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Plant Regeneration from Protoplasts of Scented-leaved Geranium

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For the first time we have succeeded in the regeneration of plants from protoplasts of a scented-leaved geranium, widely used as a fragrance plant. Protoplasts were isolated from the cultured cells of two species of scented leaved geraniums, *Pelargonium odoratissimum* Ait. and *Pelargonium crispum* L' Her, using an enzyme mixture of 0.2% Pectolyase Y-23 and 3% Cellulase Onozuka R10, and were successfully cultured in KM8P medium. Cell division began three days after the culture, and callus formation occurred within one month. When the calli were transferred to MS medium with 0.5 mg/*l* BAP and 1% sucrose, many adventitious shoots and regenerated plants were obtained.

Introduction

Approximately 280 species of a genus *Pelargonium* exist mainly in South Africa[1] and in this genus there are many kinds of crossbreeds having horticultural value. Harvey[2], Knuth[3] and van der Walt[4] divided this genus into 15 sections. The section Pelargonium includes most of scented-leaved geraniums, which have various aromatic oils similar to those of rose, apple, orange or lemon, strawberry and so on[5] Essential oils extracted from the leaves are utilized as an essence for perfumes and cosmetics. On the other hand, the section Ciconium is the ancestor of *Pelargonium* x *hortorum* BAILEY. Many kinds of crossbreeds of *P.* x *hortorum*, which are well known as horticultural plants with beautiful flowers, have been widely developed by ordinary plant breeding methods[4]. However, *P.* x *hortorum*, known as the Common Fish Geranium, has the demerit of having an unpleasant fishy odor and no research for solving this problem has been performed until now. As the crossing between the scented-leaved geranium and *P.* x *hortorum* is considered impossible, the formation of a somatic hybrid by protoplast fusion is desired. Plant regeneration from protoplasts of *Pelargonium* was performed from mesophyll protoplasts of horicultural species of *P.* x *hortorum*[6] and from protoplasts of the cell culture of *P. aridum*, *P.* x *hortorum*, and *P. peltatum*[7]. However, there is no report on protoplast culture of scented-leaved geraniums. The final objective of our study is to produce a somatic hybrid between *P.* x *hortorum* and a scented-leaved geranium. As the first step, plant regeneration from protoplasts has been carried out for *P. odoratissimum*, which has a rose like fragrance, and for *P. crispum*, which has a lemon-like fragrance.

Materials and Methods

Plant material- We obtained *P. odoratissimum* from the Kagawa Agricultural Experiment Station

Table 1. Compositions of four different media used for protoplast culture and propagation of protoplast-derived callus.

Medium No.	Basal medium	Sugar (g/*l*)	Mannitol (M)	Growth regulators (mg/*l*)			
				NAA	2. 4-D	BAP	Zeatin
1	KM8P	G: 68. 4	0	1. 00	0. 2	0	0. 5
2	KM8P	G: 68. 4	0	0. 22	0	2. 2	0
3	MS	S: 30. 0	0. 3	1. 00	0. 2	0	0. 5
4	MS	S: 30. 0	0. 3	0. 22	0	2. 2	0

G: glucose, S: sucrose

and *P. crispum* from Ehime University. However, the characteristic of *P. odoratissimum* employed in this experiment was quite different from those reported earlier[4)8)9)]. Yoshida reported *P. odoratissimum* was very similar to *P. roseum* Wild[10)]. Bailey[9)] and Demarne *et al.*[11)] considered *P. roseum* was not a species but a variety or hybrid. We also considered *P. odoratissimum* to be a variety or hybrid like *P. roseum*. *P. crispum* had the same characters as reported by van der Walt[4)]. All plants were propagated by the cutting method and cultivated in a greenhouse.

Callus formation and maintenance of cell line- Leaves or petioles of two species of scented-leaved geranium were cultured in Murashige and Skoog (MS) medium[12)] containing 30 g/*l* sucrose, 10 g/*l* agar, NAA 0. 22 mg/*l* and BAP 2. 2 mg/*l* (pH 5. 6) under 16 hr light (white fluorescent lamps, 5, 000 lux) at 25°C to induce callus formation. The callus was successively subcultured on the same medium.

Protoplast isolation- About 1 g (fw) of the calli was incubated in 20 m*l* of an enzyme solution (pH 5. 6) containing 0. 2% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 3% Cellulase Onozuka R-10 (Yakult Honsha Co. Ltd., Japan), 0. 5% potassium dextran sulfate, 0. 4 M mannitol, 10 mM 2- (N-morpholino) ethanesulfonic acid, monohydrate (MES), 2. 5 mM $MgCl_2 \cdot 2H_2O$, 1/2 diluted inorganic solution of CPW salts (Power and Chapman 1985)[13)], and agitated on reciprocal shaker (50 rpm/min) at 28°C for 4 hr in the dark. Released protoplaats were passed through a nylon sieve (pore size: 40 μm) and washed twice with a 0. 4 M mannitol solution by centrifugation (300 rpm, 10 min). They were overlayed onto a 25% sucrose solution and centrifuged at 500 rpm/min for 5 min. Protoplasts floated on the sucrose solution were collected, washed with a 0. 4 M mannitol solution and used for the following experiments.

Protoplast culture- For protoplast culture, Kao and Michayluk medium 8 P (KM8P medium)[14)] and MS medium were used and a liquid culture or an agarose culture was tested (**Table 1**). In the case of the liquid culture, protoplasts were suspended in 2 m*l* of the medium at a density of 5×10^4/m*l* and cultured in a petri dish (diameter: 35 mm). In the case of the agarose culture, protoplasts were suspended in 1 m*l* of the medium with 0. 5% w/v agarose (Type VII, SIGMA Chemical Company, U. S. A.) at a density of 5×10^4/m*l* and poured into a petri dish. After the medium was solidified, 1 m*l* of the same medium without protoplasts was overlaid. The petri dish was sealed with Parafilm and incubated at 25°C in a weak light (600-1000 lux). Fresh medium was added or replaced every week.

Results and Discussion

Calli induced from leaves or petioles were subcultured for 1-2 months. Selection of green calli, showing better growth, and transfering them to a fresh medium at weekly intervals was indispensable for succesful subculture of the calli. Approximately 1×10^6 protoplasts could be isolated from 1 g of callus and over 90% of them showed esterase activity by fluorescence analysis using FDA

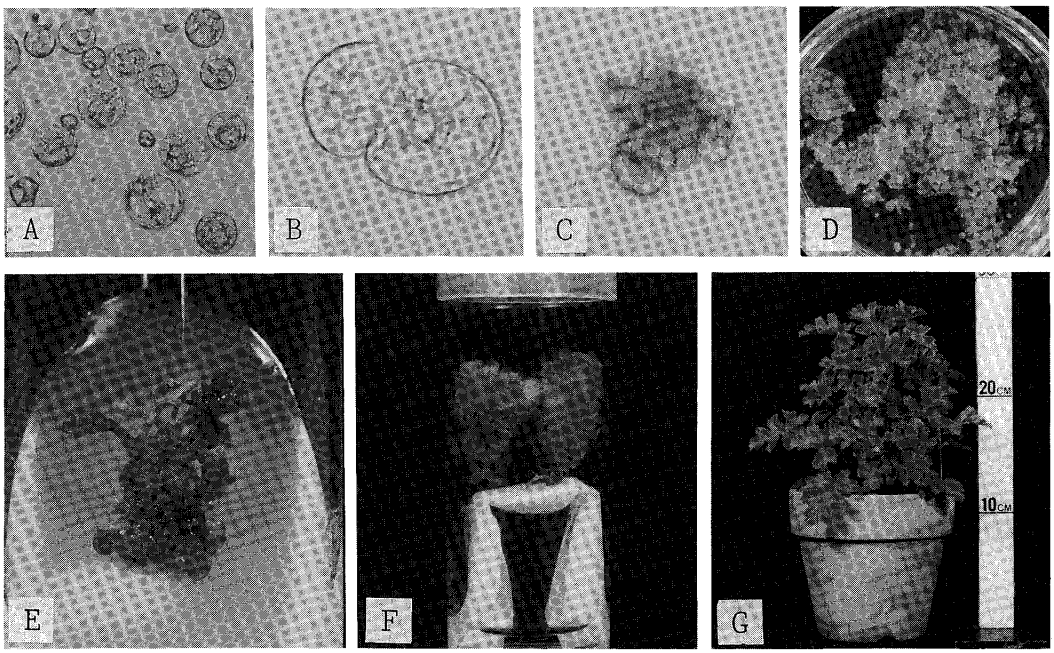

Fig. 1 Development of plants from protoplasts of *P. odoratissimum*. (A) Isolated protoplasts from callus. (B) First cell division of a protoplast in the KM8P medium No. 1 after 3 days of culture. (C) Cell clusters formed from protoplasts in the KM8P medium No. 1 after 14 days of culture. (D) Callus formed on the KM8P medium No. 1 with 0.5% agar after 2 months of culture. (E) A regenerated shoot from callus in MS medium with 0.5 mg/*l* BAP, 10 g/*l* sucrose and 1% agar after 4 months of culture. (F) A rooted plant on a paper-bridge over the MS liquid medium with 10 g/*l* sucrose after 8 months of culture. (G) A cultivated whole plant in the greenhouse.

(fluorescein diacetate) immediately after the isolation (**Fig. 1-A**). The first cell division was observed after 3 days of culture (**Fig. 1-B**). The greatest frequency of cell division (4.0%) of *P. odoratissimum* was observed after 4 days of culture in the KM8P agarose medium No. 1 (**Table 2**). The protoplasts cultured in the KM8P medium No. 1 formed many colonies after 14 days of culture (**Fig. 1-C**), but only a few colonies developed on the KM8P medium No. 2. The rate of cell proliferation was so low in the media No. 3 and 4 that no colony was obtained. Colonies formed in medium No. 1 or No. 2 developed into microcalli (diameter 0.5–1 mm) and then the microcalli were plated onto the same medium solidified with 0.5% agar (**Fig. 1-D**). The protoplast-derived calli were incubated in a light condition (fluorescent lamps, 5,000 lux, 16 hr/day). Finally, all the calli were transferred to the agar medium No. 4 without mannitol. After 3 months of culture, these calli were transferred to MS medium containing plant growth regulators, 1% sucrose and 1% agar (**Table 3**) to induce adventitious shoots (**Fig. 1-E**). Adventitious shoot formation of *P. odoratissimum* was most frequent (65%) in a medium containing 0.5 mg/*l* BAP (**Table 3**). In a liquid medium, *P. crispum* callus frequently gave multiple shoots, whereas *P. odoratissimum* callus did not form any adventitious shoots, and finally died. Adventitious shoot formation of *P. odoratissimum* was promoted when the sucrose concentration was decreased from 30 g/*l* to 10 or 20 g/*l* (**Table 4**). Regenerated plants of both species were transplanted onto a paper-bridge over the MS liquid medium to promote rooting (**Fig. 1-F**). The whole plants thus obtained were acclimated at 20°C after transplanting to vermiculite in pots and cultivated in the greenhouse. They showed longer petioles and rosette-like appearance, turned to normal appearance after prolonged cultivation in the greenhouse (**Fig. 1-G**)

Table 2. Frequency of cell division in protoplasts of *P. odoratissimum* in four different media.

Frequency cell division was measured after 4 days of culture and expressed as % of divided cells per total no. of cells observed. Two thousands protoplasts were counted in each of the 4 replications and mean ± standard deviation (SD) were calulated.

Medium No.	Frequency of cell division (%) (mean ± SD)
1	4.0±0.2*
2	1.2±0.3
3	0.1±0.1
4	0.3±0.3

* significant at 1% levels by analysis of variance.

Table 3. Effects of the plant growth regulators on shoot formation from protoplast-derived calli of *P. odoratissimum*.

Frequency of shoot formation was measured after 4 weeks on the MS medium containing 1% sucrose and 1% agar and expressed as % of no. of calli with adventitous shoots per 20 calli tested.

Growth regulators (mg/*l*)		Frequency of shoot formation (%)
NAA	BAP	
0	0	0
0	0.5	65
0	1.0	30
0.05	0	0
0.05	0.5	50
0.05	1.0	10
0.1	0	0
0.1	0.5	20
0.1	1.0	5

Table 4. Effects of sucrose concentrations on shoot formation from protoplast-derived calli of *P. odoratissimum*.

Frequency of shoot formation was measured after 4 weeks on the MS medium containing 0.5 mg/*l* BAP and 1% agar and expressd as % of no. calli with adventitous shoots per 20 calli tested.

Sucrose (g/*l*)	Frequency of shoot formation (%)
0	0
10	65
20	70
30	35

and flowered normally. Essential oils in the leaves of regenerated plants and original plants were extracted with diethyl ether and the aromatic components were analyzed by GC. A total of 8 plants of each species were examined. The essential oils of *P. odoratissimum* mainly consisted of citronellol, geraniol and their aldehydes, having a rose-like flavor and fragrance, whereas those of *P. crispum* contained chiefly cis-and trans-citral having a lemon-like flavor and fragrance. No significant differences were perceived in the main aromatic components between the regenerated plants and the original plants (data not shown here). The above methods for protoplast culture have made it possible to regenerate plants from protoplasts of *P. odoratissimum* with high experimental reproducibility. We also succeeded in plant regeneration from protoplasts of *P. crispum* by the same methods, but the experimental reproducibility was poor. We could not show the frequencies of cell

division and shoot formation of *P. crispum*. Although an interspecific hybrid between *P.* x *domestium* L. H. BAILEY and scented-leaved geranium was obtained by using the embryo culture method[15], no hybridization between *P.* x *hortorum* and scented-leaved geranium was achieved even if the embryo culture method was employed. Since plant regeneration from protoplasts has become possible not only for *P.* x *hortorum*[6,7] but also for a scented-leaved geranium, an interspecific hybrid between the two species might be obtained by cell fusion.

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《和文要約》

ニオイゼラニウムのプロトプラストからの植物体再生

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香料植物として広く利用されているニオイゼラニウムのプロトプラストから植物体を再生することに初めて成功した．2種のニオイゼラニウム *Pelargonium odoratissimum* Ait. と *Pelargonium crispum* L' Her. の培養細胞から，0.2% ペクトリアーゼ Y-23 と 3% セルラーゼオノズカ R-10 を含む酵素液を用いてプロトプラストを単離し，KM8P 培地で培養した．培養3日後より細胞分裂が見られ，1カ月後にはカルスを形成した．カルスを MS 培地に移して増殖させた後，BAP 0.5 mg/*l*, 1% しょ糖を含む MS 倍地で培養すると，両種とも多くの不定芽を形成し，植物体を得ることができた．