Tropical Agricultural Research Vol. 9 1997, 15-25

Cell and Protoplast Culture of *indica-javanica* Elite Rice Breeding Lines, IR 65597-134-2 and IR 65598-112-2

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ABSTRACT. *Rice (Oryza sativa L.) is one of the world's most important food crop species with an increasing demand. Therefore, high yielding new rice breeding lines have been produced by IRRI to raise the rice production. These breeding lines require further crop improvement. Genetic transformation provides efficient methods of crop improvement, complementing the conventional methods of plant breeding. Application of gene transfer technology requires the availability of reliable protocols for cell and protoplast culture of the particular plant. Therefore, in vitro culture systems of two indica-javanica breeding lines were established with an objective to use the developed systems in genetic transformation of these breeding lines.*

Embryogenic callus production was more than 70% from mature seed scutellum (MSS) of rice breeding lines, IR 65597-134-2 and IR 65598-112-2. Plant regeneration from these calli was achieved by inducing water stress conditions using 1% (w/v) agarose. Maltose had a positive effect on regeneration frequency over sucrose.

Regenerable protoplasts were isolated from cell suspensions initiated in AA2 medium. Protoplast yield was dependent on the genotype and the cell suspension age which showed an initial increase followed by a decrease. Protoplast-derived colonies were recovered using nurse cells. High protoplast plating densities ($1.0\text{-}2.0\times10^6$ protoplasts ml^{-1}) were required for protoplast culture. Protoplast plating efficiencies were considerably low. The highest plant regeneration frequencies of protoplast-colonies were 10.2%-14.9%. The frequencies were affected by the cell line, nurse cells and carbohydrate source of the regeneration medium.

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INTRODUCTION

World demand for rice (*Oryza sativa* L.) increases as a result of the growth in world's population. The new IRRI breeding lines have been produced in order to raise the rice production. These breeding lines require incorporation of desirable characters, such as disease and pest resistance (IRRI Reporter, 1994). Genetic transformation provides means of improving such lines in considerably shorter time frame than by conventional breeding.

Rice has been transformed using direct DNA uptake into protoplasts, biolistics and *Agrobacterium*-mediated gene transfer (Ayres and Park, 1994). Availability of reliable protocols for plant regeneration from callus or protoplasts is a prerequisite for application of these gene transfer methods to any plant species. The objective of this study was to develop reliable protocols for cell and protoplast culture of two new *indica-javanica* elite rice breeding lines [IR 65597-134-2 (IR 65597) and IR 65598-112-2 (IR 65598)] for crop improvement programmes of these lines.

MATERIALS AND METHODS

Initiation and maintenance of callus cultures

Mature seeds were surface sterilized in 30% (w/v) 'Domestos' bleach solution for 1 h, rinsed with water and cultured on LS 2.5 medium [LS medium (Linsmaier and Skoog, 1965) supplemented with 2.5 mg l^{-1} 2,4-D and 0.4% (w/v) agarose]. Cultures were incubated at 28°C in the dark. After 4 weeks, callus was separated from the original explants and maintained by subculturing to fresh LS 2.5 at 3-4 weeks intervals.

Initiation of cell cultures

Embryogenic cell cultures were initiated by suspending callus (0.5 g) in AA2 [AA medium (Müller and Grafen, 1978) supplemented with 2 mg l^{-1} 2,4-D] liquid medium (6 ml) and incubated on a rotary shaker (120 rpm) at 28°C in the dark. Culture medium (80%) was replaced with fresh medium at 3-4 days intervals for 6 weeks. The amount of fresh medium added was gradually increased with the growth of cell colonies, until 30 ml of fresh medium was used. Then cell suspensions were maintained by weekly

subculture at the ratio of (ml) 1 packed cell volume : 6 conditioned medium : 21 fresh medium.

Protoplast isolation

One gram of 2-10 month old cell suspension cells were incubated in 10 ml of filter sterilized CPW 13M solution [CPW solution (Frearson *et al.*, 1973) supplemented with 13% mannitol] containing 1.0% (w/v) cellulase RS, 0.1% (w/v) pectolyase Y 23 and 5 mM MES. The mixture was incubated for 16 h on a shaker (30 rpm, at 28°C) in the dark. The digest was filtered through nylon sieves (64, 45, 30 μm) and the filtrate was pelleted by centrifugation (60 × g, 7 min). Protoplasts were collected and washed twice with CPW 13 M solution and once with KPR medium (Abdullah *et al.*, 1986) by resuspension and centrifugation (60 × g, 7 min each). Protoplast viability was determined using the fluorescein diacetate (FDA) staining method (Widholm, 1972).

Protoplast culture

Agarose embedding method: $0.3\text{-}2.0\times10^6$ protoplasts ml were cultured in KPR medium solidified with 1.2% (w/v) Sea Plaque agarose.

Nurse culture method: *Lolium multiflorum* and Black Mexican Sweet Maize suspension cells mixed with 10 ml of KPR medium containing 0.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA, 0.5 mg l^{-1} zeatin and 0.8% (w/v) molten Sea Plaque agarose served as feeder layers. A 0.2 ml aliquot of protoplasts resuspended in KPR liquid medium at a plating density of $0.25\text{-}3.0\times10^6$ ml^{-1} was placed on a Millipore membrane filter (0.22 μm) placed on the feeder layers. Cultures were incubated at 27°C in the dark. After 3 weeks, membrane filters were transferred to KPR medium devoid of nurse cells.

Plant regeneration from tissue-derived callus and protoplast-derived colonies

Embryogenic callus at the age of 2-3 months was transferred to regeneration medium. Two plant regeneration procedures were assessed.

One-step procedure: Callus was transferred to MSKN medium [MS medium (Murashige and Skoog, 1962) supplemented with 2 mg l^{-1} kinetin and

0.5 mg l^{-1} NAA] solidified with 0.4% (w/v) agarose. Cultures were incubated in the dark at 28°C for 2 weeks and then transferred to 12 h light at 28°C for 3 weeks (Jain *et al.*, 1995).

Two-step procedure: Callus was transferred to MSKN medium solidified with 1.0% (w/v) agarose and incubated in the dark at 28°C for 2 weeks. Then callus was transferred to MSKN solidified with 0.4% (w/v) agarose and incubated under 12 h light at 28°C for 3 weeks (Tang, 1995).

5 weeks old protoplast-derived colonies were assessed for regeneration using two-step procedure.

Statistical analysis

Experiments were conducted at least three times with 3-6 replicates. Means and standard errors of means were calculated. Statistical significance between mean values of plant regeneration from MSS-derived callus was assessed using a conventional Student's *t*-test. Standard analysis of variance was used to analyse the effects of cell suspension line, feeder cells and carbohydrate source in the regeneration medium in terms of plant regeneration frequency of protoplast-derived colonies. A probability of $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Initiation of callus from explants

Mature seeds of rice are one of the most commonly used explants to initiate embryogenic callus due to their availability throughout the year (Rueb *et al.*, 1994). In this study 76% and 74% mature seed scutellum (MSS) of IR 65597 and IR 65598 respectively produced embryogenic callus in LS 2.5 medium.

Protoplast isolation

The isolated protoplasts had an average diameter of 17-20 μm and 80-90% viability. The mean protoplast yield was influenced by the genotype and the age of the cell suspension (Figure 1). The cell line of IR 65598 yielded an

increased amount of protoplasts than that of IR 65597. The yield initially increased with the age and then decreased.

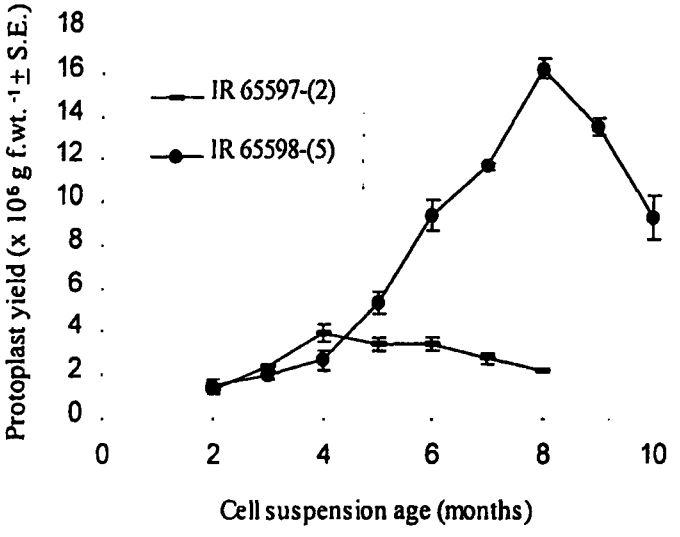

Figure 1. Effect of the genotype and the cell suspension age on protoplast yield.

[Note: Data represents the mean values of at least 3 replicate protoplast isolations. IR 65597-(2) and IR 65598-(5) cell lines were initiated from MSS-derived calli of the rice breeding lines IR 65597 and IR 65598 respectively in AA2 medium.]

Similarly, Krautwig and Lörz (1995) reported that young (less than 2 months old) suspension lines yielded low amount of protoplasts and with regular subculture, the yield increased. This is due to the increase in the population of small, cytoplasmically dense embryogenic cells with regular subculture. As observed in this study, a decreased protoplast yield after about 12 months (depending on genotype) was also reported by Tang (1995). This was caused by the increase of less densely cytoplasmic and irregular cells in older cell suspensions.

Table 1. Effect of protoplast culture density on plating efficiency.

Cell suspension age (months)	Protoplast culture density ($x10^6$ ml^{-1})	Protoplast plating efficiency (%) ± S.E. (After 35 days)	
		IR 65597	IR 65598
4	0.5	0.0	NT
	1.0	0.0098 ± 0.0008	0.0
	2.0	0.0078 ± 0.0035	NT
	3.0	0.0088 ± 0.0056	NT
5/6	0.5	0.003 ± 0.001	0.0123 ± 0.003
	1.0	0.007 ± 0.002	**0.0292 ± 0.0012**
	2.0	**0.043 ± 0.003**	0.0038 ± 0.0005
	3.0	0.032 ± 0.003	NT
7	0.5	NT	0.1665 ± 0.0065
	1.0	NT	**0.3215 ± 0.0095**
	2.0	**0.137 ± 0.004**	0.091 ± 0.016
	3.0	0.129 ± 0.007	NT

Protoplasts were cultured using membrane filter nurse (*Lolium multiflorum*) culture method. Data represents mean values of at least 3 replicate experiments with 4-6 replicates per treatments. NT = not tested.

Protoplast culture

The choice of protoplast culture method was influenced by the genotype. In both breeding lines, protoplast-derived colonies were recovered only from the membrane filter nurse culture method. Similarly, recovery of

protoplast-derived colonies only in the presence of nurse cells have been reported for several recalcitrant varieties of rice (Jain *et al.*, 1995; Tang, 1995). In general, it is considered that nurse cells help to maintain a critical density of active cells (Funatsuki *et al.*, 1992) which release growth promoting factors into the medium, promoting protoplast division and their further development (Eigel and Koop, 1989). However, it may also be possible that nurse cultures condition the culture medium by reducing or modifying certain deleterious components in the culture (Krautwig and Lörz, 1995).

Cultured protoplasts showed an increased plating efficiency with the age of cell suspension. The choice of protoplast plating density depends on the genotype. In this study, the optimum plating densities were high (2.0 and 1.0×10^6 protoplasts ml for IR 65597 and IR 65598, respectively) and differed between the tested cell lines of two breeding lines (Table 1). Such high plating densities have also been shown for protoplasts of several varieties of japonica (Xue and Earle, 1995) and indica (Tang, 1995) rice.

Table 2. Plant regeneration from MSS-derived callus.

Breeding line	Plant regeneration medium	Carbohydrate source	Plant regeneration frequency (%) ± S.E.
IR 65597	MSKN[1]	sucrose	0.0
	MSKN MSKN[2]	sucrose	8.0 ± 1.63[a]
	MSKN MSKN	maltose	**18.3 ± 3.21[b]**
IR 65598	MSKN	sucrose	0.0
	MSKN MSKN	sucrose	7.0 ± 1.9[a]
	MSKN MSKN	maltose	**21.7 ± 4.19[b]**

Data represents mean values of at least 3 replicate experiments with 3 replicates per treatment. 1= One-step procedure, 2=Two-step procedure. a and b indicate differences between media containing sucrose or maltose for each genotype are significant at $P<0.05$.

Plant regeneration

In this study, plant regeneration from callus was possible when water stress conditions were induced by the application of a high agarose concentration in the regeneration medium (two-step procedure). The use of maltose, significantly increased plant regeneration frequency from 7-8% to 18.3-21.7% in MSS-derived calli of both breeding lines (Table 2). Considering the above results, plant regeneration of protoplast-derived colonies was assessed using only the two-step procedure. Statistical analysis of results using ANOVA showed that the cell line within a rice breeding line (IR 65597) and the feeder cell species significantly ($P<0.05$) increased the plant regeneration. The use of maltose had a significant effect only in the case of plant regeneration from protoplasts of IR 65598 (Table 3).

Table 3. Plant regeneration from protoplast-derived colonies.

Breeding line	Cell suspension line	Feeder cells	Carbohydrate source	Plant regeneration frequency (%) ± S.E.
IR 65597	1	LM	sucrose	12.4 ± 0.87
			maltose	**14.9 ± 2.51**
		BMS	sucrose	8.00 ± 2.76
	2	LM	sucrose	7.57 ± 1.62
			maltose	8.00 ± 0.75
		BMS	sucrose	3.35 ± 1.39
			maltose	4.35 ± 1.58
IR 65598	1	LM	sucrose	7.10 ± 1.59
			maltose	**10.20 ± 0.47**
		BMS	sucrose	1.30 ± 0.55
			maltose	5.00 ± 1.99

Data represents mean values of at least 3 experiments each with 3 replicates per treatment. LM = *Lolium multiflorum* nurse, BMS = Black Mexican Sweet Maize nurse.

The successful use of high agarose to induce water stress is previously reported for plant regeneration from protoplast-derived calli (Tang, 1995; Sindhu and Murai, 1996) and cell suspension-derived calli (Jain *et al.*, 1996). Exposure of calli to water limiting environments causes metabolic and physiological changes in cells. It has been reported that ABA is produced and is involved in stress response of plants (Skriver and Mundy, 1990) and ABA promotes somatic embryogenesis and plant regeneration (Xu *et al.*, 1995).

The superior effect of maltose, over sucrose, in stimulating plant regeneration in rice has been reported by Gosh Biswas and Zapata (1993). The beneficial effect of maltose has been attributed to its ability to stabilize culture medium osmolality (Kuhlmann and Foroughi-Wehr, 1989) or a slow rate of maltose degradation to glucose (Finnie *et al.*, 1989).

CONCLUSIONS

Cell and protoplast culture systems of elite rice breeding lines, IR 65597-134-2 and IR 65598-112-2 were developed. MSS-derived callus was suitable for initiation of embryogenic cell suspensions which in turn provided a source of regenerable protoplasts. The presence of nurse cells, high protoplast plating densities were found to be critical factors influencing protoplast culture. An application of water stress conditions in the regeneration medium was needed for plant regeneration from MSS-derived callus. The plant regeneration frequencies of MSS callus and protoplast-derived colonies were comparable with those of recalcitrant varieties of rice (Abe and Futsuhara, 1986; Yin *et al.*, 1993).

The research protocols established in this study could be used as a baseline in future studies on improving cell and protoplast culture systems and gene manipulation studies of these rice breeding lines.

ACKNOWLEDGMENTS

Financial assistance provided by the Agricultural Research Project and the study leave granted by Coconut Research Institute for the postgraduate study are gratefully acknowledged. The support extended by the staff of Plant Genetic Manipulation Group, University of Nottingham, UK and the Tissue Culture Division, Coconut Research Institute is appreciated.

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