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# Suspension and protoplast culture of U.S. rice cultivars

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**Abstract.** Efficient protoplast culture and plant regeneration of five U.S. rice cultivars (*Oryza sativa* L.) - Mercury, Lacassine, Maybelle, Cypress, and Lemont - were obtained from suspension cells maintained in modified General Medium. Embryogenic suspension cells were developed from calli grown on the original callus induction medium for 10-20 weeks without subculture. Weekly subculture of the suspensions for five to eight weeks yielded cells suitable for protoplast isolation. After 2 weeks, rate of colony formation from protoplasts varied among the cultivars and ranged from 2.5 to 6.8%. Improvement of plating efficiencies to as high as 13.7% was obtained by conducting a second cycle of protoplast culture. A total of 525 plants were regenerated from the cultivars studied.

**Abbreviations:** BAP, 6-benzylaminopurine; CH, casein acid hydrolysate; MGM, modified General Medium; Kin, kinetin; NAA, naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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## Introduction

Efficient rice protoplast culture systems can facilitate accomplishing several research objectives, including direct gene transfer and *in vitro* screening for desired characters that are expressed at the cellular level. Reports on regeneration of plants from rice protoplasts are accumulating, including japonica types, e.g., Taipei 309 (Fujimura *et al.*, 1985) and Nipponbare (Li and Murai, 1990), and indica types, e.g., Chinsurah Boro II (Datta *et al.*, 1990), IR 54 (Lee *et al.*, 1989) and IR 72 (Datta *et al.*, 1992). However, there is little information on protoplast culture and plant regeneration from protoplast-derived calli of javanica types. According to isozyme polymorphism analysis, U.S. rice cultivars belong to a group consisting primarily of japonica and javanica types (Glaszmann, 1986). Even though regeneration of the U.S. rice cultivar Labelle has been achieved from protoplasts (Xie, 1991), reliable protoplast culture systems for a wide range of commercial U.S. cultivars have not been reported.

This report describes an efficient suspension culture, protoplast culture, and plant regeneration system for the U.S. cultivars Mercury, Lacassine, Maybelle, Cypress, and Lemont. Lemont and Cypress are leading rice cultivars in the United States. Mercury,

Lacassine, and Cypress are cultivars released by the Rice Research Station, Crowley, LA, U.S.A, in 1987, 1991, and 1992, respectively. Mercury is a medium grain cultivar while the others are long grain cultivars. Maybelle is a very early maturing cultivar grown in the southern United States. The main objective of this research was to develop a protoplast culture system from which several commercial rice cultivars could be regenerated.

## Materials and Methods

*1. Initiation of suspension cultures*

Callus was induced from immature panicles 2-3 cm in length, using MS basal medium (Murashige and Skoog, 1962) supplemented with 2 $mg \cdot l^{-1}$ 2,4-D, 0.2 $mg \cdot l^{-1}$ Kin, 0.5 $g \cdot l^{-1}$ CH, 30 $g \cdot l^{-1}$ sucrose, and 7 $g \cdot l^{-1}$ Sigma purified agar at pH 5.8 in 100x15 mm plastic Petri dishes using 1-3 panicles per dish. Culture was carried out under dark conditions at 25°C. Callus formation started two weeks after plating. After culture for 10-20 weeks without transfer from the original dish of induction medium, 1.5 g per flask of friable, embryogenic callus tissue was placed in 250-ml Erlenmeyer flasks containing 50 ml liquid General Medium (Chen, 1986) that had been modified by adding 40 $mg \cdot l^{-1}$ $FeSO_4 \cdot 7H_2O$, 100 $mg \cdot l^{-1}$ myo-inositol, 10 $mg \cdot l^{-1}$ thiamine·HCl, 1 $mg \cdot l^{-1}$ pyridoxine·HCl, and 3 $g \cdot l^{-1}$ CH at pH 5.8. Suspension cells were subcultured weekly and maintained at 25°C on a gyratory shaker at 100 rpm under a light intensity of 9 $uEm^{-2} \cdot s^{-1}$. After 5-8 weeks, rapidly proliferating suspension cells were obtained and utilized for protoplast isolation.

*2. Protoplast isolation and culture*

Protoplasts were isolated from suspension cells 3-5 days after subculturing. Enzyme solution was prepared by filter sterilizing a solution of 1 g Sigma pectinase and 4 g Sigma cellulase (6.7 units·$mg^{-1}$) in 100 ml CPW 7 (Power and Davey, 1980). Twenty ml of enzyme solution was added to digest 3 g of suspension cells. Enzymatic digestion was carried out on a gyratory shaker (30 rpm) at 30°C for 3 h. An equal amount of washing solution containing 16.6 $g \cdot l^{-1}$ $MgCl_2 \cdot 6H_2O$, 12.5 $g \cdot l^{-1}$ $CaCl_2 \cdot 2H_2O$, and 8.7 $g \cdot l^{-1}$ KCl (Harms and Potrykus, 1978), was mixed with the enzymatic digest solution before filtering through 30 um nylon mesh. The filtrate was centrifuged at 130 g for 10 minutes. The protoplast pellets were collected and washed twice by resuspension in washing solution, with centrifugation at 130 g for 5 min. Purified protoplasts were suspended in MGM containing 0.4 M sucrose.

Protoplast culture procedures followed those of Shillito *et al.* (1983), combined with the mixed nurse culture method of Kyozuka *et al.* (1987). Four ml of protoplast suspension containing $2x10^6$ protoplasts·$ml^{-1}$ were mixed thoroughly with 4 ml of warm (45°C) protoplast nutrient medium containing 25 $g \cdot l^{-1}$ FMC SeaPlaque agarose before transfer to a 100x15 mm Petri dish. When solidified, the agarose sheet was cut into square centimeter blocks. Four agarose blocks were immersed in 20 ml protoplast solution containing approximately 400 mg nurse cells (Li and Murai, 1990). Nurse cells were developed from long-term cultures of the rice variety Taipei 309 in liquid MS medium supplemented with 1 $mg \cdot l^{-1}$ 2,4-D, 0.5 $mg \cdot l^{-1}$ thiamine·HCl, and 30 $g \cdot l^{-1}$ sucrose at pH 5.8. Three weeks prior to use, nurse cells were transferred to MGM. Protoplasts were cultured on a gyratory shaker at 30 rpm for 11 days at 28°C in the dark. At the end of this period, nurse cells were removed and fresh protoplast medium was added. Protoplasts were further cultured for 2 weeks. During this period, plating efficiencies were determined. Plating efficiency is defined as the percentage of the plated protoplasts which formed colonies, and was determined by examining $cm^3$ agarose blocks of plated protoplasts. Agarose blocks with visible colonies were placed onto soft protoplast medium containing 3 $g \cdot l^{-1}$ ultra pure DNA grade agarose (Bio-Rad) and a reduced concentration of sucrose (30 $g \cdot l^{-1}$). Colony propagation was carried out in the dark at 25°C for 10 days.

*3. Plant regeneration*

Fourteen calli with a diameter of 1 mm or more were plated per dish onto regeneration medium containing N6 basal medium (Chu *et al.*, 1975) with 30 $g \cdot l^{-1}$ sorbitol, 20 $g \cdot l^{-1}$ sucrose, 0.5 $g \cdot l^{-1}$ CH, 7 $g \cdot l^{-1}$ agarose, 2 $mg \cdot l^{-1}$ BAP, and 0.5 $mg \cdot l^{-1}$ NAA at pH 5.8. Calli were subcultured monthly and maintained at 25°C in a light regime of 16/8 light/dark with a light intensity of 15 $uEm^{-2} \cdot s^{-1}$. Small plantlets that formed were transferred onto MS medium containing 20 $g \cdot l^{-1}$ sucrose and 2.5 $g \cdot l^{-1}$ Gelrite at pH 5.8. Plantlets with good root systems were transferred to 20-cm pots filled with soil, placed in the greenhouse, flooded with water, and grown to maturity.

## Results and Discussion

A key step towards the establishment of suspension culture cells that have the capacity for long-term regeneration is the utilization of embryogenic calli. Embryogenic cultures sometime form in suspension medium after a period of culture using 3-5 week old calli as inoculum. However, these cultures typically go through a period of browning, and only a small percentage form embryogenic cells. The success of this procedure also varied from one rice cultivar to another, and was inefficient for some cultivars, such as Lemont.

Alternatively, embryogenic calli could be formed during the callus induction period simply by extending the period of culture on the original callus induction medium to 10-20 weeks. The embryogenic calli that formed arose from older calli and were friable, one mm or less in diameter, and rapidly growing. While secondary calli formed on only about 3% of the primary calli, this secondary growth appeared

consistently embryogenic. Unlike suspensions derived from 3-5 week old calli, suspensions initiated from these embryogenic calli did not go through a browning period. They readily proliferated in suspension culture, and were ready for protoplast isolation 5 to 8 weeks after initiation. This method appears to be a simpler way to establish embryogenic suspension cultures compared to manually selecting small-celled, cytoplasmically dense meristematic cell colonies, as suggested by Datta *et al.* (1992). No difficulties were encountered establishing embryogenic suspension cultures utilizing this method with the 5 cultivars evaluated in this study. Additionally, the embryogenic secondary callus growth can be readily identified, reducing the need for the technical expertise normally required to distinguish embryogenic from non-embryogenic phenotypes.

Improvements in cell growth occurred following medium modifications of the liquid General Medium. The Mercury suspension cell line used had been in culture for 26 months, while suspension cells of Lacassine, Maybelle, Cypress, and Lemont were 3 months old. Growth rates of each rice cultivar varied slightly (Table 1). Six days after subculturing, the amount of initial tissue nearly tripled for Maybelle and doubled for Lemont.

**Table 1.** Fresh weight (g) of suspension cell cultures of five U.S. rice cultivars at various lengths of time after subculturing.

| Rice cultivar | Days after subculturing 0 | 2 | 4 | 6 |
|---|---|---|---|---|
| Mercury | 1.00 | 1.05 ± 0.09[a] | 2.4 ± 0.14 | 2.8 ± 0.14 |
| Maybelle | 1.00 | 1.21 ± 0.08 | 2.6 ± 0.11 | 2.9 ± 0.12 |
| Lacassine | 1.00 | 1.33 ± 0.12 | 1.8 ± 0.13 | 2.1 ± 0.13 |
| Cypress | 1.00 | 1.15 ± 0.13 | 2.0 ± 0.12 | 2.4 ± 0.15 |
| Lemont | 1.00 | 1.30 ± 0.09 | 1.9 ± 0.10 | 2.0 ± 0.09 |

[a] Mean ± standard error, repeated 5 times

Regeneration capability of Mercury suspension cells over time is presented in Table 2. High regeneration rates were maintained by these cells. This may be partially due to the low concentration of 2,4-D used in this medium (1 $mg \cdot l^{-1}$), combined with the utilization of embryogenic cell lines. Regeneration ability started to decrease, however, after nine months in culture.

**Table 2.** Plant regeneration from Mercury suspension cells after various lengths of time in culture.

| Months in culture | % regeneration |
|---|---|
| 1 | 93 ± 4.3[a] |
| 3 | 85 ± 5.4 |
| 5 | 85 ± 3.2 |
| 7 | 87 ± 3.9 |
| 9 | 90 ± 6.7 |
| 11 | 71 ± 8.1 |
| 13 | 64 ± 7.3 |
| 15 | 46 ± 8.6 |

[a] Mean ± standard error of 5 replications of 14 calli per dish

To regain high regeneration rates, secondary Mercury suspension cultures were produced. Suspension cells were plated onto N6 regeneration medium without growth regulators. Friable cells were selected and used to develop new suspension cultures. Using this method, a high regeneration rate for suspension cells in long-term culture can be maintained. An average regeneration rate of 91% has been maintained for 34 months utilizing this procedure.

Protoplast yield varied between the cultivars (Table 3). Mercury was highest ($5.3 \times 10^6 \cdot g^{-1}$) and Cypress lowest ($3 \times 10^6 \cdot g^{-1}$). High protoplast viabilities were observed after enzymatic digestion of 3 hours. First divisions of protoplasts were observed at day 5, except for Cypress which occurred at day 8. Percent colony formation (plating efficiencies) for Mercury, Maybelle, and Lemont were high and comparable to those previously reported for japonica types (Li and Murai, 1990).

It appears that plating efficiencies can be further improved, however, by adding a second cycle of protoplast isolation (Table 4). First cycle protoplast-derived colonies that formed on agarose sheets plated on soft agarose medium were used to develop new suspension cultures. Once an adequate amount of suspension cells was obtained, protoplasts

**Table 3.** Protoplast culture and plant regeneration from five U.S. rice cultivars.

| Rice cultivar | Protoplast yield ($x10^6$)[a] | Protoplast viability (%)[b] | % colony formation after:[c] 1 week | 2 weeks | No. of calli plated[d] | No. of regen. plants[e] F | S |
|---|---|---|---|---|---|---|---|
| Mercury | 16 | 96 | 0.150 ± 0.026 | 6.8 ± 0.3 | 191 | 161 | 0 |
| Maybelle | 10 | 92 | 0.050 ± 0.030 | 5.1 ± 0.5 | 180 | 62 | 5 |
| Lacassine | 11 | 97 | 0.003 ± 0.006 | 2.6 ± 0.9 | 213 | 72 | 31 |
| Cypress | 9 | 89 | 0 | 2.5 ± 0.3 | 172 | 95 | 2 |
| Lemont | 11 | 96 | 0.110 ± 0.053 | 5.9 ± 0.7 | 207 | 91 | 6 |

[a] Based on enzymatic digestion of 3 g of suspension cells, repeated three times
[b] Based on FDA staining, repeated three times
[c] Based on observation of five 1-$mm^3$ agarose blocks; experiment repeated three times
[d] Based on observation of five 1-$cm^2$ agarose blocks; experiment repeated three times
[e] F=fertile; S=sterile

**Table 4.** Percent colony formation following two cycles of protoplast isolation.

| Rice cultivar | % colony formation |
|---|---|
| Mercury | 12.9 ± 0.4[a] |
| Maybelle | 13.7 ± 1.1 |
| Lacassine | 13.1 ± 0.9 |
| Cypress | 11.6 ± 1.8 |
| Lemont | 12.4 ± 0.7 |

[a] Mean ± standard error of five 1-$mm^3$ agarose blocks; experiment repeated three times

were again isolated and cultured as previously described. Following this second cycle, first cell divisions were observed 4 days after isolation. More importantly, plating efficiencies were improved and colony formation was more homogeneous with respect to time. Concurrent weekly protoplast isolations from callus-derived suspensions during a period of 3 months yielded consistently lower plating efficiencies for each cultivar (Table 3). On three occasions during this period when a second cycle of protoplast isolation was conducted using protoplast-derived suspension cultures, colony formation rates approximately doubled (Table 4).

A total of 525 green plants and one albino were regenerated from protoplasts in this study (Table 3). Under greenhouse conditions, plant sterility depended on the cultivar and ranged from 0 (Mercury) to 30% (Lacassine).

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