식물학회지 제36권 제3호 (1993)
Korean J. Bot. 36(3) : 211~218

Plant Regeneration from Protoplasts Isolated through Embryogenic Cell Suspension Culture in Rice

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벼 현탁배양을 통하여 분리된 원형질체로부터 식물체 재분화

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ABSTRACT

Plant regeneration was accomplished from protoplast culture of rice (*Oryza sativa* L. cv. Taebaeg). Embryogenic callus was induced from mature seed on MS medium containing 5 mM proline, 2.5 mg/L 2,4-D, 30 g/L sucrose in the dark at 28℃ and used to establish embryogenic cell suspension culture. Suspension cells were subcultured every one week in N6 medium supplemented with 5 mM proline, 200 mg/L casein hydrolysate, 2.5 mg/L 2,4-D and amino acids of AA medium. Suspension cultures were composed of cells that were densely cytoplasmic, potentially embryogenic and were at least maintained for more than 6 months in liquid medium. Protoplasts were isolated from fast-growing suspension culture cells and cultured in a slightly modified KpR medium by mixed nurse culture. Isolated protoplasts began to divide within 5~7 days and thereafter, protoplast-derived calli were sequentially transferred to callus proliferating medium that soft agar MS medium contained 2 mg/L 2,4-D and produced distinct embryogenic cells. Microcolonies were then transferred to solid medium which consisted of MS medium containing 5 mg/L kinetin, 1 mg/L NAA, 1 mg/L ABA, 30 g/L sucrose and 10 g/L sorbitol under fluorescent light. Multiple shoots of 4~5 per callus emerged and were transeferred to hormone-free MS medium for root initiation. Thereafter, The plantlets were transferred to pots of soil to mature in the culture room.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops. Therefore, the studies of tissue culture and genetic manipulation in rice plant are of great importance for the agricultural industry. The cultivation and regeneration of protoplasts is important as a prerequisite for the use of various technologies for breeding program such as gene transfer and somatic cell fusion. Protoplasts are usually used in these systems and transgenic cereals have mainly been obtained by using direct gene transfer requiring competent regenerable protoplasts (Lörz *et al.*, 1985; Fromm *et al.*, 1986; Potrykus, 1990). Success of plant regeneration from rice protoplasts has been rarely achieved only within the last several years (Fujimura *et al.*, 1985; Abdullah *et al.*, 1986; Coulibably and Demarly, 1986; Yamada *et al.*, 1986; Kyozuka *et al.*, 1987; Toriyama *et al.*, 1988; Jenes and Pauk, 1989; Lee *et al.*, 1989; Wang *et al.*, 1989; Hayashimoto *et al.*, 1990) and several research groups have been concerned in the development of cultivation of rice protoplasts which are suitable for use in experiments on genetic modification of plant cells. However, the cultivation techniques of rice protoplasts have not yet been developed sufficiently and the responsive genotypes are somewhat restricted.

We investigated procedures for the establishment of

embryogenic cell suspension from mature seed-derived calli and plant regeneration from embryogenic cell suspension-derived protoplasts of recalcitrant variety by mixed nurse culture (Kyozuka *et al.*, 1987).

MATERIALS AND METHODS

Callus induction and maintenance. Mature seeds of rice (*Oryza sativa* L. cv. Taebaeg) used in this study were provided by Crop Experiment Station, RDA. Seeds were sterilized by immersion in 70% (v/v) ethanol for 10 min and in 5% (v/v) sodium hypochlorite solution for 10 min, followed by three-times rinse with sterilized distilled water. The sterilized seeds were placed on the surface of agar medium containing salts of MS (Murashige and Skoog, 1962) supplemented with 5 mM proline, 2.5 mg/L 2,4-D, 30 g/L sucrose or N6 (Chu *et al.*, 1975) medium supplemented with 200 mg/L casein hydrolysate, 2.5 mg /L 2,4-D and 30 g/L sucrose. After 2 weeks, the only callus was selected from seed and were proliferated for 4 weeks under the same conditions as those adopted for callus induction.

Embryogenic cell suspension culture. The embryogenic calli were cut into small pieces and then transferred to 250 mL Erlenmeyer flasks containing 40 mL of liquid AA (Müller and Grafe, 1978) medium supplemented with 2.5 mg/L 2,4-D or N6 medium supplemented with 2.5 mg/L 2,4-D, 5 mM proline, 200 mg/L casein hydrolysate and amino acid of AA medium for establishment of cell suspension. The suspension cultures were placed on a shaking incubator (70 strokes/min) in the dark at 28℃ and subcultured every 3 to 4 days by adding a half of fresh medium until fast growing cell suspensions were established. After 2~3 weeks, large clumps of cells were removed by sieving through 500 μm diameter stainless steel mesh and actively growing fine cells were transferred to AA or N6 medium in 250 mL Erlenmeyer flasks and then placed on a rotary shaker at 120 rpm in the dim light (6 μE/sec m^2). Mechanical separation by sieving was applied every 3 weeks to obtain a more homogeneous fine suspension. Subculturing was carried out, once established, at 7-day intervals at a 3 : 1 (inoculum : fresh medium) ratio and suspension cultures were maintained for more than 6 months in liquid medium.

Protoplast isolation. Protoplasts were isolated from embryogenic suspension cells 2 to 3 days after subculture. About one gram (fresh weight) of suspension cells were incubated in 20 mL filter-sterilized solution that contained 1.2% (w/v) cellulase "onozuka" RS (Yakalt Honsha Co., Tokyo, Japan), 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), in CPW salts (Frearson *et al.*, 1973), 0.4 M mannitol, and 5 mM MES. The pH of the enzyme solution was adjusted to 5.6 before it was filtered. The cells were incubated on a rotary platform shaker (40 rev/min) for 2 hours at 28℃, followed by 2~3 hours stationary incubation. It was filtered through a series of 50 and 25 μm diameter stainless steel mesh to remove undigested cell clumps, and an equal volume of washing solution was added after centrifugation for 7 min at 100 *g*. Protoplast were collected and washed twice in the same solution by centrifugation at 80 *g* for 5 min and finally resuspended in a protoplast culture medium. The protoplast were then suspended in 1 mL of protoplast culture medium, layered on to 5 mL of 19% sucrose, and spinning at 30 *g* for about 60 sec allowed the fine protoplasts to be floated (Larkin, 1976).

Protoplast culture. Freshly isolated protoplasts were suspended at a density of 10^6/mL of protoplast culture medium and treated heat shock (Thompson *et al.*, 1987). The conditions for heat shock were 5 min at 45℃ followed by 10 sec in 4℃. Thereafter, protoplasts were cultured using several method, especially mixed nurse culture and basal media used were modified KpR (Kao and Michayluk, 1975). Feeder cells were Nakdong (*Oryza sativa* L. cv. Nakdong) and Taebaeg rice cell suspensions, and protoplast of Nakdong rice was obtained from method described by Hwang and Hwang (1991). One of 5~6 $\times 10^6$ protoplasts in suspension was mixed gently with an equal volume of prewarmed protoplast medium containing 1.2% (w/v) sea plaque agarose (FMC). Nurse cells were added in the liquid part of the culture and about 100 mg/5 mL of nurse cells were added to each plates, and then dishes were sealed and placed in the dark at 28℃. Agarose blocks were washed in protoplast culture medium to remove nurse cells and transferred to fresh medium 10 days later. The osmotic pressure of the liquid medium surrounding agarose embeded protoplasts was reduced by the addition of osmoticum reduction of approximately one-third at every two weeks (from medium containing 90 g/L, 60 g/L, 30 g/L glucose to medium containing only the sucrose). Visible colonies were transferred onto soft agarose medium containing MS basal medium, 2 mg/L 2,4-D, 0.3% agarose and cultured under dark at 28℃. In 2~3 weeks, colonies became about 1 mm in diameter and placed on the same medium with 0.5% agarose concentration in the dark at 28℃ until the colony size reached 1-2 mm diameter.

Plant regeneration. Protoplast-derived calli were

transferred onto MS basal medium containing 30 g/L sucrose, 10 g/L sorbitol, 5 mg/L kinetin, 1 mg/L NAA, 1 mg/L ABA and 0.8% agarose and then maintained under continuous 16 h/day light condition (fluorescent light 50 μE/sec m^2) at 28℃ and subcultured every 2 weeks. As shoots became 3 cm or longer, they were tranferred to a hormone-free MS basal medium for root initiation. The number of calli producing regenerated plants counted and regenerated plants were transferred to pot.

RESULTS AND DISCUSSION

Induction and culture of callus. Calli were induced from mature seeds on MS and N6 basal medium containing 2.5 mg/L 2,4-D within 2 weeks. Callus formation frequency was almost 100% and embryogenic callus appears about 20% of vials of non-embryogenic callus. Callus appearance was compact in which large cell-clumps were liable to be made. Embryogenic callus (EC) and nonembryogenic callus (NEC) are shown in Fig. 1. Nodular structures which appeared to be highly embryogenic formed on the surface and by visual examination it has been possible to visually select embryogenic calli.

Initiation and maintenance of cell suspension. Embryogenic cell suspensions were established using callus obtained from mature seeds and initiated from cluster of callus that contained embryogenic cells. The experiment was designed to obtain best medium and 2,4-D concentration for the production of embryogenic cell suspensions. Four basal media of MS, AA, N6 and R2 (Ohira *et al.*, 1973) were tested for their effect on rapid growth of cell suspension culture and the concentration of 2,4-D was determined. Suspension cultures were sustained in modified N6 and AA medium containing 2.5 mg/L 2,4-D. Cell suspensions were only established after 2~3 months under a subculture every 7 days. However, calli inoculated in MS and R2 suspension media turned brown. Calli in N6 medium were appeared more rapidly than that of AA medium and were highly cytoplasmic. In particular, the use of N6 medium containing amino acids of AA medium enables to rapid establishment of embryogenic cell suspension culture which readily yield protoplast. Thereafter, fast growing embryogenic cells composed mostly of highly cytoplasmic and spherical cells were obtained. Embryogenic cells were composed of cells ranging from less than 10 to 100 per cluster (Fig. 2). On the other hand, the large, elongated and vacuolated non-embryogenic cells of small group were also observed. Established culture exhibited a doubling in settled cell volume every one week and was maintained in exponential growth by weekly subculturing. Lee and Kim (1991) reported that N6 medium containning 5 mg/L 2,4-D is essential for embryogenic cell suspension culture of Taebaek rice. One of the important medium constituents influencing embryogenic cell suspension cultures is the nitrogen source. The nitrogen source has been found to be important for the development of cell suspension cultures (Veliky and Rose, 1973; Thompson *et al*, 1986) and for sustained division of protoplasts (Toriyama and Hinata, 1985) of rice. The results show that N6 medium, which contains nitrate and ammonium as the nitrogen source, was effctive in reducing the browning of the suspension cultures in Taebaek rice. However, addition of amino acids in the N6 medium to improve the morphology of the cultures was useful and this medium supported fast growth. Therefore, amino acids of AA medium were essential for embryogenic cell-suspension culture of Taebaeg rice. We is considered that there is a great diversity in the nitrogen source requirement of plants.

The concentration of 2.5 mg/L 2,4-D was suitable for sustainment of embryogenic cell suspensions and this is in contrast to previous report on embryogenic cell suspension culture of Taebaek rice (Lee and Kim, 1991). The concentration of 2,4-D had a significant influence on culture morphology and long period culture (Abdullah, 1987; Muller *et al.*, 1990). In particular, higher levels of 2,4-D concentration has been appeared to cause increased morphological abnormalities and cytological instability (Varga *et al.*, 1988; Hangyel Tarczy *et al.*, 1986). In this experiment, higher levels of 2,4-D were appeared negatively to culture morphology and suspension culture during long period.

In the beginning, the selection of embryogenic cell clusters and medium constituents were essential in the establishment of an embryogenic cell suspension culture of rice. The importance of these selection procedures has also been demonstrated by other workers. (Abdullah *et al*, 1986; Lee *et al.*, 1989; Shillito *et al*.1989; Thompson *et al.*, 1986; Li and Murai, 1990; Redway *et al*, 1990). In the result, requirements for success of suspension preparation include the selection and use of embryogenic small granular callus, duration time for subculture and 2,4-D level.

Protoplast isolation from suspension culture cells. Protoplast isolation from the cell suspension cultures was possible after 3 months of maintenances in AA and N6 basal medium. To remove cell walls, the osmotic potential of the medium containing hydrolyzing enzymes (1.2%

Table 1. The effect of culture methods on plating efficiency of protoplast derived from embryogenic suspension culture cells of Taebaeg rice

Culture method	Plating efficiency (%)
Agrose layer culture	<0.1
Liquid culture	0.1
Liquid drop culture	0.1
Agrose bead culture	0.3
Feeder cell layer culture	0.5
Mixed nurse culture	2.3

(w/v) cellulase "onozuka" RS and 0.1% (w/v) pectolyase Y-23) in CPW was adjusted with mannitol and 0.4 M mannitol concentration was suitable for stabilizing the protoplast. The length of digestion time was critical for obtaining both yields and high viabililty of the protoplasts and five hours of digestion was chosen as the most effective time. The pH of the enzyme solution was very critical and a slight deviation from pH 5.6 caused marked reduction in both the yield and viability of protoplasts. This results were consistent to which described in the preceding paper (Lee and Kim, 1991).

Typical yields of $5\sim6\times10^6$ protoplasts per gram of cell suspension culture could be obtained during 2 to 3 days after subculture. The freshly isolated protoplasts appeared highly cytoplasmic and cell debris or undigested cell aggregates were rarely observed and some spontaneous fusion of protoplasts occured during digestion in the enzyme (Fig. 3). The basal media employed in suspension cultures also significantly influenced the protoplast yield. To compare the medium effect, suspensions were subcultured weekly for months in the 2 seperate liquid media and harvested cells were digested enzymatically to release protoplast. Cells cultured in N6 medium released the largest number of protoplast and cultures in AA medium produced about 20% fewer protoplast than those in N6 medium. More than 90% of protoplasts isolated from suspension cultures in N6 medium exhibited a typical spherical shape with dense cytoplasm. In contrast, protoplasts isolated from suspension cultures in AA medium contained single elongated cells and small cell clumps to various extents.

Lee *et al.* (1989) reported that addition of amino acids in the medium was effect to protoplast isolation, and also Lee and kim (1991) reported that transfer to AA medium containing amino acids before protoplast isolation was very effective. We considered that N6 medium containing amino acids may be similar effect to protoplast isolation of suspension cells.

Protoplast culture. Within 2~3 days of culture, protoplasts lose their characteristic spherical shape and this has been taken as an indication of new cell wall synthesis. First division of protoplasts was observed at day 5 and continued till day 7 (Fig. 4). These were densely cytoplamic, and some of these showed features of imminent cell division. Microcolonies were identificable within the 2 weeks (Fig. 5). Successible cell divisions resulted in the formation of small and visible colonies (Fig. 6). Division frequencies of protoplasts isolated from culture cells are listed in Table 1 and were 0.3% without nurse cell, but 2.3% after 12 days of nurse culture. Four basal media of N6, MS, AA and KpR were examined for protoplast culture and modified KpR medium is suitable for obtaining sustained rice protoplast division. The presence of nurse cells was absolutely acquired to induce division of protoplasts and reduction in the osmotic pressure of the culture medium after the onset of cell division was necessary for colony formation (Two or three feedings of fresh medium with successively). The culture method and nurse cell quantity determined to produced the highest protoplast division frequency. They were cultured by a mixed nurse culture method and the use of the agarose-bead culture was essential to obtain reproducible colony formation. When examined the feeder-cell layer method of Horsch and Jones (1980), little division was detected. However, when agarose beads were placed on the nurse cell, protoplasts formed colonies with low frequency. This is in contrast to previous report on the protoplast culture of Taebaek rice (Lee and Kim, 1991). In several experiments involving large populations of protoplasts, high frequency of initial divions was observed which later ceased dividing. The maximum protoplast division frequency was found at nurse cell concentrations between 100 and 200 mg per 5 mL of protoplast medium. Whether there is any cell line specificity in the capacity to support protoplast division was also examined (not shown data) and the results showed that only Taebaeg cells had an appreciable effect on induction of protoplast division. Jeon and Lee (1992) reported that agarose culture with AA2 medium was very effective in promoting protoplast division. This difference may be in response to genotype-specific in rice variety. Several possibilities that might operate in a higher plating efficiency from protoplasts of rice are currently being investigated. Onto soft agar MS medium contained 2 mg/L 2,4-D, 90% of protoplast-derived calli developed embryo-like struture and the calli were similar in appearrance to embryogenic

calli of explant origin.

A combination of factors was necessary to overcome the difficulties of protoplast isolation, culture and finally plant regeneration from these rice cells. Protoplast division was strongly affected by the culture medium used and type of culture and hormone content. Earlier literature indicated that the application of nurse cells was absolutely required to induce divisions of rice protoplasts (Kyozuka *et al.*, 1987, 1988; Lee *et al.*, 1989; Shimamoto *et al.*, 1989). Also, fast growing suspensions were found to be essential for efficient protoplast isolation, division and subsequent plant regeneration. Especially, the quality of the embryogenic cell suspension seems to be the key factor for the success in obtaining plants from protoplasts. This fact has also been reported for regeneration from protoplasts of other cereals (Prioli and Sondahl, 1989; Shillito *et al.*, 1989; Vasil *et al.*, 1990).

Plant regeneration. The first sign of differentiation was the rapid formation of globular embryoids from approximately 90% colonies within 4 weeks of transferred, compact and granular calli obtained. A second round of transfer to MS medium containing 5 mg/L kinetin, 1 mg/L NAA, 1 mg/L ABA, 30 g/L sucrose and 10 g/L sorbitol was necessary for continued development of most of the embryo-like structure. After 3 weeks of subculture, green spots appeared on callus (Fig. 7), green spots grew eventually forming multiple shoots of 4~5 per callus. The leaves of the regenerated plants had reached the top of the flasks (Fig. 8) and roots had produced in hormone-free MS basal medium and the plantlet were then cultured in potted for further growth (Fig. 9). A combination of several factor was important for obtaining regenerated plants from protoplasts of Taebaeg rice. A combination of kinetin plus NAA was found most suitable in stimulating plant regeneration from protoplast derived colonies. MS medium was also suitable for achieving rapid regeneration from such compact protoplast-derived colonies. However, the differentiation was not obtained from colonies on N6 medium. The difference in the total nitrogen ratio of these two medium suggested that the nitrogen source plays an important role in obtaining differentiation from rice protoplast. These results should encourge efforts to transfer agronomically useful gene into rice through the direct delivery of DNA and applicative somatic hybridization.

적 요

태백벼(*Oryza sativa* L. cv. Taebaeg)의 원형질체 배양을 통하여 식물체 재분화가 이루어졌다. 배발생 캘러스는 5 mM proline, 2.5 mg/L 2,4-D, 30 g/L sucrose가 첨가된 MS 기본배지에 성숙종자를 치상하여 28℃ 암상태에서 유기하였고, 이러한 캘러스를 벼 현탁배양에 사용하였다. 현탁배양 세포들은 5 mM proline, 200 mg/L casein hydrolysate, 2.5 mg/L 2,4-D 그리고 AA 배지의 아미노산이 첨가된 N6 기본배지에서 일주일 간격으로 계대배양하였다. 이러한 현탁배양 세포들은 구형의 세포질이 충만한 배발생 세포군으로 구성되었으며, 6개월 이상 유지되었다. 현탁배양 세포로부터 분리된 원형질체는 다소 변형된 KpR 배지로 mixed nurse culture에 의해 배양되었으며, 5~7일 이내에 첫 분열이 관찰되었다. 그후 캘러스는 2,4-D가 2 mg/L 첨가된 soft-agar MS 배지로 옮겨 배양하였으며 전형적인 배발생 세포가 형성되었다. 이러한 캘러스는 5 mg/L kinetin, 1 mg/L NAA, 1mg/L ABA, 30 g/L sucrose and 10 g/L sorbitol이 첨가된 MS 배지에 옮겨졌으며, 캘러스당 4~5의 multiple shoot가 형성되었다. 그후 hormone-free MS 배지로 옮겨, 뿌리 분화를 유도한 다음 pot로 이식하여 성숙시켰다.

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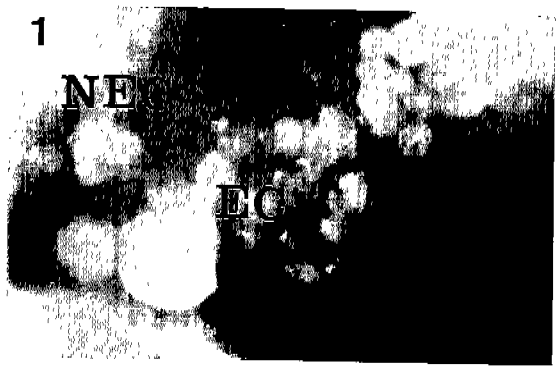
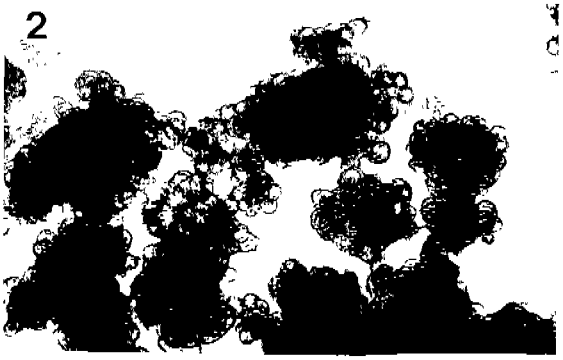
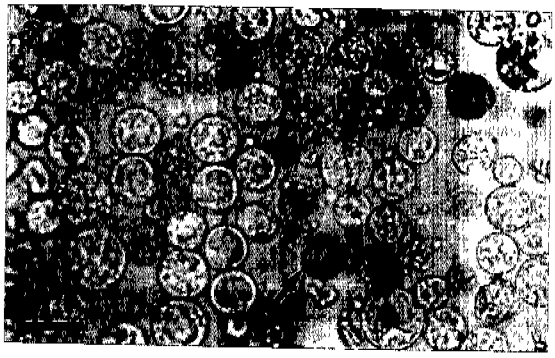
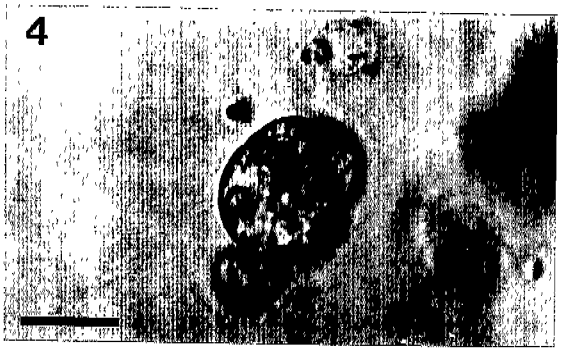
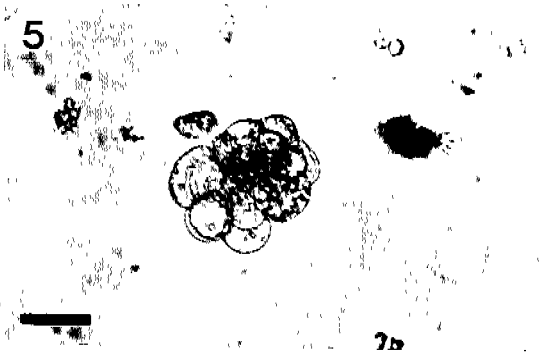
(Received April 10, 1993)

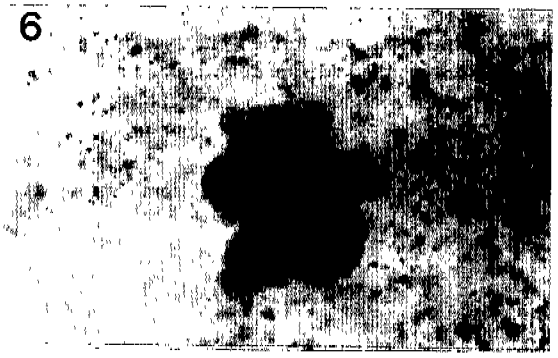












Explanation of Figures

Fig. 1. Comparision of embryogenic callus (EC) and non-embryogenic callus (NEC) induced from matured seed of rice. EC appears white, compact whereas NEC appears yellow to translucent and crystaline.

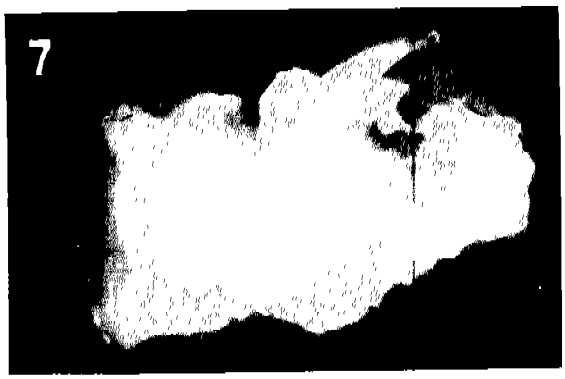
Fig. 2. Cells from embryogenic cell suspension cultures of rice. Embryogenic suspension cultures were established with selected embryogenic callus and were composed of densely cytoplasmic, spherical cells. Scale: 100 μm.

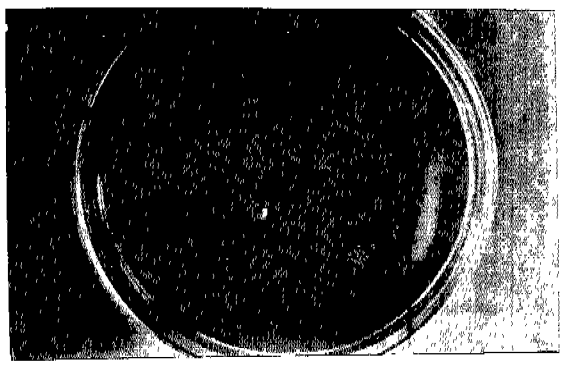
Fig. 3. Freshly isolated protoplasts from embryogenic cell suspension cultures of rice with the enzyme solution of 1.2% cellulase "onozuka" RS and 0.1% pectolyase Y-23. Damaged protoplasts were darkly stained with Evans blue 0.025% (w/v). Scale bar represents 30 μm.

Fig. 4. Initial cell division of rice protoplast after 5 days of culture. Scale bar represents 30 μm.

Fig. 5. Small cell cluster formed as cell division occurs without separation of the sister cells after 14 days. Scale bar represents 50 μm.

Fig. 6. A microscopic view of protoplast derived colony in an agarose block after 4 weeks. Scale: 100 μm.





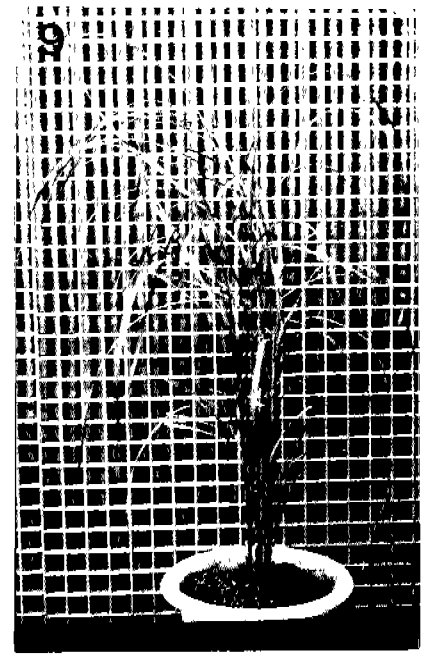


Fig. 7. Green spot was formed on embryogenic callus and grew eventually forming shoot. Embryogenic callus was cultured on solidfied MS medium containing 5 mg/L kinetin, 1 mg/L NAA, 1 mg/L ABA, 30 g/L sucrose and 10 g/L sorbitol under continuous 16 h/day light condition at 28℃.

Fig. 8. Overview of regenerated plant in flask. Shoots were formed on embryogenic callus growing on solid MS medium with hormones.

Fig. 9. Mature rice plants derived from protoplasts growing in soil.