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The Filter Membrane Culture Procedure with Feeder Cells in Rice Protoplast Culture

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Filter membrane과 feeder 세포를 이용한 벼의 원형질체 배양

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To investigate the response on feeder cell cultures, protoplasts isolated from cell suspensions initiated from mature seed scutellum-derived callus of the Japonica rice variety Taipei 309, were cultured on filter membranes under various conditions. The effects of various factors, such as gelling agents, feeder cell and protoplast densities, species of feeder cells and heat shock treatment, have been investigated to improve protoplast plating efficiencies on filter membranes. Maximum protoplast plating efficiencies were obtained when protoplasts were cultured on KPR medium semi-solidified with Sea Plaque agarose at a density of 5×10^5 ml^{-1} protoplasts in the presence of *Lolium multiflorum* as feeder cells (0.5 ml pcv per 10 ml of protoplast culture medium). Pre-culture heat shock treatments for 1 min. and 5 min. to the protoplasts did not give any appreciable increase on the plating efficiency of protoplasts in the presence of feeder cells. Maltose-supplemented medium was superior for plant regeneration from protoplast-derived colonies compared with medium containing only sucrose. The plants were transferred to the glasshouse, flowered and were fertile.

Key words: Carbohydrate, nurse culture, plant regeneration, *Oryza sativa* L.

INTRODUCTION

There is considerable interest for crop improvement through protoplast-based technologies, such as somatic hybridization via protoplast fusion and genetic transformation using direct gene transfer into protoplasts (Cocking and Davey, 1987). Reliable and efficient protoplast culture procedures for higher plating efficiency and subsequent plant regeneration from protoplasts is essential in order to utilize such technologies (Davey and Lynch, 1990). Since, Abdullah et al. (1986) demonstrated an efficient and reproducible regeneration of fertile rice plants from cell suspension-derived protoplasts via somatic embryogenesis, a number of laboratories has reported regeneration of fertile rice plants from protoplasts of several varieties of rice. However, the procedures used for protoplast culture were substantially different in each laboratory, and the results also varied. It may be the reason that many interacting factors, such as genotypic differences, the nutritional components and their balance in the medium, supplementation of media with phytohormones and their combination and concentrations, the presence of nurse or feeder cells, influence the various developmental stages in the process of fertile plant regeneration from protoplasts (Hodges et al., 1990).

Nurse cultures or feeder culture techniques have been used for a number of plant regeneration systems from protoplasts of cereals (Shillito et al., 1989; Funatsuki et al., 1992). In rice, Kyozuka et al. (1987) reported plant regeneration from protoplast-derived callus at a frequency of 17-50% using the agarose embedding method with feeder cells. They did not observe sustained division in the absence of feeder cells. A much more efficient procedure for protoplast culture and plant

regeneration, using the same procedure as Kyozuka et al. (1987) has been reported by Li and Murai (1990). Cell suspension cultures of the two Japonica varieties, Nipponbare and Taipei 309, were initiated from mature seed-derived callus. The protoplasts were cultured in a general medium based on N6 medium. Plating efficiencies were 13.7% and 17.3%, respectively, when 100 mg of nurse cells was in 5 ml of general medium. Li and Murai (1990) also reported a total of 141 regenerated plants (67% of plant regeneration frequency) from only 30 protoplast-derived callus by their improved procedure.

A filter membrane culture procedure with feeder cells has been used for protoplast culture of rice varieties Radon, Baldo, Nortai and Miara (Wen et al., 1991: Su et al., 1992: Guiderdoni and Chair, 1992). Protoplasts were isolated from cell suspensions initiated from immature and mature embryo-derived and anther-derived callus, repectively, and cultured with cell suspensions of IR52 and IR54 as feeder cells. Plant regeneration frequencies were from 21 - 32% in Radon, 33 - 35% in Baldo, 4.3% in Nortai, and 37.3 - 58.6% in Miara, respectively.

Our primary aim in this study was to assess the effect of the filter membrane culture procedure with feeder cells on protoplast plating efficiency and plant regeneration in rice.

MATERIALS AND METHODS

Plant Material

Seeds of the Japonica rice variety Taipei 309 used in this study were supplied by the International Rice Germplasm Center (IRGC), International Rice Research Institute (IRRI), Manila, Philippines. Cell suspension cultures of *Lolium multiflorum*, used as feeder cells, were obtained from Dr. Guiderdoni (IRAT-CIRAD, Montpellier, France).

Callus Initiation and Maintenance

Linsmaier and Skoog (1965) medium 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, was used for callus initiation (designated as LS2.5 medium). Dehusked seeds were surface sterilized and plated onto LS2.5 medium. The dishes were sealed with Nescofilm and incubated in the dark at 27°C. Embryogenic callus was selectively transferred to fresh LS2.5 medium after 4 weeks initiation. Subsequently, embryogenic callus was subcultured at monthly intervals.

Initiation and Maintenance of Cell Suspension Cultures

Suspension cultures were initiated from friable, globular embryogenic callus. Approximately 1 g fresh weight of callus was transferred into a 100 ml conical flask containing 20 ml of AA2 liquid medium (Müller and Grafe, 1978). The cultures were incubated on a rotary shaker at 120 rpm in the dark at 27°C. During the initiation period, 80% of the culture medium was replaced at 3-4 day intervals. After 6 weeks, a 7 ml aliquot of the cell suspension (1 mL of pcv and 6 mL of old medium) was subcultured with 21 mL of fresh AA2 liquid medium in a 100 ml conical flask. The culture was maintained as above. Subculture was subsequently carried out at weekly intervals. *L. multiflorum* cell suspensions, used as feeder cells in this study, were maintained by weekly subculture in N6 medium (Chu et al., 1975).

Protoplast Isolation

Protoplasts were isolated from cell suspensions, in exponential growth phase between 4-5 days after subculture, by enzymatic digestion through overnight incubation. The rest of the isolation procedures were followed as described by Abdullah et al. (1986).

Preparation of Feeder Cell Plates

Cell suspensions of *L. multiflorum* were harvested 3-4 days after subculture and mixed with molten KPR Sea Plaque agarose (0.8%, w/v) in the ratio of 0.5 pcv per 10 mL of medium. Ten ml of aliquots of agarose and cell suspension mixture were dispensed into 9 cm diameter plastic Petri dishes. The plates were sealed with Nescofilm and then incubated in the dark at 27°C. The feeder cell plates were set up 1 day prior to protoplast isolation.

Protoplast Culture

Whatman cellulose nitrate filter membranes (0.2 μm pore size, 47 mm in diameter) were placed on the top of the surface of each solidified feeder culture plate (one full size and two half sizes per Petri dish). Protoplasts at a density of 5 × 10^5 mL^{-1} were dropped onto the full size (200 μl) and the half size (100 μl) filter membranes, and uniformly spread over

the membrane surface area using a sterilized plastic inoculation loop. Petri dishes were sealed with Nescofilm and incubated in the dark at 27°C. After 3 weeks of culture, the membranes supporting the growth of protoplast-derived colonies were transferred onto a feeder cell-free LS2.5 medium and incubated in the dark at 27°C for 2-3 weeks. The plating efficiency was calculated as the total number of both transferred and non-transferred compact protoplast-derived callus obtained 5 weeks after protoplast plating.

Plant Regeneration

Protoplast-derived callus, 1-2 mm in diameter, were transferred to 25 compartment dishes with one colony in each compartment, containing 2 mL of Murashige and Skoog (1962) medium solidified with 0.4% (w/v) agarose (Sigma Type I) and supplemented with 2 mg/L^{-1} kinetin and 0.5 mg/L^{-1} NAA (MSKN medium). To study the effect of carbohydrate source on plant regeneration, sucrose (3.0%, w/v) was either fully replaced with maltose (3.0%, w/v) or partially replaced with maltose [both sucrose and maltose were used at 1.5% (w/v) concentration]. Plant regeneration frequency was recorded after 28 days as the percentage of protoplast-derived colonies forming plants. Protoplast-derived plantlets were transferred to 0.4% (w/v) agarose-solidified MS medium supplemented with 2 mg/L^{-1} BAP and 5% (w/v) sucrose (MSBP medium) and incubated at 27°C in the light for 28 days. Shoots were then individually transferred to the MS medium contiaing 1.5 mg/L^{-1} NAA and 3% (w/v) sucrose (MSN1.5 medum).

Green plants, approx. 12 cm in height, were removed from the MSN1.5 medium and washed to remove agarose from the roots. The plants were transferred to plastic pots containing rice compost and plants were grown to maturity.

RESULTS

Initiation and Maintenance of Cell Suspension Cultures

Callus was formed at the surface of the scutellum of mature seed on LS2.5 medium (Fig. 6a). In general, embryogenic callus was compact, dry, globular in appearance and white-yellow in colour. Suspension cultures were initiated from embryogenic callus. Single cells and small cell groups were dissociated from the callus during the first 3-4 weeks of initiation, and the regular replacement of 80% of the fresh AA2 medium at the 3-4 day intervals every week for 6 weeks was an essential factor for the successful production of cell suspension cultures. After 3-4 weeks, the suspension cultures were composed mainly of fast dividing cell clumps, which consisted of isodiametric cells with dense cytoplasm and thin walls (Fig. 6b). In order to maintain embryogenic suspension cultures, it was necessary to keep the cultures in the exponential growth phase. A 7-day subculture interval was found suitable for the maintenance of embryogenic cell suspensions. In addition, the pcv was critical during subculture in order to maintain the growth of suspension cultures. One ml pcv of small cell groups, together with 6 mL of the existing culture medium plus 21 mL of fresh AA2 medium, was optimum for the maintenance of embryogenic suspension cultures.

Protoplast Isolation

Protoplasts were isolated from cell suspensions by enzymatic digestion through overnight incubation. The viability of freshly isolated protoplasts was about 90%, and most of the protoplasts was densely cytoplasmic (Fig. 6c). The yield of protoplasts was 6.21 ± 0.47 × 10^6 per gram fresh weight, and was not influenced by the age of the suspension after over 1 year of subculture.

Effect of Gelling Agents on Plating Efficiency

Plant tissue culture media generally contain nutrients and, frequently, a gelling agent to support the tissues. The type of gelling agent in the medium has been shown to affect the growth of tissues in culture. A study was conducted to evaluate the effects of gelling agents on plating efficiency during rice protoplast culture. Gelling agents used were Sea Plague agarose (FMC Bio Products, Rockland, ME, USA) at 0.8% (w/v), Sigma Type I agarose (Sigma Chemical Co. Ltd.) at 0.4% (w/v), Phytagel™ (Sigma Chemical Co. Ltd.) at 0.2% (w/v) and agar (Gum Agar, Sigma Chemical Co. Ltd.) at 0.4% and 0.8% (w/v), and Difco-Bacto agar (Difco Laboratories, Detroit, Michigan, USA) at 0.4% and 0.8% (w/v), mixed with *L. multiflorum* cell suspensions (0.5 pcv per 10 mL of KPR medium) as feeder cells. Protoplasts, at a density of 5 × 10^5 mL^{-1}, were cultured on the filter membrane. After 1 week of culture, microscopic colonies were observed on Sea Plaque and Sigma Type I agaroses and Phytagel™, at similar plating efficiencies under the microscope. However, microscopic colonies were not formed on

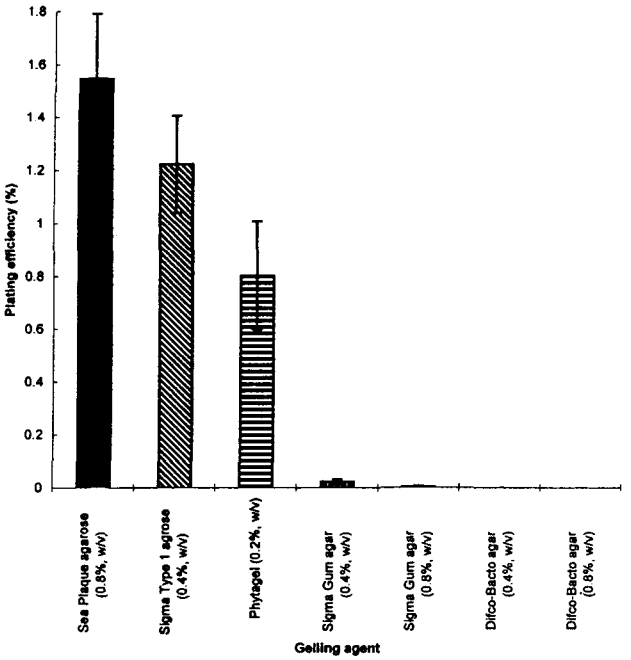

Figure 1. Effect of gelling agents on protoplast plating efficiency. Cell suspension-derived protoplasts of Taipei 309 were cultured in the presence of *L. multiflorum* as feeder cells (0.5 pcv/10 mL) with KPR medium solidified with various gelling agents. Plating effciency was recorded after 5 weeks of culture. The values represent the mean of 3 independent ezperiments. Bars repressent standard errors.

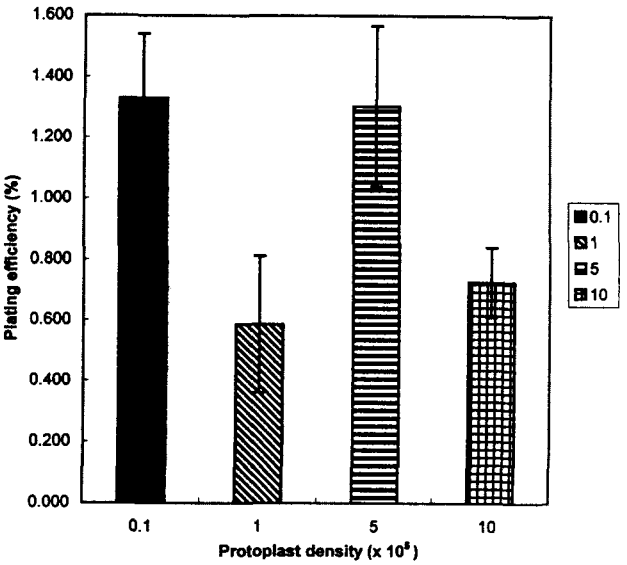

Figure 2. Effect of plating density on protoplast plating efficiency. Protoplasts were isolated from mature seed-derived suspensions and cultured using the filter membrane culture procedure with *L. multiflorum* cells as feeder cells. The plating efficiency was recorded after 5 weeks of culture. The values represent the mean of 3 independent experiments. Bars represent standard errors.

both 0.4% and 0.8% (w/v) Difco-Bacto agar and 0.8% (w/v) Sigma agar. A few microscopic colonies were formed on 0.4% (w/v) Sigma agar medium. After 3 weeks of culture, a large number of visible microcolonies were obtained from protoplasts on Sea Plaque and Sigma Type I agaroses and Phytagel™, with higher plating efficiencies, but there were only a few microcolonies on 0.4% (w/v) Sigma agar (Fig. 6e). After 5 weeks of culture, plating efficiencies were calculated. Maximum plating efficiency was obtained when protoplasts were plated on medium semi-solidified with Sea Plaque agarose, followed by Sigma Type I and Phytagel™ (Fig. 1).

Effect of Protoplast Density on Plating Efficiency

Protoplast plating density influences initial protoplast division, as well as sustained division. In order to assess the effect of low densities of rice protoplasts in combination with feeder cells, protoplasts were plated at densities of 0.1-10 × 10^5 ml^{-1}. Plating efficiency was not affected by protoplast density (0.1 × 10^5 mL^{-1}), with a similar plating efficiency at this concentration compared with protoplasts at the normal density (5 × 10^5 mL^{-1}) in the presence of feeder cells (Fig. 6f). However, a higher density of protoplasts (10 × 10^5 mL^{-1}), decreased the plating efficiency (Fig. 2).

Effect of Feeder Cell Density on Plating Efficiency

The presence of feeder cells in protoplast culture increased protoplast division and, subsequently further growth. In order to determine the effect of feeder cell density on the plating efficiency, protoplasts at a density of 5 × 10^5 mL^{-1} were cultured with various pcv of *L. multiflorum* suspensions as feeder cells (0 mL pcv, 0.1 mL pcv, 0.5 mL pcv, 1 mL pcv and 1.5 mL pcv in 10 mL of the medium). The feeder cells improved protoplast plating efficiency, depending on the feeder cell density (Fig. 6g). The highest plating efficiency was obtained at a feeder cell density of 0.5 mL pcv per 10 mL of protoplast culture medium (Fig. 3). However, when feeder cells were not employed, microscopic colonies were observed within 1 week of culture, but, subsequently, sustained cell division did not occur and further development to microcolony ceased. Protoplast plating efficiencies decreased when the feeder cell density was increased to more than 0.5 mL pcv per 10 mL of the medium.

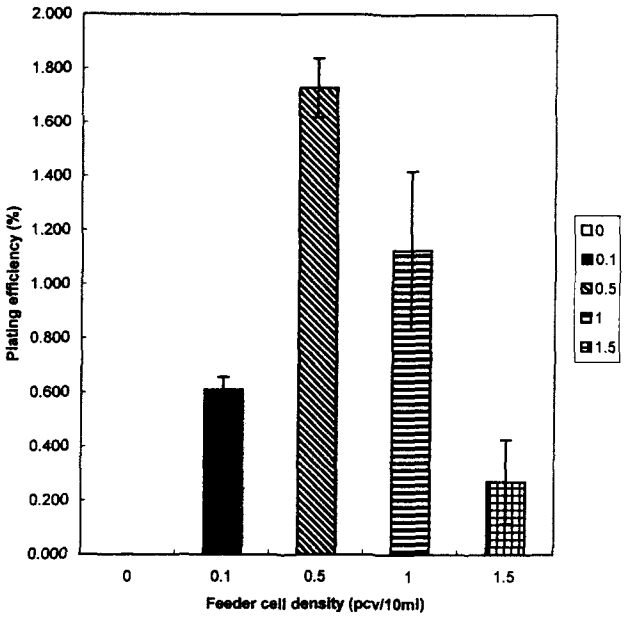

Figure 3. Effect of feeder cell density on protoplast plating efficiency. Protoplasts were cultured with various pcv of *L. multiflorum* cell cuspensions as feeder cells. Plating efficiency was recorded after 5 weeks of culture. The values represent the mean of 3 independent experiments. Bars represent standard errors.

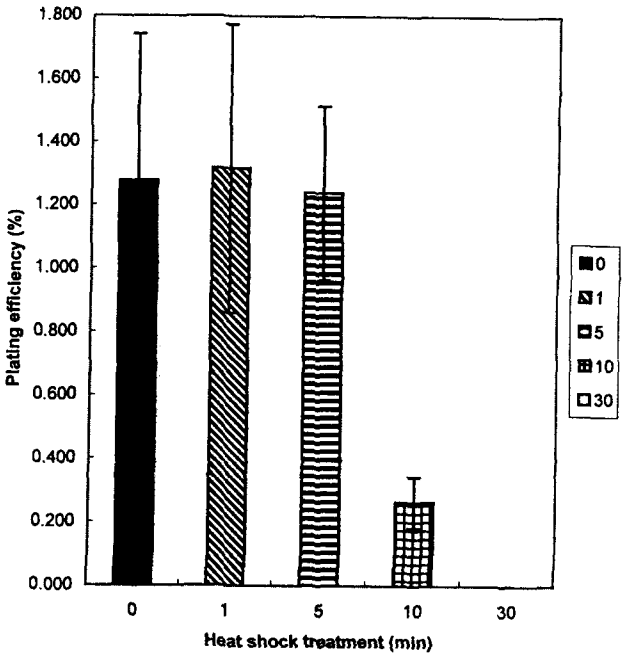

Figure 4. Effect of pre-culture heat shock treatment on protoplast plating efficiency. Freshly isolated protoplast pollets were resuspended in the medium and treated heat shock and cultured then with *L. multiflorum* cell suspensions as feeder cells using membrane filter culture procedure. Plating efficiency were recorded after 5 weeks of culture. The values represent the mean of 3 independent experiments. The bar represents a standard error.

Effect of Heat Shock Treatment on Plating Efficiency

To evaluate the effect of pre-culture heat shock on the plating efficiency of protoplasts plated over feeder cells, freshly isolated protoplast pellets were resuspended in KPR liquid medium and heat shocked at 45°C for 1-30 min, followed by 10 sec in ice (except for the 1 min. treatment to avoid cold shock).

Protoplasts, at density of 5×10^5 mL^{-1}, were cultured with 0.5 mL pcv of *L. multiflorum* as feeder cells. Pre-culture heat shock treatments for 1 min or 5 min did not give any appreciable increase in the protoplast plating efficiency in the presence of feeder cells. Protoplasts exhibited a similar plating efficiency to untreated protoplasts. However, the plating efficiency decreased dramatically when a heat shock treatment in excess of 10 min was applied to protoplasts (Fig. 4).

Effect of Species of Feeder Cells on Plating Efficiency

To investigate whether there was any species specificity in the capacity to improve rice protoplast plating efficiency in the presence of feeder cells, protoplasts at a density of 5×10^5 mL^{-1} were cultured with cell suspensions of various species (*Oryza sativa*, *Lolium multiflorum*, *Zea mays* and *Stylosanthes guianensis*) as feeder cells. After 1 week of culture, a large number of microscopic colonies were observed in the presence of *O. sativa* and *L. multiflorum* feeder cells. Some microscopic colonies were also observed in the presence of S. guianensis feeder cells under the microscope. However, microscopic colonies did not develop on *Zea mays* feeder cells. Subsequently, in the case of *S. guianensis* feeder cells, microscopic colonies were inhibited in their further development. After 3 weeks of culture, visible microcolonies were obtained from protoplasts only in the presence of both *O. sativa* and *L. multiflorum* feeder cells (Fig. 6h). After 5 weeks, plating efficiencies were calculated, and similar plating efficiencies were obtained with both *O. sativa* and *L. multiflorum* cell suspensions as feeder cultures (Fig. 5).

Plant Regeneration from Protoplast-derived Microcallus

To investigate the effect of carbohydrate source on plant regeneration, protoplast-derived colonies grown originally at a density of 5×10^5 mL^{-1} on KPR medium semi-solidified with 0.8% (w/v) Sea Plaque agarose with *L. multiflorum* cell

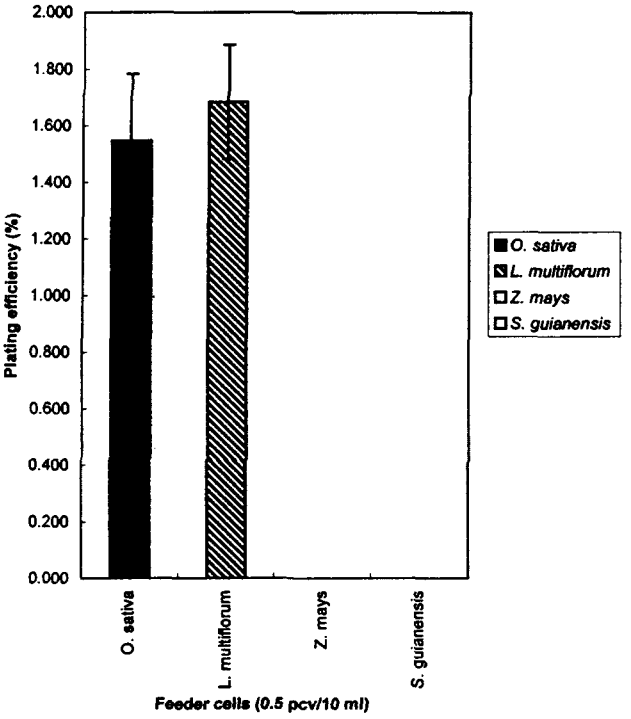

Figure 5. Effect of feeder cells on protoplast plating efficiency. Photoplasts were cultured with cell suspensions of various species as feeder cells using the filter membrane procedure. Plating efficiency was recorded after 5 weeks of culture. The values represent the mean of 3 independent experiments. Bars represent standard errors.

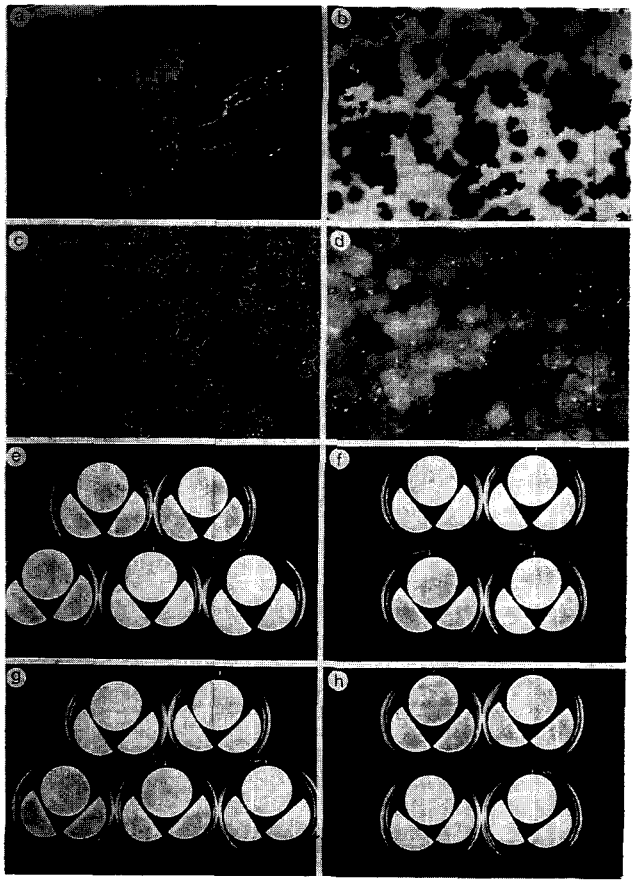

Figure 6. Callus production, initiation of cell suspensions and protoplast culture in the Japonica rice variety Taipei 309 using the filter membrane culture procedure. a) Callus initiation from a mature seed after 3 weeks of culture on LS 2.5 medium. b) Cell suspension cultures in AA2 medium after 2 months from initiation. c) Freshly isolated cell suspension-derived protoplasts. d) Protoplast-derived colonies growing on the surface of a filter membrane after 28 days of culture. e) Effect of gelling agents on plating efficiency after 28 days of culture: Sea Plaque agarose (0.8%, w/v) (top, left), Phytagel™ (0.2%, w/v) (top, right), Sigma Type I agarose (0.4%, w/v) (bottom, left), Sigma Gum agar (0.4%, w/v) (bottom, middle) and Difco-Bacto agar (0.4%, w/v) (bottom, right). f) Effect of protoplast density on plating efficiency after 28 days of culture: 0.1 × 10^5 mL^{-1} (top, left), 1 × 10^5 mL^{-1} (top, right), 5 × 10^5 mL^{-1} (bottom, left) and 10 × 10^5 mL^{-1} (bottom, right). g) Effect of feeder cell density on plating efficiency after 28 days of culture: 0 pcv (top, left), 0.1 pcv (top, right), 0.5 pcv (bottom, left), 1 pcv (bottom, middle) and 1.5 pcv (bottom, right). h) Effect of source of feeder cells on plating efficiency after 28 days of culture: *Lolium multiflorum* (top, left), *Oryza sativa* (top, right), *Zea mays* (bottom, left) and *Stylosanthes guianensis* (bottom, right).

suspensions as feeder cells (Fig. 6d), were transferred onto plant regeneration medium with either sucrose or maltose, or a mixture of both, as carbohydrate source. Protoplast-derived colonies produced plantlets within 2-4 weeks of culture on regeneration medium semi-solidified with 0.4% (w/v) agarose (Sigma Type I). Plant regeneration frequency varied considerably with carbohydrate source. Maltose-supplemented medium (Fig. 7b) was superior for regeneration of green plants from protoplast-derived colonies compared with medium containing only sucrose (Fig. 7a). The mean percentage of protoplast-derived colonies exhibiting plant regeneration on sucrose, maltose and a mixture of both these carbohydrates were 33.3%, 52.6% and 46.6% respectively, from 2 independent experiments with 3 replicates in each experiment (Table 1). Thus, maltose alone or combination with sucrose (1:1: w/w) as carbohydrate source in plant regeneration medium increased the plant regeneration frequency 1.5 fold compared with sucrose alone. Plantlets regenerated from protoplasts produced multiple shoots in MSBP medium (Fig. 7c). Individual shoots were transferred to MSN1.5 rooting medium, and finally to pots containing a compost-perlite mixture. The fertile plants developed in the glasshouse (Fig. 7d).

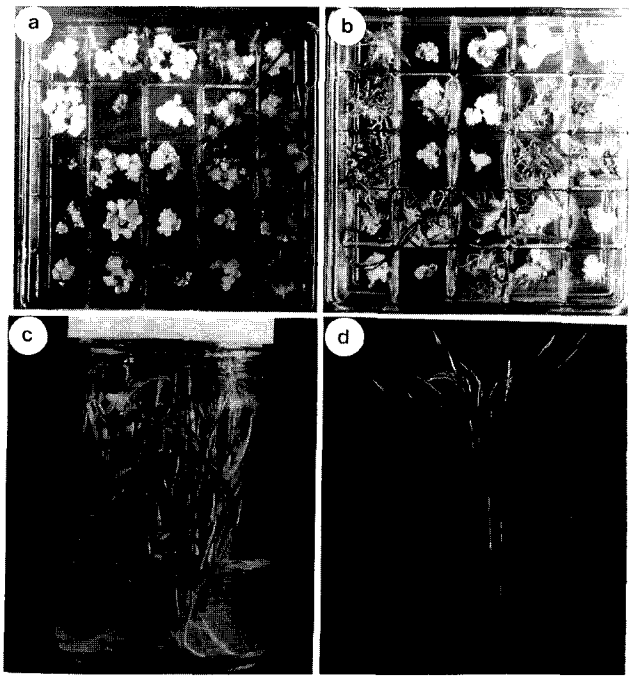

Figure 7. Plant regeneration from cell suspension-derived protoplasts of the Japonica rice variety Taipei 309. a) Differentiation of plantlets from protoplast-derived callus after 28 days of culture in MSKN medium with 3.0% (w/v) sucrose and maltose (b) as carbohydrate source. c) Development of young plants from protoplast-derived callus after transfer to MSBP medium. d) A fertile plant regenerated from protoplasts after transfer to the glasshouse.

Table 1. Effect of carbohydrate source on plant regeneration from protoplast-derived colonies of the Japonica rice variety Taipei 309

Experiment number	Percentage of protoplast-derived colonies exhibiting plant regeneration		
	Sucrose (3%, w/v)	Sucrose (1.5%, w/v) + Maltose (1.5%, w/v)	Maltose (3%, w/v)
1	33.3 ± 3.5	41.3 ± 7.4	52.0 ± 10.6
2	33.5 ± 1.3	52.0 ± 6.1	53.3 ± 3.5
Mean	33.3 ± 2.4	46.6 ± 6.7	52.6 ± 7.0

Mean ± S.E. of 3 replicates

DISCUSSION

Nurse culture or feeder cell culture techniques have been widely used for protoplasts of cereals (Shillito et al., 1989: Funatsuki et al., 1992). It is considered that such nurse cultures generally help to sustain cell division and development in cultured protoplasts, either through the production of physiologically-active compounds or through the prevention of leaching of vital components out of the cells (Hall et al., 1993: Peeters et al., 1994). The principle action of feeder cells is presently unclear, but is likely that living cells supply growth factors which promote the sustained growth of neighbouring cells (Dix, 1986).

As the filter membrane culture procedure, protoplasts were cultured on the surface of a filter membrane positioned on top of agarose-solidified protoplast culture medium in which the feeder cells were embedded. This procedure was widely and successfully adopted in protoplast culture of rice (Guiderdoni and Chair, 1992: Su et al., 1992: Jain et al., 1995). Feeder cells for nurse culture systems were essential to induce sustained cell division and colony formation from protoplasts of Indica rice. However, feeder cells were not necessary to induce protoplast division and subsequent colony formation in the protoplast culture of some varieties of Indica rice, namely Chinsurah Boro II (Datta et al., 1990), Tetep (Ghosh Biswas and Zapata, 1991), and IR72 (Datta et al., 1992). In the present investigation, feeder cells were not absolutely required for sustained protoplast division and colony formation in the culture of Japonica rice protoplasts. However, the use of feeder cells using filter membranes increased significantly plating efficiency.

The choice of feeder cells was also found to influence plating efficiency during the culture of protoplasts. Several feeder cells from various species have been used previously to support cell division and subsequent formation of colonies in rice. In the present study, protoplasts of the Japonica rice Taipei 309 were cultured on the filter membranes with or without cell suspensions of various species (*Oryza sativa*, *Lolium multiflorum*, *Zea mays* and *Stylosanthes guianensis*) as feeder cells. Visible microcolonies were obtained from protoplasts of Japonica rice only in the presence of both *O. sativa* and *L. multiflorum* feeder cells. Protoplasts failed to divide when plated on the surface of the filter membranes without feeder cells. The same result was also reported by Kyozuka et al. (1987) and Jain et al. (1995). *L. multiflorum* cell suspensions, used in this study, have also been used as feeder cells to support protoplast division in banana (Megia et al., 1992) and in rice (Jain et al., 1995).

Protoplast plating density is important for initiating and sustaining division of protoplast-derived cells. It has also a significant effect on plating efficiency. Protoplasts are generally cultured at a density which varies from 10^4 to 10^6 mL^{-1}. In the present study, 5.0×10^5 mL^{-1} protoplasts ml^{-1} for the filter membrane culture with feeder cells were successfully usd to spport protoplast division and colony formation. Same density of protoplasts was cultured on filter membranes with feeder cells in Indica rice (Lee et al., 1989: Su et al., 1992). The filter membrane culture procedure with feeder cells to

allow a low plating density culture of rice protoplasts was also successfully developed. Plating efficiency was not affected by a lower protoplast density (0.1×10^5 mL^{-1}) with a similar plating efficiency at this concentration compared with protoplasts at the normal density (5×10^5 mL^{-1}) on the filter membrane when cultured in the presence of feeder cells. The results showed that the utilization of feeder cells for low plating density of protoplast culture significantly enhanced the plating efficiency. However, a higher density of protoplasts (10×10^5 mL^{-1}) decreased the plating efficiency.

Packed cell volume (pcv) of feeder cells in the medium has a major effect on the frequency of protoplast division. In the present study, the feeder cells improved the protoplast plating efficiency, depending on the feeder cell density. The highest plating efficiency was obtained at a feeder cell density of 0.5 mL pcv in 10 mL of protoplast culture medium. However, Gupta and Pattanayak (1993) reported that a 1.5 mL pcv of feeder cells in 10 mL of medium produced approximately two times more colonies than half dense feeder cells (0.75 mL pcv in 10 mL medium). However, doubling the feeder cell density (3 mL pcv in 10 mL medium) had a negative effect on rice mesophyll protoplasts in culture.

The improvement in protoplast plating efficiency with the use of a heat shock treatment in rice has been reported by Thompson et al. (1987). They evaluated the effect of heat shock treatment on rice protoplast viability, and reported an improvement in the stability and division of cultured rice protoplasts by combining the application of a heat shock treatment with agarose embedding culture without feeder cells. Heat shock treated protoplasts exhibited an effective doubling in both the frequency of plated protoplasts dividing and in their sustained division, compared to untreated protoplasts. In the present investigation, however, heat shock treatment before culture did not give any appreciable increase in the protoplast plating efficiency in the presence of feeder cells. Protoplasts exhibited a similar plating efficiency to untreated protoplasts. A similar observation was reported by Gupta and Pattanayak (1993), who found that heat shock treatment to the mesophyll protoplasts of rice prior to plating did not give any appreciable increase on the plating efficiency when applied feeder cell culture.

In vitro procedures routinely incorporate nutrients, growth regulators, a carbon source and, frequently, a gelling agent to support plant tissues. The type and concentration of gelling agent have been shown to affect the growth of tissues in culture (Bhattacharya et al., 1994: Wetzstein et al., 1994: Huang et al., 1995). In rice, KPR medium semi-solidified with Sea Plague agarose was suitable for sustaining protoplast division (Thompson et al., 1986). This was the first report of rice protoplast culture in Sea Plaque agarose medium. The present study was conducted to evaluate the effects of gelling agents on plating efficiency during rice protoplast culture on the filter membrane using feeder cells. Five different gelling agents, such as Sea Plaque agarose, Sigma Type I agarose, Phytagel™, Sigma Gum agar and Difco-Bacto agar, were assessed. Maximum plating efficiency was obtained when protoplasts were plated on medium semi-solidified with Sea Plaque agarose, followed by Sigma Type I agarose and Phytagel™.

In summary, maximum protoplast plating efficiencies on the filter membrane culture procedure with feeder cells in rice were obtained when protoplasts were cultured on medium semi-solidified with Sea Plaque agarose at a density of 5×10^5 mL^{-1} protoplasts in the presence of *Lolium multiflorum* cell suspension as feeder cells with 0.5 mL pcv per 10 mL of protoplast culture medium.

적 요

Japonica 벼 품종 Taipei 309 성숙 종자의 배반에서 유도된 캘러스로부터 유기 시킨 세포 현탁 배양체에서 원형질체를 분리하여 filter membrane과 feeder 세포를 이용한 여러 가지 조건에서 배양하였다. 이러한 조건들은 gelling agents, feeder 세포와 원형질체 밀도, feeder 세포의 종류 및 heat shock 처리 등이며 이들이 filter membrane 배양 방법에서 원형질체 평판 효율에 미치는 효과들을 조사하였다. 원형질체 평판 효율은, *Lolium multiflorum*을 feeder 세포로 사용하고 (10 mL의 원형질체 배양 배지당 0.5 mL pcv) 원형질체를 mL 당 5×10^5 개로 하여 Sea Plague agarose 배지에 원형질체를 배양 했을때 최고치를 얻었다. 원형질체에 heat shock 처리를 했을 때 원형질체 평판 효율은 변화가 없었다. carbohydrate source로서 sucrose 대신에 maltose를 사용했을 때 식물체 재분화율이 높았으며 원형질체로부터 재분화된 이들 식물체들은 임성을 나타내었다.

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