7 M glucose and 0.15% seaplaque agarose and cultured in dunuts surrounded by feeders.

Feeders were prepared from suspension cells of RCPL 1-2C following the method of Pattanayak and Gupta (1993). Protoplasts were cultured either at a density of 1 x 106/ml (Japonica genotypes) or 2 x 10 6/ml (Indica genotypes). Control plates of feeders were plated with liquid N6 PCMZ to monitor leakage of feeder cells. Protoplasts were cultured in 5 replicates in the dark at 27±2oC. After initiation of division cultures were fed with fresh medium every 7th day with reduction of osmoticum of about 1/3rd at each feeding reaching to a final concentration of 0.17 M glucose. Macrocolonies, visible after 25 - 30 days were further proliferated on filter papers moistured N6 protoplast cultured media supplemented with 2.5 mg/l 2,4-D and 0.5mg/l ABA and 0.17M glucose. Protoplast derived calli of 2-3 mm size were transferred to MSB medium (Gupta and Pattanayak, 1993) for regeneration and maintained under 16/8 hour photo-period. Regenerated plantlets were transferred to bottles containing ½ strength MS medium for rooting. Plantlets with profuse roots were transferred to pots and maintained in the green house.

RESULTS AND DISCUSSION

Callus induction and establishment of suspension

Frequency of callus induction ranged from 53 - 91% in mature and immature embryos and 5.2 - 35.5% in anthers of various genotypes. Immature embryos were, however, more responsive. After 3-4 subcultures in N6M medium friable embryogenic calli were obtained. When tested for regeneration 6- -

65% of these calli showed plant regeneration. These calli when transferred to AA4 medium and grown on shaker dissociated slowly and within about 2 months fine suspensions were obtained. But in IR64 the suspension became brown and mucilaginous. Addition of proline (100 mg/ml) to AA4 medium helped in growth of the cells and after about 10 weeks fine cell suspensions were obtained in this genotype. Similar effects of praline on cell suspensions were reported in maize (Armstrong and Green, 1985) and barley (Rengel and Jelaska, 1986).

Source of calli played an important role in the successful initiation and establishment of cell suspension. It was observed that immature embryo-derived calli and calli obtained from anthers dissociated faster than calli obtained from mature seeds. Calli obtained from Japonica genotypes dissociated at a much faster rate compared to those obtained from Indica rices. Thus, from 1.0g of immature embryo-derived calli of Japonica genotypes 1.0ml PCV (packed cell volume) of <500mm size cells was obtained within 20-30 days of initiation of suspension while in Indica genotypes (IR64, IR72 and IR74) it took 60-80 days. In Japonica genotypes, calli-derived from mature embryos produced 1.0ml PCV of <500mm size cells within 30-45 days while in Indica genotypes they took 80-90 days . Although, anther-derived calli dissociated well and the suspensions obtained were comparable to those obtained from immature embryo-derived calli (in Japonica genotypes); these suspensions lost their embryogenic potential within another 60 days. In Indica genotypes, the extent of browning of anther-derived calli in suspension was much higher compared to the suspensions initiated from both mature seed and immature embryo-derived calli of the same genotype.

Protoplast isolation, colony formation and plant regeneration

Protoplast yield ranged from 0.8 - 1.3 x 107/g cells with 90 - 95% viable protoplasts. Freshly isolated protoplasts were round and densely cytoplasmic with prominent starch granules, however, some non-cytolasmic protoplasts were also seen. Both cytoplasmic and non-cytoplasmic protoplasts regenerated cell wall upon culture. But, cell division occurred only in the cytoplasmic protoplast and first divisions were seen between 4 - 7 days in japonica genotypes and between 6 - 10 days in Indica genotypes. After cell division started in the reconstituted cell addition of fresh medium with lower concentration of sugar helped in subsequent division of cells leading to formation of 32 - 64 cell micro colonies. These micro colonies became macroscopic in about 4 weeks time. No cell division was seen in Indica genotypes when protoplasts were cultured without nurse, while in japonica genotypes very low frequency of division was seen in Megha Rice 2 and RCPL 1-2C (0.001 - 0.0004%). Between the other two methods of protoplast culture, plating efficiency was higher (1.33 - 5.66%) in culture method III (Table - 1) irrespective of the source of protoplast.

Source of suspension cells from which protoplasts were isolated showed conspicuous effect on plating efficiency. Plating efficiencies were higher in protoplasts isolated from immature embryo-derived and anther derived cell suspension (Table 2).

Protoplast derived calli, on transfer to MSB medium, produced white embryo like structure on their surface within 7 - 10 days of culture in the dark. These calli on transfer to 16/8 hr. photoperiod differentiated into shoots and roots. In general, plant regeneration frequency from anther derived calli was lower. In addition, many albino plants (10 - 17%) were produced. On the contrary, in protocalli obtained from mature and immature embryo-derived suspension cells, albinos were obtained (3 - 8%) only when cell suspensions were more than 12 months old. Plantlets of 2 - 3 cm size rooted profusely in ½ MS medium and upon transfer to soil produced fertile plants.

Results presented here show that source of suspension cells strongly influence subsequent protoplast division and plating efficiency. Although there is no previous reference of a comparative study, immature embryos were generally used in rice (Abdullah et.al. 1986; Lee et.al., 1989; Bhattacharjee et.al., 1998) while anther-derived calli were also used by some workers (Gupta et.al., 1993). The results of the present investigation also showed that feeder cells are essential for division of protoplasts of Indica genotypes.

This is in agreement with the findings of Lee et.al. (1989) and Bhattacharjee et al. (1998).
The protocol described here can be used as a general protocol for culture of protoplasts of suspension cells obtained from various explants. Although cells obtained immature embryos are best source of calli, anthers also can be used with good success.

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Table 1. Effect of culture method on plating efficiency (%) of protoplasts isolated from immature embryo-derived suspension of different genotypes.

Genotype	Plating efficiency (%)		
	Liquid only (1)	Liquid + nurse (3)	Liquid + 0.15% agarose + nurse (4)
Megha Rice 1*	ND	3.12	3.66
RCPL1-2C*	0.004	4.85	5.66
RCPL1-3C*	ND	3.45	4.33
Megha Rice 2*	ND	3.41	3.92
IR64**	ND	1.98	2.23
IR72**	ND	1.19	1.33
IR74**	ND	1.75	1.90

ND = no division

*Plating density = 1 x 106 /ml; **Plating density = 2 x 106

Table 2: Effect of source of suspension cells on plating efficiency of protoplasts of different genotypes cultured in liquid N6PCMZ with nurse.

	Plating efficiency from different source (%)		
Genotype	Immature embryo	Mature embryo	Anther
RCPL1-1C*	3.12	2.75	2.95
RCPL 1-2C*	4.85	4.12	4.33
RCPL 1-3C*	3.45	2.79	2.98
RCPL1-6C*	3.41	2.86	3.05
IR 64**	1.98	1.52	1.75
IR 72**	1.19	0.95	1.03
IR 74**	1.75	1.33	1.33

* Plating density 1 x 106 protoplasts/ml

** Plating density 2 x 106 protoplasts/ml