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Callus Formation and Root Differentiation by Cotyledon Protoplasts of Melon (*Cucumis melo* L.)

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Protoplasts were isolated from cotyledons of melon (*Cucumis melo* L.) seedlings grown in vitro. When young unexpanded cotyledons were a source material, 60% of protoplasts were viable and 40% of them had divided after 7 days of culture. Some of the protoplasts continued to divide to form callus colonies, and the differentiation of roots, but not of shoots, was induced in the protoplasts-derived calli.

Regeneration of whole plants is one of the essential steps for improving crop plants through protoplast fusion or transformation. A number of papers have appeared on plant regeneration from protoplasts, but many of them deal with species of Solanaceae.[1] The family Cucurbitaceae contains important vegetable and fruit crops, but there are few reports on the culture of protoplasts of this family.[2,3] In the present work, protoplasts derived from cotyledons of melon were cultured to form callus colonies, and roots were induced in the protoplast-derived calli.

Materials and Methods

Plant material. Seeds of *Cucumis melo* L. cv. *Boruga* were soaked overnight in distilled water. The seeds were then surface-sterilized for 10 min with 2.5% (w/v) sodium hypochlorite solution, washed twice with distilled water and germinated on 0.8% agar plates in the dark. After 3-5 days the seedlings were transferred to Murashige and Skoog[4] (MS) agar medium (no phytohormone), and grown at 28°C with 12 hr light (2,000 lux) period. Seedlings at two different stages were used; younger (6-8 day old) seedlings with unexpanded cotyledons (**Fig. 1**) and older (15-20 day old) seedlings with fully expanded cotyledons.

Protoplast isolation and culture. Cotyledons from the axenic seedlings were cut into 3-5 mm wide strips with a razor blade. The strips of about 0.5 g fresh weight were transferred into a screw-capped 100 ml Erlenmeyer flask, and vacuum-infiltrated for 2 min with 20 ml of a filter-sterilized enzyme solution. Enzyme mixtures listed in **Table 1** were tested for protoplast isolation. The enzymes were dissolved in 0.4 M mannitol containing 0.1% (w/v) $CaCl_2 \cdot 2H_2O$ and 0.5% (w/v) potassium dextran sulfate (S content 18.1%, inherent viscosity 0.013 dl/g, Meito Sangyo Co., Nagoya, Japan) and pH was adjusted to 5.7. The flasks containing the strips and the enzyme solution were reciprocally shaken in a water bath at 30°C at a rate of 50 strokes/min (amplitude 3 cm). After incubation for 1-2.5 hr, the mixture was filtered through a nylon sieve (mesh opening 50 μ), centrifuged at 100 g for 2 min, and the pelleted protoplasts washed with 0.4 M mannitol solution. The washed protoplasts were suspended in 0.4 M mannitol solution and counted with a haemocytometer.

Protoplasts were cultured at a cell density of $1 \times 10^5 ml^{-1}$ in 30 × 15 mm plastic dishes (Falcon 1008)

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Table 1. Release of protoplasts from fully expanded cotyledons of melon by enzyme mixtures.

Enzyme	EM 1	EM 2	EM 3	EM 4	EM 5	EM 6
	Enzyme concentration (%)					
Macerozyme R-10[a]	1.0	1.0				
Pectolyase Y-23[b]			0.1	0.1	0.1	
Cellulase Onozuka R-10[a]		2.0			1.5	
Cellulase Onozuka RS[a]	1.0		1.0			
Cellulase YC[b]				0.5		
Meicelase P-1[c]						4.0
Incubation (hr)	2.5	2.5	1.5	1.5	1.5	2.5
Yield of protoplasts ($\times 10^6$/g fr wt)	—	—	4.5	4.2	6.4	—

[a] Yakult Honsha Co. Ltd., Tokyo, Japan.
[b] Seishin Pharmaceutical Co. Ltd., Tokyo, Japan.
[c] Meiji Seika Kaisha, Ltd., Tokyo, Japan.

containing 2.0 ml of liquid media of various compositions. The dishes were placed in a plastic box to prevent desiccation and incubated at 28°C in diffuse light. Protoplasts and the cells derived therefrom were examined under a light microscope, and those with apparently intact plasmalemma and normal intracellular structures were considered to be viable.

Results and Discussion

In contrast to the report on the isolation of protoplasts from melon leaves,[2] Cellulase Onozuka R 10 alone did not release protoplasts from cotyledons of melon, suggesting that cotyledon cells differ from leaf cells in the composition of walls.

Various combinations of pectolytic and cellulolytic enzymes were tested for their ability to yield protoplasts from fully expanded cotyledons of older seedlings (**Table 1**). Protoplasts were released by using enzyme mixtures of EM 3, EM 4 and EM 5. The highest yield (6.4×10^6 cells/g fr wt) and viability (90%) of protoplasts were obtained with EM 5, followed by EM 4 and EM 3. In contrast, cotyledon tissues were hardly digested by the enzyme mixtures of EM 1, 2 and 6 even after incubation for 2.5 hr (**Table 1**). $CaCl_2$ included in the enzyme mixture was effective in stabilizing protoplasts and improving their plating efficiency, as has been reported by Gamborg et al.[5] However, $CaCl_2$ affected protoplast viability at concentrations higher than 1.0%.

Several culture media were tested with supplements of 0.5 mg/*l* NAA, 1.0 mg/*l* kinetin, 1% sucrose and 0.4 M mannitol by using cotyledon protoplasts of older seedlings isolated with EM 5. The best results (4–6% of protoplasts dividing after 7 days) were obtained with 1/2 strength MS medium, followed by full strength MS medium and the medium of White.[6] The medium of Nagata and Takebe[7] gave lower plating efficiency, and little or no division occurred in B 5 medium of Gamborg et al.[8]

When the unexpanded cotyledons of younger seedlings were used, protoplast yield and viability were similar to those from fully expanded cotyledons, but division frequency was significantly improved. In 1/2 strength MS medium with the aforementioned supplements, the first cell division occurred within 3–4 days (**Fig. 2**), and after 7 days 60% of cells were viable and 40% of them had divided. The osmotic pressure of the medium was then reduced stepwise by adding 0.5 ml of the same medium without mannitol at 2–4 day intervals. Visible colonies appeared within 3–4 weeks. These were then evenly spread over the surface of MS agar medium containing 0.5 mg/*l* NAA and 0.1 mg/*l* kinetin, and the plates were maintained under the conditions for growing the seedlings. Minicalli (2–3 mm in diameter) developed after 7 days (**Fig. 3**), and these were now transferred to MS agar medium containing 0.1 mg/*l* NAA or IAA. Root formation was observed after 10 days in nearly all calli (**Fig. 4**). Overall frequency of callus formation from protoplasts of unexpanded cotyledon was about 0.2%.

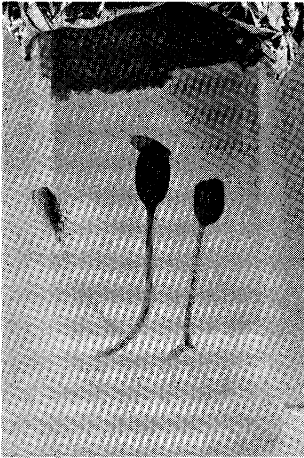

Fig. 1. Melon seedlings axenically grown for 6–8 days. Note unexpanded cotyledons.

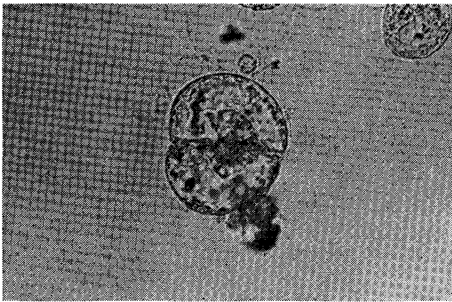

Fig. 2. First cell division in cultured cotyledon protoplasts of melon (4 days after the start of culture).

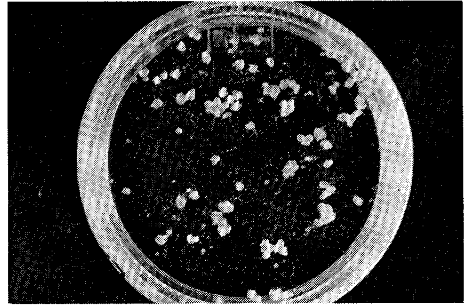

Fig. 3. Calli from protoplasts 7 days after transfer to the solid MS medium.

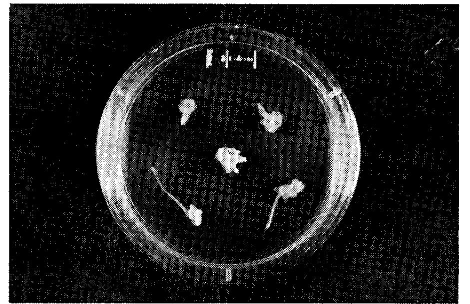

Fig. 4. Root formation by protoplast-derived calli.

We tested a number of combinations of auxin (IAA, NAA) and cytokinin (BA, kinetin) at different concentrations (0. 1–5. 0 mg each per l), but were unable to induce shoot differentiation in the protoplast-derived calli of melon. The phytohormone conditions of Moreno et al.[9] also faild to induce shoot formation. Explant-derived calli of Cucurbitaceae species readily differentiate roots but not shoots (unpublished results), although shoot formation in the presence of relatively high concentrations of kinetin (6. 0 mg/l) was reported for cotyledon-derived calli of melon.[9] Moreover, plants of this family tend to form roots rather than galls when inoculated with octopine type *Agrobacterium tumefaciens*.[10] It would thus appear that plants of Cucurbitaceae have a peculiar balance between endogenous auxin and cytokinin in favor of the former. Alternatively, these plants might be more responsive to auxin than to cytokinin.

References

1) Evans, D. A., J. E. Bravo, 1983. In "Plant Protoplast" (ed. by Giles, K. L.), Intern. Rev. Cytol. Suppl., 16, p. 33–53, Academic Press, New York.
2) Moreno, V., L. Zubeldia, L. A. Roig, 1984. Plant Sci. Lett., **34** : 195–201.
3) Orczyk, W., S. Malepszy, 1985. Plant Cell Rep.,**4** : 269–273.
4) Murashige, T., F. Skoog, 1962. Physiol. Plant., **15** : 473–479.
5) Gamborg, O. L., J. Shyluk, K. K. Kartha, 1975. Plant Sci. Lett., **4** : 285–292.
6) White, P. R., 1951. Ann. Rev. Plant Physiol., **2** : 231–244.

7) Nagata, T., I. Takebe, 1971. Planta, **99**: 12–20.
8) Gamborg, O. L., R. A. Miller, K. Ojima, 1968. Exp. Cell Res., **50**: 151–158.
9) Moreno, V., M. Garcia-Sogo, I. Granell, B. Garcia-Soga, L. A. Roig, 1985. Plant Cell Tissue Organ Cult., **5**: 139–146.
10) Matsumoto, S., Y. Machida, I. Takebe, 1986. Plant Mol. Biol. Rep., **4**: 42–47.

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メロン子葉プロトプラストからのカルス形成と根の分化

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無菌的に発芽させたメロンの芽生えの子葉のプロトプラストの単離および培養条件を検討した．若い芽生えの子葉から単離したプロトプラストは，NAA 0.5 mg/*l*, kinetin 1.0 mg/*l*, 0.4 M マンニトールを含む 1/2 MS の液体培地で培養した場合，7日目の生存率は70%で，その中の約40%が分裂した．約1カ月後に生じたコロニーを NAA または IAA 0.1 mg/*l* を含む MS の寒天培地に移したところ，根の分化が見られた．しかし，オーキシンとサイトカイニンの種類と濃度を色々に組み合わせても，茎葉分化は見られなかった．