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Protoplast Culture and Plant Regeneration of Rice

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ABSTRACT : Embryogenic calli were induced from mature seed scutella of anther culture-derived rice variety Zhonghua 8. Cell suspension cultures were initiated from friable embryogenic calli and utilized as source material for protoplast isolation. Generally, the older and finer cell suspensions gave higher protoplast yields than younger suspension cultures. Protoplasts exhibited sustained cell division and formed microcalli when cultured in KPR medium supplemented with 0.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA and 0.5 mg l^{-1} zeatin using the agarose embedding procedure without feeder cells. Protoplast plating efficiencies ranged from 0.20 to 0.54%. Microcalli were transferred to MS medium supplemented with 2.0 mg l^{-1} kinetin and 0.5 mg l^{-1} NAA for plant regeneration. The regeneration frequencies were 2 to 12%, depending on the cell suspension lines of Zhonghua 8. The plants were transferred to the glasshouse and were fertile.

Key words : Rice, Protoplast culture, Plant regeneration.

Rice breeding started in the early decades of the present century with the primary objective to generate new varieties. In most developing countries, increased rice production is always the ultimate goal in order to be able to be self-sufficient in food. This is, however, not an easy task because of several factors, such as a reduction of fertile land for rice growing, crop diversification and crop failure caused by insect pests and diseases. Recent advances in biotechnology may provide solutions to these problems through tissue and protoplast culture, somatic hybridization and transformation.

Anther culture is the most extensively used tissue culture technique in breeding programmes of rice, because this technique is relatively simple and is an efficient method to obtain haploid cells, tissues and whole plants, the latter being homozygous breeding lines (Pinson & Rutger[22]. Plant regeneration from anther-derived callus culture of rice, using various media and procedures, has been reported by a number of laboratories (Datta et al.[5] Sathish et al.[24] The Japonica rice variety Zhonghua 8 used in this experiment has been bred in China through anther culture procedures as a new variety (Li et al.[16] Ra-

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ina [23].

Since 1985, substantial progress has been achieved in plant regeneration from protoplasts of Japonica rice. In most cases, it has utilized embryogenic cell suspensions initiated from either immature embryo-, mature seed scutella-, or leaf base-derived calli as source material for the isolation of large numbers of totipotent protoplasts (Abdullah et al.[2], Kyozuka et al.[14], Masuda et al.[19], Jenes and Pauk,[13], Jain et al.[12] The protoplast culture procedure has been an important factor in increasing protoplast plating efficiency and subsequent plant regeneration. The method of agarose embedding has been found beneficial in Japonica rice as a mean of improving protoplast plating efficiencies, the formation of embryolike structures and plant regeneration from protoplast-derived calli (Thomson et al.[27, 28] Jenes and Pauk[13], Liu et al.[18] Ghosh Biswas and Zapata[7]).

The objective of the work described in this experiment was to establish embryogenic cell suspension cultures and an efficient plant regeneration system from protoplasts of the anther culture-derived rice variety Zhonghua 8, using the agarose embed-ding culture procedure.

MATERIALS AND METHODS

1. Plant material

Seeds of *Oryza sativa* var. Zhonghua 8, a variety derived through anther culture, were obtained from the International Rice Germplasm Center (IRGC), International Rice Research Institute (IRRI), Philippines.

2. Callus initiation and maintenance

Medium for callus initiation and maintenance was based on the formulation of Linsmaier and Skoog (1965), semi-solidified with 0.4% (w/v) agarose (Sigma Type I) supplemented with 3% (w/v) sucrose, 2.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} thiamine HCl (LS2.5 medium). The medium was freshly prepared by mixing equal volumes of autoclaved liquid, double strength LS2.5 medium with 0.8% (w/v) aqueous molten agarose. The mixture was then dispensed as 20 ml aliquots into 9 cm diameter Petri dishes. Seeds were dehusked and surface sterilized in 30% (v/v) 'Domestos' bleach (Lever Industrial Ltd., Runcorn, Cheshire, UK) for 30 min and rinsed several times in sterile tap water.

The seeds were placed onto the LS2.5 medium with 10 seeds in each Petri dish (the plumule-radicle side in contact with the medium and scutellum side up). The plates were sealed and incubated in the dark at 27℃. Embryogenic callus, which formed at the surface of scutella of mature seeds, was separated from the explant tissue and any surrounding non-embryogenic callus, and transferred to fresh LS2.5 medium. Subculturing was then carried out at monthly intervals with transfer of only the embryogenic callus. Cultures showing severe browning or organogenesis were removed during each subculture.

3. Initiation and maintenance of cell suspension cultures

Embryogenic callus was identified by regular observation of cultures using a stereo dissecting microscope. Callus was teased apart into smaller pieces from a compact tissue, but cutting the callus was avoided, as this leads to excessive browning of the cultures, affecting the subsequent suspension initiation process. About 1 g fresh weight of

callus (approximately 3 months old) was transferred into a 100 ml conical flask containing 20 ml of liquid AA2 medium (Müller and Grafe[20]) supplemented with 2.0 mg l^{-1} 2, 4-D, 0.2 mg l^{-1} kinetin and 0.1 mg l^{-1} GA_3. The cultures were incubated on a rotary platform shaker at 120 rpm in the dark 27℃.

During the initial stage of initiation of the suspension cultures, two thirds of the culture medium was replaced with fresh AA2 medium every 3~4 days. After 6 week from initiation, 7 ml aliquot of cell suspension [1 ml packed cell volume (pcv) with 6 ml old medium from the existing culture] was subcultured into 21 ml fresh AA2 medium in a 100 ml conical flask, using a wide-bore plastic pipette, and the resulting culture maintained as before. Established cell suspensions were cultured in AA2 medium in 100 ml conical flask under the same conditions as for suspension initiation. Subculture was carried out weekly by adding 7 ml of inoculum, containing 1 ml of pcv, to 21 ml of the fresh medium.

4. Protoplast isolation and culture

Protopasts were isolated from suitable established cell suspension cultures in their exponential growth phase, 4~5 days after subculture. The cell suspension cultures were sieved through a 500μm nylon mesh sieve into a 9 cm diameter plastic petri dish, to obtain small cell groups suitable for enzymatic digestion.

The liquid medium was removed using a sterile Pasteur pipette or a sterile 10 ml plastic pipette. About 1.0 g fresh weight of the cells was resuspended in 20 ml enzyme-CPW 13M mixture (5 ml enzyme solution for each gram fresh weight of cells diluted in 15 ml of CPW 13M solution). The enzyme solution was 1.0% (w/v) Cellulase RS (Yakult Honsha Co. Ltd., Nishinomiya, Japan), 0.1% (w/v) Pectolyase Y23 (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) and 5 mM MES (Sigma Chemical Co., Ltd., Poole, UK), dissolved in CPW 13M solution (Frearson et al.[6]) at pH 5.8. The Petri dish was sealed with Nescofilm and incubated in the dark at 25℃ with gentle shaking for 14 h (40 rpm), followed by 1 h stationary incubation at 25℃. After digestion, the enzyme-cell mixture was filtered through a series of nylon sieves of 64, 45 and 30 μm pore sizes to remove undigested cell groups and large protoplasts resulting from spontaneous fusion, and collected in a 9 cm diameter Petri dish.

The protoplast-enzyme mixture was gentle pipetted into 16 ml capacity round-bottomed glass screw-capped centrifuge tubes, using a sterile Pasteur pipette, and pelletted by centrifugation at 700 rpm for 5 min. The protoplasts were washed 3 times with CPW 13M solution and finally resuspended in 10 ml of filter sterile KPR protoplast culture medium supplemented with 0.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA and 0.5 mg l^{-1} zeatin. Protoplasts resuspended in KPR medium were heat shocked by immersion in a 45℃ water bath for 5 min, followed by plunging into crushed ice for 10 seconds. Protoplasts were counted using a modified Fuchs Rosenthal double chamber haemocytometer of 0.2 mm depth (Weber Scientific International Ltd., Toddington, Middlesex, UK). Protoplast viability was determined using the fluorescein diacetate (FDA) staining method (Widholm[30]).

Protoplasts were cultured in KPR medium, semi-solidified with 1.2% (w/v) of Sea Plaque agarose (FMC Bio Products, Rockland, ME, USA) at 3.5×10^5 protoplasts

ml^{-1}. The dishes were cultured in the dark at 27℃. After 7~14 days of culture, the agarose in each dish was cut into 4 segments and transferred to a 5 cm diameter Petri dish containing 3 ml of liquid KPR medium. The cultures were maintained in the dark at 27℃ until microcalli, 1~1.5 mm in diameter, were obtained. The number of macroscopically visible colonies at day 28, was counted in randomly chosen areas using a microscope and recored as the protoplast plating efficiency.

5. Plant regeneration from protoplast-derived colonies

Protoplast-derived microcalli (1~2 mm in diameter) were transferred individually into 25 compartment dishes. Four colonies were placed in each compartment containing 3 ml of agarose-solidified [0.4% (w/v), Sigma Type I] Murashige and Skoog (1962) medium supplemented with 2.0 mg l^{-1} kinetin and 0.5 mg l^{-1} NAA (MSKN medium). The dishes were sealed with Nescofilm and incubated in the dark at 27℃. After 4 weeks, the shoots were transferred to 6 oz Powder Round glass jars each containing 50 ml of agarose-solidified MS medium supplemented with 2 mg l^{-1} BAP and 5% (w/v) sucrose (MSBP medium), and then transferred to light (55 μmol m^{-2} s^{-1}, daylight fluorecent tubes) with a 16 h photoperiod at 27℃.

Multiple shoots of micropropagules, which developed on tissue cultured on this medium, were separated and transferred to agarose-solidified MS medium supplemented with 1.5 mg l^{-1} NAA and 3% (w/v) sucrose (MSN1.5 medium). Green plants, approximately 12 cm in height, were removed from culture vessels, washed carefully to remove agarose from the root and transferred to 10 cm diameter plastic pots containing Levington M3 soil-less compost (Fisons plc., Ipswich, UK) and perlite (Silvaperl Ltd., Gainsborough, Lincolnshire, UK) and grow in the glasshouse.

RESULTS

1. Callus initiation

The cultured seeds became swollen and germinated within several days, and callus was gradually formed on the surface of scutella and coleoptiles on LS2.5 medium (Fig. 1a). Two types of callus were produced; embryogenic callus which was dry, compact, globular in appearance and white-yellow in colour, and non-embryogenic callus, which was watery and friable in appearance.

2. Initiation and maintenance of cell suspension culture

Cell suspension cultures were initiated from embryogenic callus. During the first 2~3 weeks of suspension initiation, single cells and cell groups derived from callus were dissociated in the liquid medium. Regular replacement of 80% of the liquid medium every 3 to 4 days for the first 4~5 weeks was essential for the successful production of suspension cultures. After 4~5 weeks of medium replacement, suspension cultures were composed of small cell groups, consisting of densely cytoplasmic cells and thin cell walls, which were actively dividing. At this stage, selective subculture of suspension cultures was performed. After 2 weeks of subculture, the suspension cultures grew actively (Fig. 1b).

In order to maintain the embryogenic cell suspension culture, it was necessary to keep

the cultures in the exponential growth phase. A 7 day subculture interval was found suitable to maintain embryogenic cell suspensions.

3. Protoplast isolation

Totipotent protoplasts were isolated from embryogenic cell suspension cultures by enzymatic digestion of the cultured cells using an overnight incubation procedure. The yield of protoplasts varied from 2.20 to 7.90×10^6 per gram fresh weight of cells in two cell suspension lines, Za-1 and Za-2, depending upon the age of the suspension cultures (Table 1).

Freshly isolated protoplasts were densely cytoplasmic (Fig. 1c) and their viability was about 85% (Fig. 1d). Two months old suspension cultures were suitable for efficient protoplast isolation. In general, the older and finer suspensions gave higher protoplast yields than younger suspension cultures (Table 1).

Table 1. The effect of age of suspension cultures on protoplast yield

Suspension line	Age of suspension line (months)	Protoplast yield ($\times 10^6$/g.f.wt)
Za-1	2	2.4±0.08
	3	3.2±0.07
	6	5.4±0.12
	7	6.1±0.19
	10	6.3±0.15
Za-2	2	2.2±0.08
	3	2.9±0.10
	6	5.7±0.17
	7	6.9±0.24
	10	7.9±0.27

Mean ± S.E. of 3 replicates

4. Protoplast culture

The protoplasts embedded in culture medium became swollen within 24 h of culture, and the first (Fig. 1e) and second divisions (Fig. 1f) of protoplasts were observed within a week. Micro-colonies of approximately 16 cells formed after 10 d of culture (Fig. 1g) and, at this stage, it was necessary to transfer the agarose sections containing the protoplasts to fresh liquid KPR medium for further development.

Visible colonies (approximately 0.5 to 1.0

Table 2. Plating efficiency and plant regeneration frequency from cell suspension-derived protoplasts

Suspension line	Age of suspension line (months)	Plating efficiency at 28 d (%)	Microcalli regenerating shoots (%)
Za-1	2	0.36±0.04	4
	3	0.28±0.01	6
	6	0.42±0.01	12
	7	0.40±0.02	8
	10	0.54±0.02	10
Za-2	2	0.20±0.03	2
	3	0.28±0.02	5
	6	0.26±0.03	8
	7	0.38±0.05	7
	10	0.44±0.04	7

Mean ± S.E. of 3 replicates
Plating efficiency was expressed as the percentage of protoplasts forming visible colonies after 28 days of culture

mm in diameter) were obtained from protoplasts of both cell lines after 3 weeks of culture (Fig. 1h). Plating efficiencies ranged 0.20 to 0.54% in both cases (Table 2), and was affected by the age of the suspension cultures; the older suspensions gave higher plating efficiencies than younger suspensions. Colonies of 1.0~1.5 mm in diameter were transferred to shoot regeneration medium, and those less than 1 mm in diameter were kept in the medium for further growth untill they were large enough to be transferred.

5. Plant regeneration from protoplast-derived callus

The colonies grew faster on shoot regeneration medium. White embryo-like structures became visible on the surface of the protoplast-derived calli after 10 days of culture. This led to the development of plantlets within 3 weeks (Fig. 1i). In most cases, shoots developed before the formation of roots.

The plantlets were transferred to MSBP micropropagation medium for further growth under continuous light (Fig. 1j). The plant regeneration frequencies were 4 to 12% in line Za-1 and 2 to 8% in line Za-2, respectively (Table 2). It was found to vary for the different suspension cell lines and also to be dependent on the age of the suspensions. After 2 weeks of culture, regenerated shoots were transferred to MS1.5 rooting medium, and finally to compost, where mature, fertile plants developed (Fig. 1k).

DISCUSSION

In order to maximize yields of viable protoplasts and subsequent plant regeneration from such protoplasts, embryogenic callus induction and establishment of cell suspensions from such callus were the primary priority. Although embryogenic callus has been obtained from several explants of rice, such as anthers and microspores (Datta et al.[5], Guiderdoni and Chaïr[10]), roots (Sticklen[25]), leaf bases and coleoptiles (Thompson et al.[28], Baset et al.[3,4]) and young inflorescences (Ghosh Biswas and Zapata,[7]), both mature seeds (Abdullah et al.[2], Kyozuka et al.[14], Ghosh Biswas and Zapata[8], Jain et al.[12]) and immature embryos (Lee et al.[15], Wu and Zapata[31]) have also been used frequently to initiate embryogenic callus and cell suspension cultures with the subsequent regeneration of plants from cell suspension-derived protoplasts.

The establishment of fine embryogenic cell suspension cultures was very important point in order to obtain cytoplasmically dense protoplasts and successful plant regeneration from such protoplast in rice. It is likely that composition of culture medium was one of the important factors in establishing fine embryogenic suspension cultures (Abdullah[1]). In the present study, cell suspension cultures of the Japonica rice were initiated and maintained in AA2 liquid medium (Müller and Grafe[20]) as reported by Abdullah et al.[2]. Although several media have been used for the initiation and establishment of cell suspension cultures in rice, including R2 (Ghosh Biswas and Zapata[8]), LS (Jenes and Pauk[13]), MS (Masuda et al.[19]) and N6 (Wen et al.[29]), AA2 medium, which consists of 4 amino acids as the sole nitrogen source, has been largely used for the initiation of fine embryogenic cell suspension cultures in rice (Abdullah et al.[2], Guiderdoni

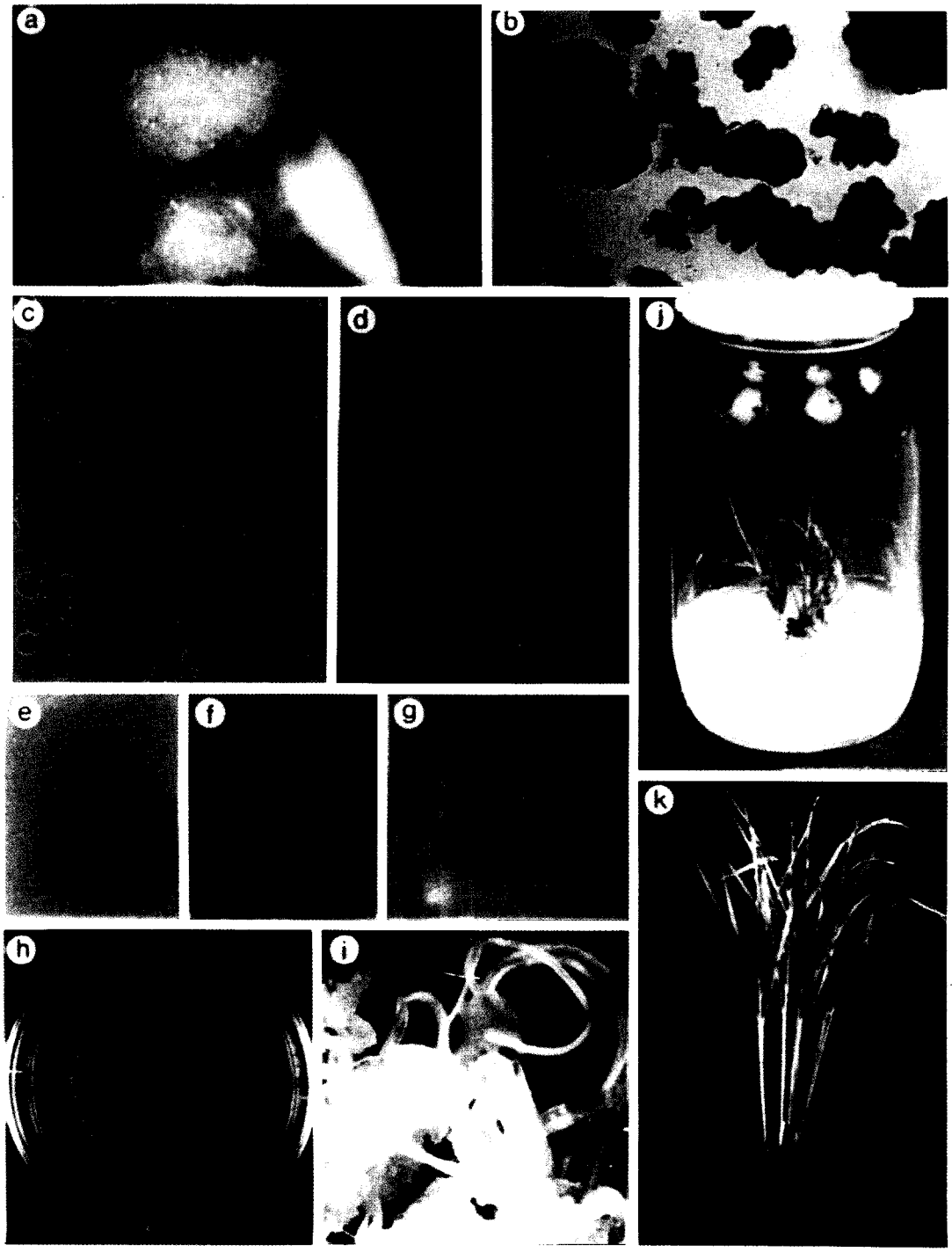
and Chalr[10], Jain et al.[12]). In most cases, embryogenic cell suspensions derived from either immature embryos, mature seed scutella, or leaf base callus has been used as source material for the isolation of large numbers of totipotent protoplasts (Abdullah et al.[2], Kyozuka et al.[14], Jenes and Pauk[13], Masuda et al.[19], Jain et al.[12]), although protoplasts were isolated directly from tissues, such as calli from immature embryos and mature seeds, scutellar tissues of mature embryos and mesophyll cells (Wu & Zapata[31], Ghosh Biswas et al.[19], Gupta and Pattanayak [11]).

Protoplast yield depended on the age of cell suspension cultures. In general, the older and finer suspensions gave a higer protoplast yield than younger suspension cultures, because younger suspension cultures were normally composed of large cell clumps, consisting of irregular and elongated cells. Similar observations were reported by Abdullah et al.[2]. Efficient protoplast isolation was possible up to 19 months after the initiation of suspension cultures. The overnight incubation of suspension cells using a diluted enzyme solution to release protoplasts over a 14 h incubation with gentle shaking, followed by one h stationary incubation without shaking, has been used routinely to isolated rice protoplasts.

In present study, densely cytoplasmic protoplasts were isolated from embryogenic cell suspension cultures of the Japonica rice variety Zhonghua 8 by enzymatic digestion of the cultured cells using an overnight incubation procedure. The overnight incubation procedure has been successfully used by many research groups for isolating protoplasts from cell suspensions in rice (Liu et al.[18], Masuda et al.[19], Baset et al.[3,4], Jain et al.[12]). The protoplast culture procedure has been an important factor in increasing protoplast efficiency and subsequent plant regeneration. Protoplast culture in media solidified with agarose has been found to be beneficial in a number of plant species as a means of improving plating efficiencies, the formation of embryo-like structures and plant regeneration from protoplast-derived calli.

Rice protoplast culture, using the agarose embedding method, has been developed in Plant Genetic Manipulation Group, University of Nottingham (Abdullah et al.[2], Thomson et al.[27,28]) and adopted by other research groups for protoplast culture of Japonica rices (Jenes and Pauk[18], Liu et al.[18], Masuda et al.[19], Ghosh Biswas and Zapata[7]). Although the real role of agarose for the improvement of protoplast division is not well understood, embedding of protoplasts in agarose medium reduced protoplast expansion

Fig. 1. Callus production, initiation of cell suspension and culture of protoplasts and plant regeneration from protoplast-derived callus of the Japonica rice variety Zhonghua 8.
a) Callus initiation from a mature seed after 2 weeks of culture on LS2.5 medium.
b) Embryogenic cell suspension cultures after 2 months from initiation in AA2 liquid medium.
c) Freshly isolated cell suspension-derived protoplasts.
d) Viable protoplasts stained with FDA.
e) First division of protoplasts after 3 days of culture in agarose-solidified KPR medium.
f) Second division of protoplasts after 5 days of culture in agarose-solodified KPR medium.
g) Micro-colony derived from a protoplast after 10 days of culture in agarose-solidified KPR medium.
h) Protoplast-derived colonies growing in the agarose-solidified KPR medium after 3 weeks of culture.
i) Differentiation of plantlet from protoplast-derived callus after 3 weeks of culture on MSKN medium.
j) Development of a young plant from protoplast-derived callus after transfer to MSBP medium.
k) A ferile plant regeneration from protoplasts after transfer to the glasshouse.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and consequent budding during the early stages of culture. It has been also suggested that surrounding protoplasts with agarose be reduced the diffusion of cell wall precursors and other metabolites through the medium (Takeuchi and Komamine,[26] Thomson et al.[27]).

In the present study, protoplasts of the Japonica rice variety Zhonghua 8 were isolated from embryogenic cell suspension cultures, and cultured using the agarose embedding method. Microcalli were recovered from protoplasts and subsequently regenerated into plants. The plants were transferred to the glasshouse and were fertile. These results indicate the feasibility of using protoplast-based technologies, such as somatic hybridization through protoplast fusion and the direct gene transfer into protoplasts, in rice breeding.

적 요

약배양을 통해 유도된 벼의 품종 Zhonghua 8의 종자로부터 배발생 캘러스를 유기한 캘러스로부터 현탁배양을 실시하였다. 원형질체 분리는 이러한 현탁배양된 캘러스를 사용하였으며, 일반적으로, 오래되고 미세한 현탁배양세포를 사용했을 때 어린 현탁배양세포보다 원형질체 나출율이 증가되었다. 원형질체는 feeder cell 없이 agarose embedding 방법에 의해 0.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA와 0.5 mg l^{-1} zeatin이 첨가된 KPR 배지에서 배양하였을 때 세포분열이 일어났으며 microcalli가 형성되었다. 원형질체의 plating 효율은 0.20~0.54% 범위로 나타났으며, 원형질체로부터 유도된 microcalli는 식물체 재분화를 위해 2.0 mg l^{-1} kinetin과 0.5 mg l^{-1} NAA가 첨가된 MS 배지에 옮겨 주었다. 식물체 재분화 빈도는 현탁배양의 line에 따라 2~12% 였다. 원형질체로부터 재분화된 식물체들은 온실에서 종자를 맺었다

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