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Arginine Enhancement of Cell Dissociation in Suspension-Cultured of Aromatic Rice Cells (*Oryza sativa* L. var Khao Dawk Mali 105)

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ABSTRACT

Suspension culture of *Oryza sativa* L. var. Khao Dawk Mali 105 was initiated from embryogenic region of callus derived from mature seeds cultured in the modified N_6 medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l kinetin, 2 % sucrose, 5 mM proline and various nitrogen sources. Three types of suspension-cultured medium (SM) with the supplementation of inorganic nitrogen (SM_1), or organic nitrogen plus arginine (SM_2), or inorganic nitrogen plus arginine (SM_3) were used.The suspension-cultured of rice callus in the SM gave cytoplasmically dense cell and homogeneous aggregation cell size larger than 1 mm, while those in the SM_2 produced cytoplasmically clear cell and aggregated cell size smaller than 1 mm. The addition of arginine to SM with inorganic nitrogen (SM_3) seemed to combine the desirable characters of finely dispersed cell aggregate and cytoplasmically dense cell. It was revealed by scanning electron micrographs indicating a difference in intercellular layer while cultured in 1 mM arginine containing SM medium was less deeply embedded in intercellular layer than in arginine-free medium. A finely dispersed cell suspension was obtained from rice callus tissue in SM supplemented with 1mM arginine. These results suggested that the suspension-cultured medium with inorganic nitrogen plus arginine should be suitable for generating an established suspension cell line for selection experiment as well as protoplast isolation.

Key words: arginine, cell aggregation, suspension culture, rice, Khao Dawk Mali 105

INTRODUCTION

A strategy in the field of plant cell culture is to isolate single cell without removal of cell walls, permitting selection of cells with wall and the control of cell growth and differentiation by synchronization of the culture. Because the single cells with walls may regenerate their normal walls more easily than those without walls (Hayashi *et al*., 1986) and grow normally to form clonal colonies and embryos. This objective should be useful for genetic manipulation via selection of transformants, mutation selection, superinduction model for gene expression (Bostock *et al*., 1999) and be a major contribution to plant biotechnology.

Hayashi and Yoshida (1988) reported that a combination of colchicine and galacturonan induced single cell separation in suspension-cultured soybean cells. The effects of colchicine and galacturonan were also observed in suspension-cultured carrot, tobacco and hibiscus cells. However, colchicine was inactive on suspension-cultured monocotyledonous cells, probably because the wall composition varies from that in dicotyledon (Burke *et al*., 1974). This indicates that the compounds and/or receptors binding cells in monocotyledons

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are not the same as those in dicotyledons. In addition, the level of cell aggregation in callus tissue of monocotyledons is usually much higher than that in dicotyledons.

There are various media favorable for suspension cultures include N_6 (Lee *et al*., 1989; Wang *et al*., 1989), AA (Abdullah *et al*., 1986; Toriyama *et al*., 1986; Yamada *et al*., 1986), R_2 (Fujimura *et al*., 1985; Kyozuka *et al*., 1988), LS (Abdullah *et al*., 1986) and B_5 (Fujimura *et al*., 1985). Amino acid medium (AA medium) which is composed of aspartic acid, arginine, glycine, and glutamine as a nitrogen source, has been known to evoke a finely dispersed cell suspension in suspension-cultured rice cells (Abdullah *et al*., 1986; Toriyama *et al*., 1986; Yamada *et al*., 1986). AA medium was effectively established suspension-cultured cells for japonica rice (Toriyama and Hinata, 1985; Yamada *et al*., 1986). For indica rice, a number of suspension-cultured media, such as N_6 medium (Lee *et al*., 1989), R_2 medium (Kyozuka *et al*., 1988) and AA medium (Datta *et al*., 1990; Sun *et al*., 1990) were used.

Toriyama and Hinata (1985) used AA medium for rice suspension culture to obtain protoplast at the yields of more than 90%; although only a few percent were obtained as protoplasts from an inorganic nitrogen medium such as B_5 or N_6 medium. Nevertheless, an inorganic medium is essential for the organogenesis and embryogenesis of rice cells (Toriyama and Hinata, 1985; Ozawa and Komamine, 1989) and for increasing cytoplasmic density of cultured cell (Yin *et al*., 1993). Hayashi *et al*. (1994) studied the effects of AA medium on the dissociation of rice callus tissue by examined a finely dispersed cell in suspension-cultured cells. Here we describe a simple procedure for the establishment of homogeneous suspension-cultured of aromatic rice cell by using medium containing arginine in conjunction with inorganic nitrogen or organic nitrogen.

MATERIALS AND METHODS

Establishment of suspension-cultured cells

Suspension-cultured cells were initiated from mature seed scutellum of rice [*Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML 105)]. Cultures of friable embryogenic callus obtained in the modified N_6 medium containing 2% sucrose, 1.5 mg/l 2,4-D, 10 mM proline, 10 mg/l $AgNO_3$ and 32 mg/l cystein were collected from large cell- aggregated (<2 mm) and transferred to suspension-cultured medium (SM) comprised various nitrogen sources. Three modified SM formulae were applied. SM_1 medium contained 2830 mg/l KNO_3 and 46.3 mg/l $(NH_4)_2SO_4$ as an inorganic nitrogen source. SM_2 medium resembled AA medium, contained 6 mM glutamine, 2 mM aspartic acid, 1 mM arginine and 0.1 mM glycine as an organic nitrogen source. SM_3 medium contained 1 mM arginine, 2830 mg/l KNO_3 and 46.3 mg/l $(NH_4)_2SO_4$ as a nitrogen source. All media contained 1.5 mg/l 2,4-D and 0.25 mg/l kinetin as a plant hormone, 20 mg/l sucrose as a carbon source plus minerals and vitamins at the same level (Table 1). Suspension-cultured cells were placed on a gyratory shaker at 120 rpm and weekly subcultured at 1:4 dilution with fresh medium. The effect of nitrogen from inorganic or organic source was indicated by the qualities of suspension-cultured cells in terms of growth rate, cell viability, densely cytoplasmic and cell dispersion after inoculation for 2 weeks.

Electron microscopic observation

Microcalli arising from SM_1, SM_2 and SM_3 were prefixed in 25% butyl alcohol, rinsed 3-4 times in phosphate buffer, dehydrated in an ethanol series (30-100% v/v), and critical-point dried. Dried specimens were coated with gold-palladium alloy and examined in a Joel JSM-35 cf scanning electron microscope at 15 kv.

Cell aggregate sizing

Suspension-cultured cells were subcultured at 7 days interval. After subculturing for 21 days,

Table 1 Composition of suspension culture medium (SM) consisted of the N_6 basal salt and various nitrogen sources for suspension-cultured rice cells of KDML105.

Composition	Medium		
	SM_1	SM_2	SM_3
Micronutrients	N_6	N_6	N_6
Macronutrients (KNO_3 and $(NH_4)_2SO_4$ not included)	N_6	N_6	N_6
Vitamins	N_6	N_6	N_6
Amino acid (mM)			
-Proline	5.0	5.0	5.0
-Glutamine	-	6.0	-
-Aspartic acid	-	2.0	-
-Arginine	-	1.0	1.0
-Glycine	-	0.1	-
KNO_3 (mg l^{-1})	2830.0	-	2830.0
$(NH_4)_2SO_4$ (mg l^{-1})	46.3	-	46.3
Sucrose (g l^{-1})	20.0	20.0	20.0
2,4-D (mg l^{-1})	1.5	1.5	1.5
Kinetin (mg l^{-1})	0.25	0.25	0.25

cell-aggregated were sieved through stainless screen with 1000 μm pore size. They were placed on sterilized paper towels to remove water and then weight was recorded.

Cell viability

Cell viability was estimated by staining cells with FDA (Fluorescein diacetate) and Evans blue. The percentage of living cells stained with 0.01% FDA and of dead cells stained with 0.1% Evans blue were recorded.

Monitoring cell growth

Growth was monitored at 4 days after subculturing cells on the basis of fresh weight. A 10 ml aliquot of uniformly distributed suspension cells was vacuum filtered through whatman filter paper no. 4. Fresh weight of the retained cells was calculated from the weight of water soaked filter disc minus wet paper weight.

RESULTS AND DISCUSSION

Optimization of suspension-cultured medium

Suspension-cultured cells were established 3-4 months later. Microcalli were transferred to the suspension-cultured medium (SM) for 3 weeks and supplemented with various nitrogen sources designated as SM_1 (resembled N_6 medium), SM_2 (resembled AA medium) and SM_3 (resembled N_6 medium plus arginine), respectively (Table 1). The differences of cell-aggregated size and cytoplasmic density of cells were visible. 78% of fine suspension cell-aggregated size less than 1 mm while culturing in the SM_2 medium was observed (Figure 1A). Culturing in the SM_1 medium, approximately 63% of large cell clumps (>1mm) was obtained (Figure 1B), however, the SM_1 medium seemed to increase densely cytoplasmic cells (Figure 1C). Small cell-aggregated in the SM_2 and SM_3 were easily dispersed and finally developed a fine suspension-

cultured cells (Figure 1 D, 1E and 1F). Arginine in the SM_3 medium indicated a favorable combination effect of the SM_1 and SM_2 media. It gave 73% of cell-aggregated developed to more finely and composed of densely cytoplasmic cells (Figure 2A and 2B).

Considering the significance of the nitrogen source in the culture medium, 3 modified media were devised to investigate the effect of nitrogen source on the quality of suspension-cultured cells (Table 2). The estimation of cell viability showed that cell-cultured with inorganic nitrogen (KNO_3 and $(NH_4)_2SO_4$) in the SM_1 and SM_3 media, revealed a higher amount of dead cells than those with amino acids treatment (SM_2). This suggested that inorganic nitrogen has a more toxic potential to the cultured cells. The nitrogen in culture medium has been known to play a key role on the qualities of rice cell in tissue culture (Koetje *et al*., 1989; Yin *et al*., 1993; Hayashi *et al*., 1994). High concentration of NH_4^+ is harmful to the cultured cells (Chu *et al*., 1975; Yamada *et al*., 1986). In addition, there were several reports suggesting that the difference in nitrogen ratio in medium-cultured causes the

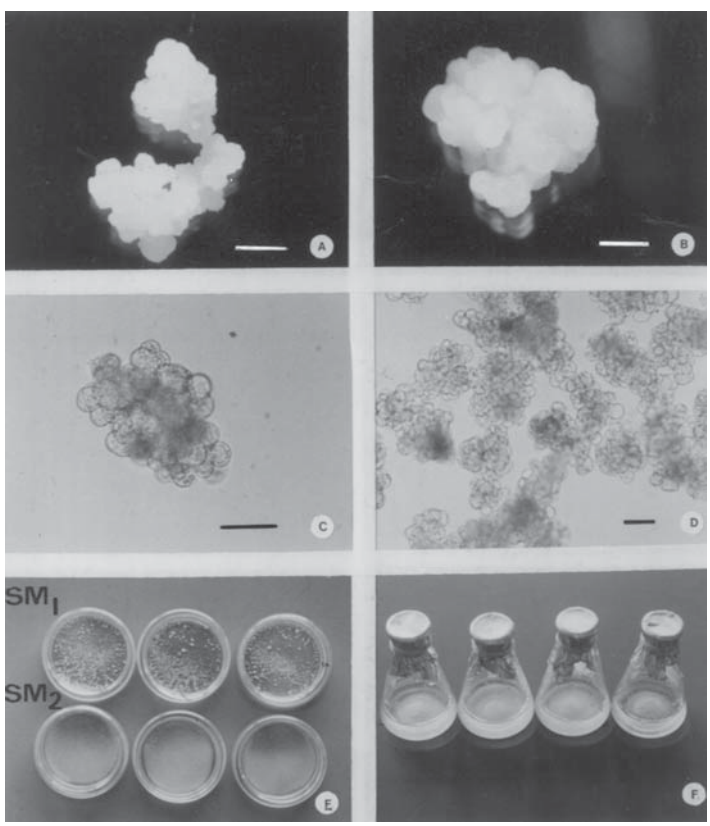

Figure 1 The microcalli of rice cv. Khao Dawk Mali 105 proliferated in the SM_2 and SM_3 culture media composed of friable nodular-like structures (bar = 1 mm) (A) which was smaller than those appeared in the SM_1 medium (bar = 1 mm) (B). Most of cell aggregations in the SM_1 consisted of highly cytoplasmic cells (C). Small cell aggregates in the SM_2 and SM_3 easily dispersed and finally developed a fine suspension culture (bar = 0.05 mm) (D). Compare suspension-cultured rice cells in the SM_1 medium (upper) with the SM_2 medium (SM plus arginine, lower) (E). Fine cell suspensions consisted of small and densely cytoplasmic cell colonies in the SM_3 medium (F).

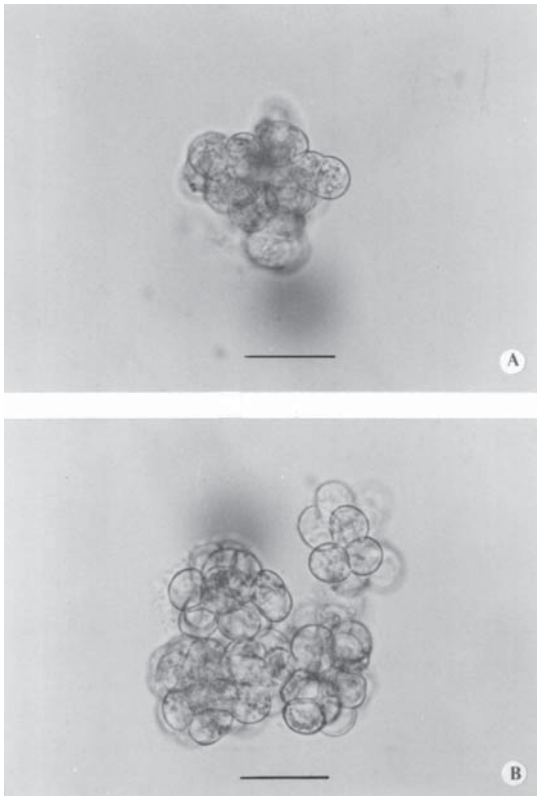

Figure 2 The cell-aggregated of rice cv. Khao Dawk Mali 105 in the N_6 medium containing 1 mM arginine (SM_3) composed of more highly cytoplasmic cells (A) than those cultured in the amino acid-containing N_6 medium (SM_2) (B) (all bar = 0.05 mm).

deviation of nitrogen metabolism especially NH_3 induced towards polyamine which is an important source of H_2O_2 that provides to the generation and accumulation of high toxic radical OH° (Angelini and Federico, 1989; Le Dily *et al*., 1983). Therefore a dramatic increase in the number of dead cells in the presence of inorganic nitrogen may be due to the adverse effect of NH_4^+in the medium.

Effects of arginine on rice cell-cultured

From scanning electron micrographs, they indicated the variation in cell-aggregated size among various culture media. Microcalli grown in the SM_2 and SM_3 media were obtained. They composed of homogeneous nodular-like structures which were smaller than those appeared in the SM_1 medium (Figure 3A and 3 B). The appearance of cell-cultured also represented a distinct difference in the intercellular layer of cells. Cells cultured in the SM_1 medium (inorganic nitrogen medium) were more deeply embedded (Figure 3D) than those in the SM_2 medium (organic nitrogen plus arginine) and the SM_3 medium (inorganic nitrogen plus arginine) (Figure 3C). This indicated that cell-cultured in the SM_1 medium contained more wall material between cells than those in the SM_2 and SM_3 media. It also implied that arginine in medium-cultured plays a key role in inhibiting wall material accumulation

Table 2 Effect of the organic nitrogen source in 3 SM media on the quality of suspension-cultured rice cells of KDML 105.

Medium	*Quality of suspension-cultured rice cells			
	Growth rate	Dispersion	Densely cells cytoplasmic	Dead cell percentage
SM_1	++	++	+++	17
SM_2	++	+++	+	5
SM_3	++	+++	+++	15

* + = low; ++ = medium and +++ = high
SM_1 = inorganic nitrogen medium
SM_2 = organic nitrogen plus arginine
SM_3 = inorganic nitrogen plus arginine

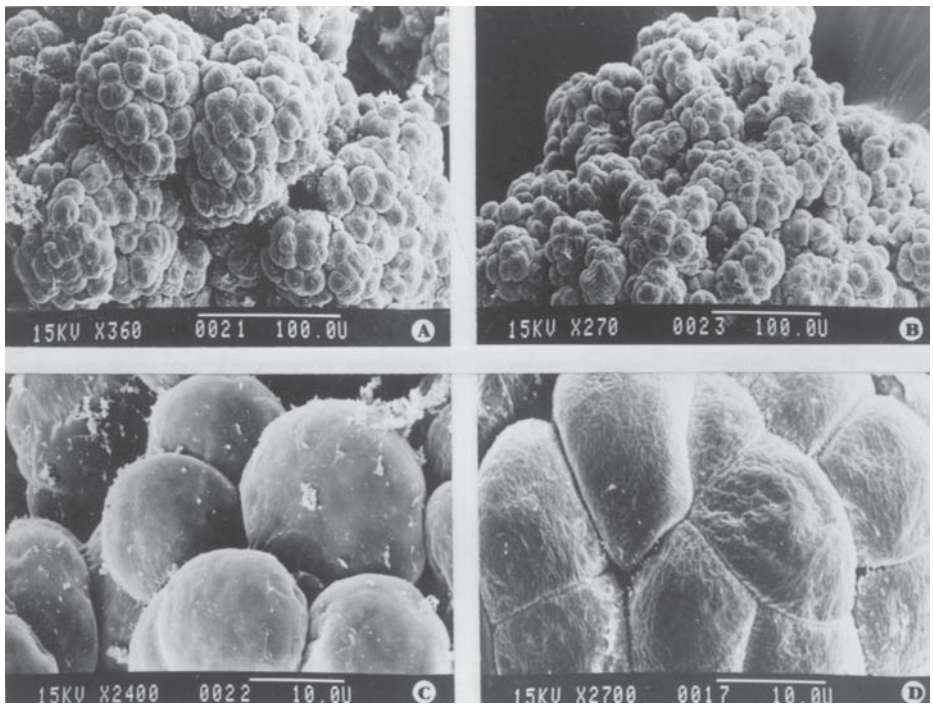

Figure 3 Scanning electron micrographs of embryogenic calli of rice cv. Khao Dawk Mali 105, cultured in the SM_1 medium (A) homogeneously composed of nodular structures which were larger than those appeared after culturing in the SM_2 and SM_3 media (B). The cultured cells in the SM_2 and SM_3 media (C) revealed less deeply embedded in intercellular of cells than those in the SM_1 medium (D).

which may support the explanation for easily dispersed small aggregated cell in the SM_2 and SM_3 media. The ability of arginine to interfere with the accumulation of wall material was reported and suggested by Hayashi *et al*. (1994) that arginine in AA medium is metabolized in a rice cell to form urea and that urea solubilizes insoluble proteins of polysaccharides by inhibiting the formation of hydrogen bond.

Cells observed on both media-cultured with inorganic nitrogen (SM_1 and SM_3) were small, round and densely cytoplasmic which also released round and densely cytoplasmic protoplasts. This suggested that inorganic nitrogens, KNO_3 and $(NH_4)_2SO_4$ were needed to increase cytoplasmic density of cell-cultured but they tend to form large cell clumps. Cell-aggregated in the SM_1 medium was decreased by several subcultures with the SM_2 medium. This implied that the SM_2 medium induces the dissociation of cell aggregates. It is likely that the dissociation observed in the SM_2 medium localized on the surface of cell-aggregated, where cell division occurs. This result agrees with the observation in AA medium (Hayashi *et al*., 1994). The addition of arginine could evoke cell dispersion the same as a combination of amino acid in the AA medium and appears to be useful for dissociating cell-aggregated. Its effect was also observed in suspension-cultured barley and rice cells (Hayashi *et al*., 1994) and vetiver cells (unpublished data). The established cell suspension could increase the efficiency of electroporation, biolistic and cell selection in monocotyledonous plants. However, we have not succeeded in separating cell-aggregated

into a single cell and the aggregated size is approximately 700-1000 mm, which is similar to that in cell suspension of dicotyledons such as soybeans, carrot and poplar.

In conclusion, arginine addition showed to assist N_6 medium in revealing the most prominent appearances to cell-cultured in terms of cell dispersion and increasing densely cytoplasmic cells which lead to a high density of healthy protoplasts. The optimal suspension-cultured medium of rice cv. Khao Dawk Mali 105 is modified N_6 medium supplemented with 1 mM arginine. From Table 2, the following points could be summarized:

-The addition of KNO_3 and $(NH_4)_2SO_4$ to suspension-cultured medium increased densely cytoplasmic cell-cultured. At the same time, large cell clumps were also formed which made protoplast isolation become more difficult.

-The addition of inorganic nitrogen was harmful to cell-cultured, this may due to an adverse effect of NH_4^+.

-The addition of amino acids to suspension-cultured medium evoked cell dispersion.

-The addition of 1 mM arginine played a key role in promoting cell dissociation.

Yin *et al.* (1993) considered the significance of nitrogen source in MS medium for cell-cultured indica rice and found that the ratio of KNO_3/ NH_4NO_3 plays a critical role in determining the quality of suspension-cultured cells. In their cases, the combination of MS with AA medium was found to be the most effective for maintaining suspension-cultured cells.

Based on this study, N_6 basal medium with the presence of 1 mM arginine was suitable for developing rice cv. Khao Dawk Mali 105 cell-cultured used for protoplast isolation and genetic manipulation. The cell line which was established by this system exhibited a rapid growth rate, uniformly small cell-cultured, tiny, round and densely cytoplasmic cells. These cultured procedure should be applied to use with other monocotyledonous plants.

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