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Culture of Isolated Tobacco Mesophyll Cells

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Since regeneration of cell walls are considered to be a prerequistite for the cytokinesis in protoplast culture,[1] we thought it would be easier to culture free or isolated cells (that have cell walls) than that of wall-less protoplasts. Furthermore, isolated cells are an interesting material for transformation studies in plants because their cell walls are porous enough to allow introduction of foreign substances such as viral RNA into cells in response to electric pulses.[2,3] Although protoplast culture has been widely researched, few studies have been conducted on the culture of isolated cells.[4,5] Here we report the culture of enzymatically isolated mesophyll cells of tobacco.

Individual mesophyll cells were isolated from fully expanded leaves of three-month-old *Nicotiana tabacum* "Samsun." Plants were grown in a greenhouse, under natural light at 25±5℃. Leaves were cut into 6 pieces and then surface-sterilized by using 70% ethanol for 10 sec, 0.2% benzalkonyl chloride for 10 min, 1% sodium hypochlorite for 5 min and then rinsed 3 times. After surface sterilization, the lower epidermis of the leaf section was peeled off and the leaf section was placed into a filter-sterilized solution of 0.5% Macerozyme R-10 (Yakult Yakuhin Kogyo Co.), 25 mM 2-(N-morpholino) ethane sulfonic acid (MES), 5 mM $MgCl_2$ and 400 mM glucose at pH 5.6. Cultures were then incubated at 26℃, in the dark, with continuous agitation (50 rpm) for a total of 4 hr. Halfway through the incubation period (2 hr) the enzyme solution was replaced by fresh enzyme solution. After incubation, the mixture was filtered through 4 layers of gauze. The isolated mesophyll cells were washed three times by centrifugation (37 g for 1 min) with an appropriate culture medium (as described in **Table 1**). After washing, approximately 1×10^5 cells were cultured in 1.0 ml of medium in a plastic petri plate (35 mm i.d.). Culture incubation was carried out at 26℃, without agitation, and in the dark. **Table 1** lists various modified Kao-8p[6] media tested. All media contained 1.6×10^{-5} M 1-naphthaleneacetic acid (NAA) and 4.4×10^{-6} M 6-benzyladenine (BA).

Viable cells were assayed by staining with 1% Nigrosine B (Chroma Co.). Viable and dead cells were counted in three different microscopic fields (total of approximately 50 cells per field) and percent viable cells was calculated. Cells in a dividing cell cluster, if any, were counted individually (see below).

Previously we had determined that the percentage of viable cells after 1 day was not dependent upon the kind of the media used, but dependent upon the type of osmoticum. Viability of cells grown on medium containing 0.4 M glucose, mannitol or sucrose was 30-32%, 23-28% and 10-15%, respectively (all media were filter-sterilized). Therefore in this study, glucose was used as an osmoticum.

Table 2 shows the percentage of viable cells after 2 or 7 days. After 2 days, viable cells were observed in all cultures with the highest percent viability in cultures containing IM-4 medium. Interestingly, after 7 days, viable cells were observed only in IM-4 cultures. The presence of activated charcoal did not affect or decreased cell survival after 2 days but increase viability after 7 days.

Evidence of cell division is demonstrated by the percentage of viable cells in IM-4 medium plus charcoal which was greater after 7 days than after 2 days (**Table 2** and **Fig. 1C**).

Table 1. Various modified Kao-8 p basal media.[a]

Media	Modifications in Kao-8 p basal medium
IM-1	1/2 coconut water and 1/2 casamino acids
IM-2	1/10 NH_4NO_3 and 1/2 Fe
IM-3	1/4 organic acids and amino acids, nucleic acid bases, coconut water and casamino acids were omitted
IM-4	1/10 NH_4NO_3, 1/2 Fe and 1/4 organic acids and nucleic acid bases, coconut water and casamino acids were omitted
IM-5	same as IM-4 except that vitamins and sugars were omitted while thiamine HCl (0.4 mg/*l*) and myoinositol (100 mg/*l*) were added

[a] Each medium contains 1.6×10^{-5} M NAA and 4.4×10^{-6} M BA.

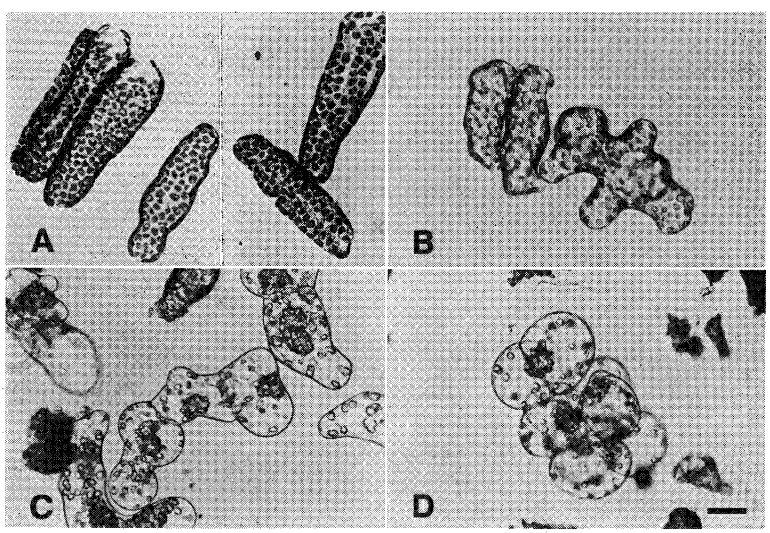

Fig. 1. The time course of the culture of isolated tobacco mesophyll cells. The cells were cultured in IM-4 medium containing 0.4 M glucose with 1% activated charcoal in the dark without shaking at 26°C. A, freshly isolated mesophyll cells. B, mesophyll cells after 5-day culture. C, small cell colonies after 7-day culture. D, small cell colonies after 18-day culture. Black patches in D are the pieces of activated charcoal solidified with agar medium. Bar represents 30 μm.

Figure 1 shows the time course of the culture of isolated mesophyll cells. Freshly isolated mesophyll cells were long and narrow and green in color with very dense chloroplasts (A). After 5 days in culture, cells changed their shape and became light green with sparsely distributed chloroplasts (B). During 6 or 7 days cell division occurred and small cell colonies of about five cells formed (C). Chloroplast in these cells were located around the nucleus. Dead cells were plasmolyzed, shrunken and brownish. Small colonies of 10 to 15 cells had formed after 18 days in culture (D). These colonies continued to develop to be 100 cells large after 30 days in culture.

The maximal frequency of colony formation of the isolated cells in the present study was estimated to be about 20%, which is lower than the reported values for tobacco mesophyll protoplasts.[4] The causes for this are not fully understood yet. It is possible, however, that various lytic enzymes contained in Macerozyme were not washed out from the surface of the plasmamembrane (because of the presence of cell walls) or from cell wall matrix after isolation of free cells and that these enzymes inhib-

Table 2. Percentage of viable cells of tobacco after culture in various media in the presence or absence of activated charcoal

Basal media	Activated[a] charcoal	Percentage of viable[b] cells after culture for	
		2 days	7 days
IM-1	−	5.1±1.2	0
	+	3.9±1.9	0
IM-2	−	13.7±3.3	0
	+	8.1±2.2	0
IM-3	−	0.7±1.0	0
	+	4.7±0.7	0
IM-4	−	32.0±2.2	25.4±3.8
	+	21.7±4.4	43.9±8.4
IM-5	−	9.4±1.2	0
	+	8.2±0.6	0

[a] One slice (*ca.* 160 mg fresh weight) of the culture medium containing 0.6% agar and 8% activated charcoal was added to 1 ml of culture medium (final concentration of activated charcoal was about 1%).

[b] The numbers represent average of three determinations±standard deviation.

ited cell division. It is well known that cell walls regenerated during an early stage of protoplast culture differ in their composition and structure from those of intact tissues or cultured cells.[7] Thus, it is also possible that nascent cell walls of the isolated cells do not support cell division.

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≪和文要旨≫

タバコ葉肉遊離細胞の培養

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タバコ（*Nicotiana tabacum* var. Samsun）葉肉遊離細胞の培養について研究した．その結果，細胞を 1.6×10^{-5} M ナフタレン酢酸，4.4×10^{-6} M ベンジルアデニン，0.4 M グルコース，1% 活性炭を含む改変 Kao-8P 基本培地で培養すると6～7日目に分裂を開始し，18日目には 10～15 細胞集塊を形成することがわかった．