Plant Cell, Tissue and Organ Culture **44**: 155–159, 1996.

Growth and regenerability of long-term suspension cultures of the U.S. rice cultivar Mercury

Herry S. Utomo, Timothy P. Croughan & Suzan S. Croughan*
Rice Research Station, Louisiana State University Agricultural Center, P.O Box 1429, Crowley, LA 70527–1429, U.S.A. (requests for offprints)*

Received 29 December 1994; accepted in revised form 9 November 1995

Key words: Oryza sativa L., regeneration, tissue culture, rejuvenation

Abstract

Suspension cultures of the U.S. rice cultivar Mercury have been maintained in modified General Medium for more than 3 years. These suspensions have continued to have high and relatively stable regeneration rates. Two different explants, immature panicles and seeds, were compared during the development of these embryogenic suspensions. Initial formation of secondary embryogenic callus from immature panicles on induction medium was greater than that from seeds. Suspensions of these two cell lines, however, did not differ morphologically and maintained similar regeneration rates. After 5 months in culture the rates of regeneration began to decline. The suspensions were plated onto regeneration medium without growth regulators for 2 weeks and then embryogenic cells were manually selected and used to develop secondary suspensions. Through this simple rejuvenation procedure, the suspensions retained high and stable regeneration rates. Variability in suspension growth, however, was observed during the culture period. Slower growth occurred at weeks 13, 15, 27, and 29 and was associated with a decrease in regeneration rates. Reproductive fertility of regenerated plants remained high for 3.5 years but then declined.

Abbreviations: CH – casein (acid hydrolysate); 2,4-D – 2,4- dichlorophenoxyacetic acid; MS – Murashige & Skoog basal medium; SE – standard error

Introduction

The use of suspension cells of rice (*Oryza sativa* L.) can facilitate the accomplishment of several research objectives. They have been utilized to generate transformed rice plants, through both particle bombardment and polyethylene glycol treatment (Cao *et al.*, 1992; Lee *et al.*, 1991), and for *in vitro* screening (Yamada *et al.*, 1986). Rice suspension cells also have been widely utilized as a source for protoplasts that are used for somatic hybridization (Kyozuka *et al.*, 1989), the production of protoclones (Kanda *et al.*, 1988), transformation (Zhang & Wu 1988; Li *et al.*, 1992; Shimamoto *et al.*, 1989; Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Davey *et al.*, 1991), and *in vitro* screening (Xie, 1991; Utomo, 1994). In other species, suspension cells have been used for rapid and continuous production of uniform biomass for studies of basic plant cell physiology (Fry, 1990), enzymology (Herzbeck & Hüsemann, 1985), and secondary metabolites (Hall *et al.*, 1988).

Establishment, selection, and preferential culture methodology of embryogenic callus have been recognized as critical factors in maintaining long-term morphogenetic potential in cultures (Vasil & Vasil, 1984). In recent years, the establishment of embryogenic cultures has been achieved with several rice genotypes previously regarded as recalcitrant (Lee *et al.*, 1989; Datta *et al.*, 1990; Datta *et al.*, 1992). This resulted from better recognition of the conditions required to produce embryogenic calluses (Vasil & Vasil, 1991; Datta *et al.*, 1992). However, characterization and maintenance of long-term embryogenic rice suspensions has not been reported. This paper describes the characteristics of embryogenic suspension cells of the U.S. rice cultivar Mercury that have been in culture for over 3 years.

Table 1. Frequency of the formation of secondary embryogenic callus from the rice cultivar Mercury from immature panicle and seed-derived cultures that were developed 10–20 weeks after initial callus induction on the original callus induction medium[1].

Original explant	Number of explants plated	Mean±SE of secondary embryogenic calluses formed	Frequency of secondary embryogenic callus formation (%)
Immature panicles	400	12±4	3.00
Seed	400	7±3	1.75

[1] based on 3 replicates

Materials and methods

Explant sources

Initial calluses were induced from two different explants, immature panicles and mature seed. The immature panicles were obtained from either greenhouse- or field-grown plants at the early boot stage when immature panicles were 2–3 cm in length. Stems were surface sterilized in 22.5 g l^{-1} NaOCl for 30 min, followed by rinsing 3 times in sterile water. Immature panicles were aseptically removed and chopped into one-cm pieces or smaller, before transfer to 100×15 mm disposable plastic Petri dishes containing MS basal medium (Murashige & Skoog, 1962) supplemented with 9.05 μM 2,4-D, 0.93 μM Kinetin, 0.5 g l^{-1} CH (Sigma; Cat. # C-9386), 30 g l^{-1} sucrose, and 7 g l^{-1} purified agar (Sigma; Cat. # A-7049) at pH 5.8. Cultures were incubated in the dark at 25 °C for 10–20 weeks without subculture.

For seed-derived callus initiation, Mercury seeds were dehulled, shaken in 70% ethanol for 45 sec and surface sterilized in 22.5 g l^{-1} NaOCl with vigorous shaking for 20 min. After rinsing 3 times with sterile water, ten seeds per dish were plated onto callus induction medium. Following 10–14 days of culture, roots, shoots, and endosperm were removed from each seedling. The remaining root/shoot interface was transferred to fresh medium and further cultured in the dark at 25 °C for an additional 8–18 weeks without subculture.

Between the period of 10–20 weeks after initial plating, a frequency of secondary embryogenic callus formation from the two explants was determined. The frequency was calculated based on the number of cal-

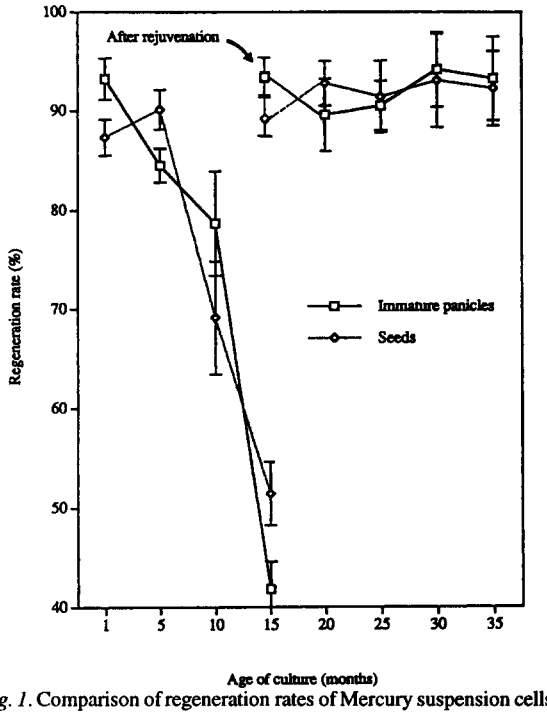

Fig. 1. Comparison of regeneration rates of Mercury suspension cells derived from immature panicles and seeds. Values are means based on five replicates. Bars indicate standard error.

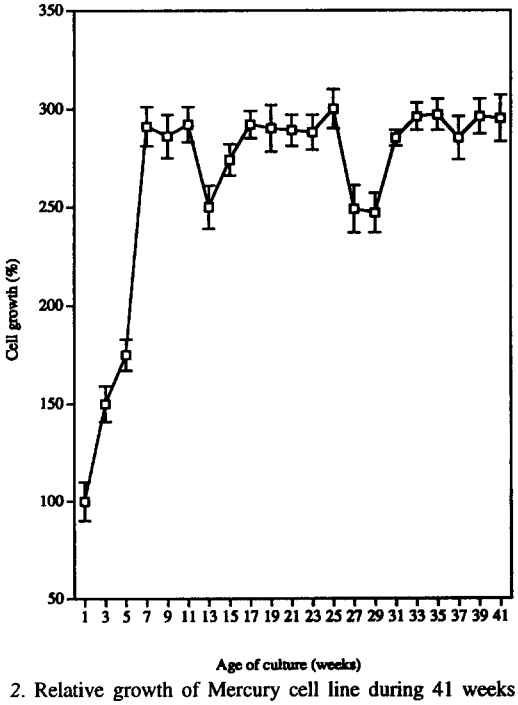

Fig. 2. Relative growth of Mercury cell line during 41 weeks of culture. Values are means based on five replicates. Bars indicate standard error.

Table 2. Growth of Mercury suspension cells derived from immature panicles and seeds after 5 and 36 months of culture.

Days after subculture	Fresh weight (g)[1] Mean±SE			
	Immature panicle derived		Seed derived	
	5 months	36 months	5 months	36 months
1	3.1±0.56	3.2±0.41	3.0±0.44	3.1±0.46
2	3.6±1.10	3.9±0.87	3.5±0.90	3.8±0.79
3	4.3±0.87	4.5±0.64	4.0±0.77	4.6±0.69
4	5.4±1.21	5.5±0.98	5.3±0.93	5.7±1.01
5	8.0±0.92	7.8±0.88	7.7±0.72	8.1±0.81
6	8.6±0.75	9.1±0.87	8.6±0.95	8.9±0.66

[1] based on 3 replicates.

luses which gave rise to secondary embryo calluses per number of initial calluses plated. The secondary embryogenic calluses consisted of yellowish cell granules, one mm in diameter or less, that vigorously grew on top of the primary calluses.

Suspension cultures

An inoculum of friable, compact callus tissue weighing 1.5 g was used to initiate each suspension culture. Calluses were placed in sterile 250 ml Erlenmeyer flasks containing 50 ml liquid General Medium (Chen, 1986; Li & Murai, 1990) that had been modified by adding 40 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 100 mg l^{-1} myo-inositol, 10 mg l^{-1} thiamine·HCl, 1 mg l^{-1} pyridoxine·HCl, 3 g l^{-1} CH, and 2.25 μM 2,4-D at pH 5.8. Flasks were capped with sterile aluminum foil, placed on a gyratory shaker at 100 rpm, and cultured under a fluorescent light intensity of 9 μmol m^{-2} s^{-1}, with a 16:8 h light:dark regime, at 25 °C. Suspensions were subcultured weekly by decanting the spent medium from each flask and replacing with fresh medium. Growth of suspension cells was determined based on fresh weight. The fresh weight was determined following removal of the liquid medium with a sterile pipette.

Plant regeneration

To regenerate plants, cell clumps were plated onto N6 basal medium (Chu *et al.*, 1975) supplemented with 30 g l^{-1} sorbitol, 20 g l^{-1} sucrose, 0.5 g l^{-1} CH, 7 g l^{-1} ultra pure DNA grade agarose (Bio-Rad; Cat # 162–0125), 8.89 μM 6-benzylaminopurine and 2.68 μM naphthaleneacetic acid at pH 5.8. Cultures were maintained with monthly subculture at 25 °C under a fluorescent light regime of 16:8 light:dark with a light intensity of about 15 μmol m^{-2} s^{-1}. Small plantlets that formed were transferred to MS medium containing 20 g l^{-1} sucrose and 2.5 g l^{-1} Gelrite at pH 5.8. Calluses that had not yet developed plants after one month were subcultured to fresh regeneration medium to encourage plantlet formation. Regeneration rates were determined after 2 months of culture, based on the number of calluses giving rise to plantlets per number of initial calluses plated. Plantlets were transferred to soil in 20 cm pots, placed in the greenhouse, flooded with water, and grown to maturity.

Table 3. Fertility of plants derived from suspension cultures after various lengths of culture.

Age of culture (months)	Percent fertility
1	100
5	100
10	100
15	96.3
20	100
25	100
30	99.1
35	100
40	96.8
45	68.7

Results and discussion

Secondary embryogenic calluses

With prolonged culture on the initial callus induction medium, the majority of calluses that formed turned

Table 4. Time needed to produce plantlets and the regeneration rate of suspension cells sampled during high and low cell growth periods.[1]

Suspension cells	Formation of the first plantlets (weeks)	Total regeneration period (months)	Regeneration rate (%)[2]
Cells sampled during a high cell growth period	2	1–2	89
Cells sampled during a low cell growth period	3	2–4	55

[1] based on 3 replicates.
[2] Regeneration rates were determined after two month-long passages on regeneration medium, based on the number of calluses giving rise to plantlets per number of initial calluses plated.

brown and eventually died. A small number of calluses, however, produced secondary embryogenic calluses. The frequency of secondary embryogenic callus formation was low: 3% and 1.75% for immature panicles and the root/shoot interface, respectively (Table 1). The secondary embryogenic callus consisted of cell granules one mm in diameter or less. It was compact, yellowish, and grew vigorously on top of the primary callus. This callus rapidly proliferated without browning when used to initiate suspension cultures. The formation of secondary embryogenic callus might be induced by starvation or other micro environmental stress due to the relatively long period of culture on the original medium without subculture. This may be similar to enhancement of the regeneration ability of indica rice through partial desiccation (Rance *et al.*, 1994). The importance of using embryogenic callus for tissue culture has been described (Vasil & Vasil, 1984). In this study, the capacity of suspension cells to maintain high regeneration rates for an extended period of culture appears due in part to the use of secondary embryogenic cell lines as inoculum.

Characteristics of Mercury suspension cultures

The growth of established Mercury suspension cells after weekly subculture followed a typical cell growth pattern; an initial lag phase of less than 2 days followed by exponential, linear, and stationary phases (Table 2). Doubling times were 4 to 5 days. By the end of a subculture period, cell fresh weight nearly tripled. There was no statistical difference in growth of suspension cells sampled at 5 and 36 months in culture. The suspensions did not differ morphologically.

Long-term monitoring of regeneration rates

Suspensions developed from secondary embryogenic calluses had high initial regeneration rates (Fig. 1). However, regeneration rates for suspensions from both explant sources started to drop after 5 months. Morphological examination indicated that this drop was accompanied by the formation of irregular shaped clumps that were very small, rapidly dividing, and less friable clumps. The problem, however, could be overcome by plating the suspension cells onto regeneration medium lacking growth regulators for 2 weeks. Embryogenic calluses were individually picked and used to initiate fresh suspensions. The embryogenic callus that formed on the solidified medium had morphological characteristics similar to the secondary callus that formed on the callus induction medium. This relatively simple rejuvenation procedure improved the regeneration rate of the suspensions to the levels comparable to younger cultures. More importantly, the secondary suspensions developed through this procedure maintained relatively stable regeneration rates through 3 additional years in culture. The study suggests that this procedure may serve to maintain high regeneration rates in long-term suspensions of rice and possibly other species. A very low level of sterility was observed among regenerated plants derived from Mercury suspesion cells during 40 months of culture (Table 3), but after 45 months sterility increased.

Long-term monitoring of cell growth

Suspension growth after the rejuvenation procedure was monitored every other week for 41 weeks by measuring the fresh weight of cells before subculturing. Changes in growth occurred over time (Fig. 2). Cell growth was marked by a period of early acclimatization that appeared to occur during the first 7 weeks. This process may entail selection for cells within the inoculum population that grow rapidly in suspension culture. Once these cells apparently predominate, growth rates remain relatively stable. Slight reductions of cell growth at weeks 13, 15, 27, and 29 were, however, observed. This reduction coincided with the production of larger clumps of cells within the cultures. Following this period, a rapid production of fine clumps was observed and cell growth subsequently returned to the normal rate. An observation associated with this change in growth within the cultures was that cells transferred to regeneration medium during periods of low cell growth rates regenerated fewer plants and at a slower rate than suspension cells with higher growth rates (Table 4). Once more highly dispersed, faster growing cultures were again observed, these were accompanied by recovery of regeneration capability.

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