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Suspension Culture of *Gardenia jasminoides* Ellis Cell for Production of Yellow Pigment

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Gardenia callus was induced in MS medium containing 10 μM of 2,4 diphenoxy acetic acid (2,4-D), 1 μM kinetin, and 3% sucrose in the dark. B_5 medium was identified to be the most adequate medium for cell growth. Indole-3-acetic acid (IAA) was better growth regulator than 2,4-D not only for cell growth but slso for carotenoid production. Ligt also played a critical role on synthesis of carotenoid. Gardenia cells grown in B_5 medium could utilize a polysaccharide, soluble starch, as a carbon source. The cell growth was stimulated in B_5 medium fortified with 0.2% yeast extract. The optimum pH for cell growth was 5.7. High density cultures can be maintained by increasing inoculum size and medium concentration accordingly. Specific growth rate and mass doubling time were 0.095 day^{-1} and 7.3 days, respectively. The cell immobilized in alginate tends to formulate more enlarged vacuoles containing yellow pigment compared with those of suspended cell. Carotenoid content of immobilized cell was about 264.4 μg/g fresh weight (F.W.) corresponding twice of the content of suspended cell (112.08 μg/g F.W.). The color of gardenia cell was shifted from yellow to red when carbohydrase-secreting fungus, *Trichoderma reesei*, was co-cultivated with gardenia cells.

Gardenia jasminoides Ellis is an evergreen bush that grows in temperate zone and belongs to a species of the *Rubiaceae*. Yellow pigment contained in the fruit of *G. jasminoides* has been used as a coloring reagent of food in our country for a long time, and the dry fruit, named SAN-CHI-JA in Chinese medicine, is also used as a sedative, a diuretic, and a hemostatic. The component of this yellow pigment is mainly α-crocin [8,8′-diapo-ψ,ψ-carotenedioic acid bis (6-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester, $C_{44}H_{66}O_{24}$, MW. 977.00], which is chemically classified as carotenoids (1). Their protective role is essential for the survival of chloroplasts under light/aerobic conditions and they also contribute to light harvesting in photosynthesis.

Chemical and microbial methods have been mainly employed for production of various chemicals, however, plants are also very important as a source of various chemicals, such as, alkaloids, antibiotics, volatile oils, resins, tannines, cardiac glycosides, and saponins (5, 23). Especially about 25% of medicines are presently derived from plants. There have been many new approaches to produce these chemicals through plants cell suspension culture in commercial scale. Shikonin of *Lithospermum erythrorhizon* represented the first commercial chemical produced from large-scale plant suspension culture (2).

The preceding researches on *G. jasminoides* can be roughly divided into two groups. One is the study of chemical compositions of the fruit, stem,and flower (1, 8, 10, 13, 15, 28, 31) and the application of above chemicals to medicine and coloring reagents (11, 17). The other application is mainly related to tissue or cell culture, e.g. *in vitro* propagation (6, 7), the biosynthesis of iridoid glucoside (14), and the biotransformation of esculetin (27).

In this work the optimal medium and culture conditions of *Gardenia jasminoides* cell for cell growth and carotenoid production have been investigated, including (a) effects of various medium components on cell growth

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and carotenoid synthesis, (b) effects of culture condition on cell growth and carotenoid synthesis, and (c) application of special cell culture techniques, e.g. effect of immobilization and co-cultivation of plant cell with microorganism on the amount of carotenoid formation and color change.

MATERIALS AND METHODS

Callus Induction

Dry fruit of *Gardenia jasminoides* Ellis was surface-sterilized by immersing in 2% sodium hypochlorite in vacuum for 40 min, and then germinated on solid Murashige and Skoog (MS) basal medium (23) containing 1% agar at 28℃ under 16 hr photoperiod supplemented by fluorescent light. The two-month-old seedlings were dissected into explants of size about 1 cm^2 and punched with a needle, and then placed on a solid MS medium supplemented with 2,4-D or kinetin alone, and a combination of both of them, and 1% agar.

Media

The basal media tested for callus growth were those of MS (Murashige and Skoog) (23), LS (Linsmaier and Skoog) (18), B_5 (Gamborg, Miller, and Ojima) (9), and WPM (Lloyd and McCown) (19), which were supplemented with the some growth regulators, and 3% (w/v) sucrose.

Bacterium, *Agrobacterium tumefaciens*, was cultivated on YMB medium (0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l NaCl, 10 g/l Mannitol and 0.4 g/l Yeast extract, pH 7.0) at 27℃. Fungi, *Trichoderma reesei* and *Aspergillus fumigatus*, were cultured on 15% potato dextrose agar (PDA) and CYC-10 medium (33.4 g/l Czapek-Dox liquid medium powder, 2.0 g/l Yeast extract, and 6.0 g/l Czapek-Dox liquid medium powder, 2.0 g/l Yeast extract, and 6.0 g/l Casamino acid, pH 7.2), respectively.

Suspension Culture

Rapidly growing friable white callus was used to establish the cell suspension culture of gardenia. 3.0 g of callus was placed in a 250 m*l* conical flask containing 50 m*l* of liquid B_5 medium supplemented with 10 μM IAA and 1.0 μM benzylaminopurine (BA). The suspension culture was carried out in a reciprocal shaker (90 rpm) at 27℃ in the dark. After every 14-day culture, subculture was carried out by transferring 25 m*l* of suspension to 30 m*l* of fresh medium.

Cell Growth Measurement

Cell growth was determined by measuring packed cell volume (PCV) and fresh weight. 10 m*l* of suspension was transferred to a 15-m*l* graduated conical centrifuge tube, and centrifuged at 2,000 rpm for 10 min, and then checked the volume of spin downed cells. After PCV determination in a preweighed graduated centrifuge tube, the supernatant was removed and stood for 2 h with inverted position. The wet weight was determined by weighing the tube containing cells, and then calculating the mass of remnant cells.

Immobilization of Gardenia Cell in Alginate Bead

Cell of a 7-day-old suspension was filtered through 200 μm pore size nylon net. Filtered cell (10.0 g fresh weight) was suspended in a flask containing 50 m*l* of 3% alginate dissolved in B_5 medium, and then suspended cell was added dropwise to 50 mM $CaCl_2$ solution for bead formation. The beads were maintained in this solution for 1 h under the stirring condition for stabilization, and then washed with B_5 medium twice.

After 18-day culture, the beads were placed to be dissolved in a flask containing a 0.05 M potassium phosphate solution of pH 7.5, and agitated at 125 rpm for 100 min.

Co-culture of Gardenia Cell with Bacterium and Fungi

The cultured bacterial and fungal cells were harvested after 2 days of culture, and part of them were directly inoculated in 10 day-old gardenia suspension. The remained harvested microbes were dried in a dry oven (70℃) overnight and then 0.5 g of each dry cells was ground into powder in a mortar and pestle, and then dissolved in 10 m*l* of distilled water. Each 10 μ*l* of the sterilization with millipore (diameter; 0.2 μm, Gelman sciences Inc.), was inoculated into each 10 day-old gardenia cell culture.

Quantification and Identification of Carotenoid

0.5 g of cell cultured in different conditions was extracted with 3 m*l* of 80% cold acetone. 1.0 g of powder of gardenia dry fruit was also extracted with 100 m*l* of 80% acetone and 70℃ hot water separately and the remnant was removed by filtration.

The quantity of carotenoids in the 80% acetone was determined by measuring the absorbance at 540 nm. β-carotene dissolved in 80% acetone was used as a standard.

The yellow pigment was identified by spectrophotometric method. The absorbance of each sample was scanned with a UV-Visible spectrophotometer (PYE UNICAM PU8800, Phillips), from wavelength of 350 nm to 700 nm. The carotenoid was identified by comparing with spectral data of standard materials, crocetin and β-carotene (15).

RESULTS AND DISCUSSION

Optimization of Medium and Culture Conditions for Cell Growth and Pigment Production

Growth regulator on callus induction: Growth

regulators, especially auxin and cytokinin, have profound effect on plant cell in terms of callus induction, cell growth, and secondary metabolite formation (4, 16), even though their modes of action at the molecular level are not clearly understood. Therefore the effects of growth regulators on callus induction were investigated by using MS medium supplemented with various concentrations of 2,4-D and kinetin. As shown in Table 1, the best hormonal combination for callus induction was identified to be 10 μM of 2,4-D and 1 μM of kinetin. The use of 2,4-D (10 μM) or kinetin (5 μM) alone tended to induce roots and shoots, respectively.

Growth regulator on cell growth and carotenoid production: The callus was cultured on MS medium containing single or a combination of various growth regulators to determine the effect of growth regulators on cell growth and carotenoid production. IAA was identified to be the best for cell growth among several growth regulators, including 2,4-D, as can be seen in Fig. 1.

In general, 2,4-D stimulates cell growth but depresses secondary metabolite formation (32). However, Table 2 shows that the different effect of 2,4-D and IAA was not recognized on carotenoid production.

Table 1. Effect of growth regulator on callus induction of *Gardenia jasminoides*

Kinetin (μM)	2,4-D (μM)			
	0	0.1	0.5	10
0	−	+	+(R)	+(R)
1	+(S)	+	+	++
5	S	+	+	+

+; Callus, S; Shoot, R; Root, +(R); Callus and Root, +(S); Callus and Shoot

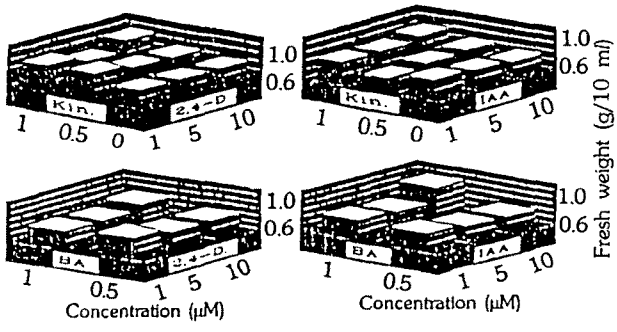

Fig. 1. Effect of growth regulator on gardenia callus growth.
0.5 g of callus was inoculated on Murashige and Skoog (MS) medium. Fresh weight was measured after 14-day culture.

Salt composition: To identify the most suitable salt component for cell growth and carotenoid formation, four basal media which have characteristic salt compositions were examined. B_5 medium was identified to be the most suitable medium for cell growth as illustrated in Fig. 2.

On the other hand, MS medium was suitable for carotenoid production as shown in Table 3. Light did not affect cell growth significantly, but critically affected fria-

Table 2. Effect of growth regulator on carotenoid formation of gardenia cell

(Absorbance at 540 nm)

Light	Growth regulator					
	D,K	D,B	I,K	I,B	B	K
Dark	0.18	0.11	0.11	0.14	0.27	0.17
Light	0.25	0.23	0.23	0.32	0.19	0.17

D; 10 μM 2,4-D., I; μM IAA, K; 1 μM Kin., B; 1 μM BA

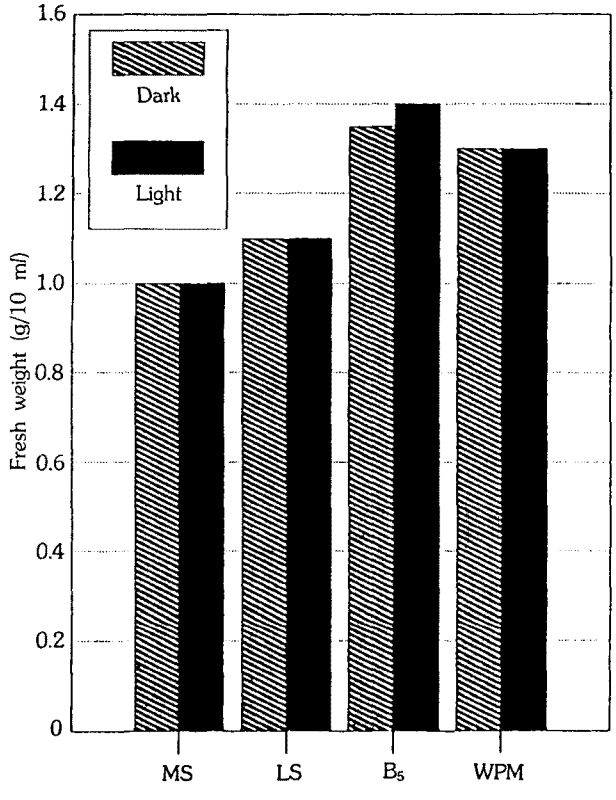

Fig. 2. Effect of basal media on gardenia callus growth.
MS (23), LS (18), B_5 (9), and WPM (19) were used for callus culture. Each medium was supplemented with 10 μM IAA and 1.0 μM BA. 0.5 g of callus was inoculated and the cell growth was monitored by measuring fresh weight after 14-day culture.

Table 3. Effects of various basal media and light on carotenoid formation of gardenia cell

(Absorbance at 540 nm)

Media	MS	LS	B_5	WPM
Dark	0.04	0.04	0.03	0.04
Light	0.09	0.05	0.06	0.07

MS (23), LS (18), B_5 (9), and WPM (19)

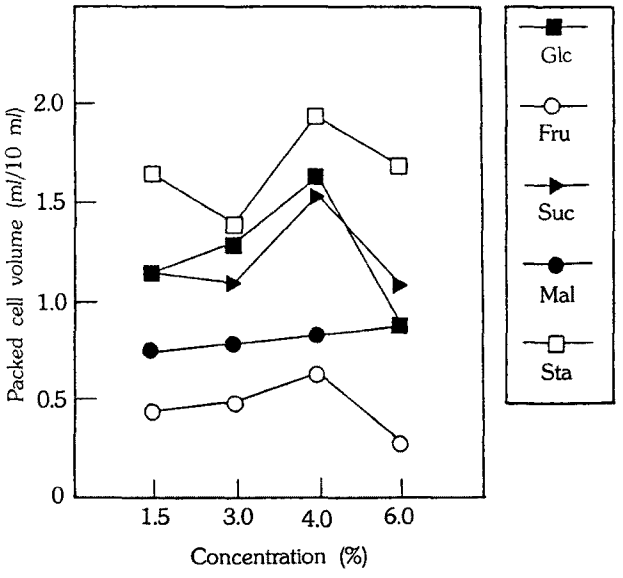

Fig. 3. Effects of carbon sources on gardenia cell growth.

12.0 m*l* (PCV/10 m*l*) of suspension cultured cell was inoculated in 50 m*l* of B_5 medium suppleminted with different concentration of several carbon sources. Packed cell volume was measured after 14-day culture.

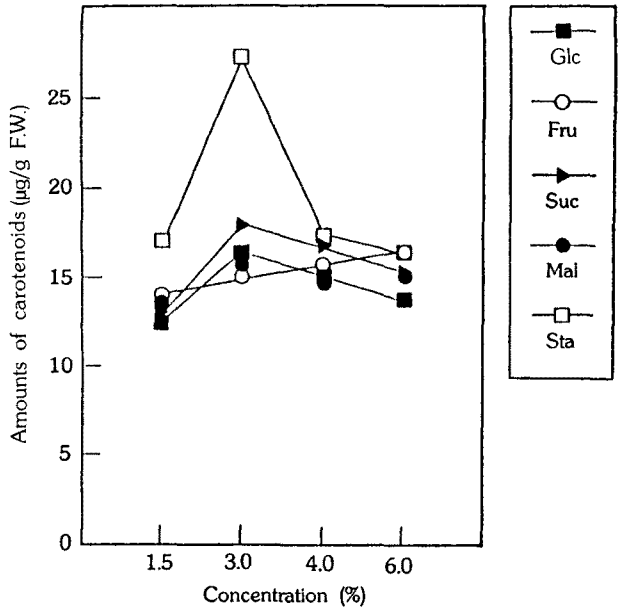

Fig. 4. Effects of carbon sources on carotenoid synthesis of gardenia.

12.0 m*l* (PCV/10 m*l*) of suspension cultured cell was inoculated in 50 m*l* B_5 medium supplemented with different concentration of several carbon sources. 0.5 g of each cell cultured in different conditions was extracted with 3 m*l* of 80% cold acetone.

bility of cell and carotenoid synthesis. The cells grown in the brighter light showed the less friability, but the more carotenoid production.

Carbon source: Some plant cells can utilize various polysaccharides for their growth along with mono- and di-saccharides (20, 29). The response of plant cells to each carbohydrate for growth and product formation depends sensitively on plant species (3). Gardenia cell was cultured in B_5 medium fortified with various carbohydrates and then the effect of carbon sources illustrated in Figs. 3 and 4. Soluble starch was found to be the best for cell growth and carotenoid formation. It is contrasted with the known fact that sucrose and its monosaccharides, glucose and fructose, are generally more useful for plant cell culture as an adequate carbon source.

Organic nitrogen sources: The gardenia cell grew more rapidly in B_5 medium supplemented with several organic nitrogen sources compared with those of control (1.1 g F. W./10 m*l*) not supplemented. Especially the cell mass in B_5 medium fortified with 0.2% yeast extract was almost doubled in comparison to that of control as shown in Fig. 5.

pH: pH has influence on nutrient utilization, cell growth, and product formation as well (21). Initial pH of B_5 medium was adjusted from 4 to 8 to determine the optimum initial pH for gardenia cell culture. The pH of B_5 medium was lowered after autoclaving regardless of its initial pH like the reported result obtained from MS medium (25), as shown in Table 4. The optimum pH for cell growth was 5.70, and pH was adjusted about 5.75 at actively growing phase (day 14) regardless of initial pH.

Dense Cultivation of Gardenia Cell and Growth Characteristics

Dense cultivation: It is essential to maintain cell density above a critical level for the sake of cell division. The cell cultures at high cell density are also beneficial to control the culture conditions and down-stream processing. For dense cultivation, the amount of inoculum size was proportionally increased according to B_5 medium concentration so as to obtain high density cell mass. The cell mass was proportionally increased around three times by increasing the inoculum size and medium

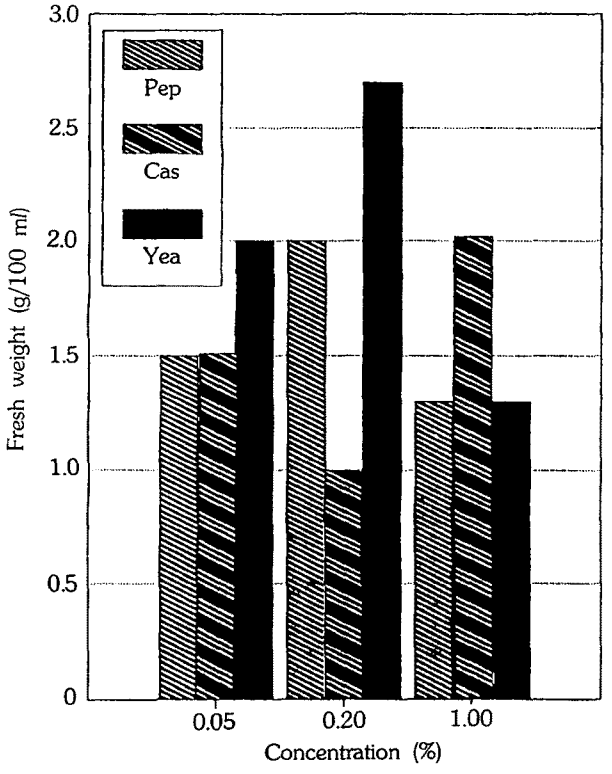

Fig. 5. Effects of organic nitrogen sources on gardenia cell growth.
3.0 g F.W. of suspended cell was inoculated in 35 ml of B_5 medium enriched with various concentration of organic nitrogen sources. After 14-day culture, suspension was centrifuged at 2,000 rpm for 10 minutes and then spin downed cell was weighed. Control (1.1 g F.W./10 ml 14 day)

Table 4. Effect of initial pH on gardenia cell growth and pH at active growth phase

Initial pH	4	5	5.7	6.5	8
pH after autoclaving	3.5	4.4	5.17	5.9	7.08
F.W. (g/10 ml)	1.5	1.35	1.75	1.3	1.0
pH (Day_{14})	6.0	5.7	5.8	5.75	5.8

F.W.; fresh weight, pH (Day_{14}); pH after 14 days of subculture

concentration as illustrated in Fig. 6.

Growth characteristics: 10 ml of cell suspension was inoculated in a flask containing 100 ml of B_5 medium to determine cell mass doubling time and packed cell volume, and the result is shown in Fig. 7. The pH of suspension broth was initially dropped to 4.5 after three day culture, and then maintained about pH 5.6 thereafter. The specific growth rate(u) of gardenia cell was 0.095 day^{-1}, corresponding to mass doubling time of 7.3 days.

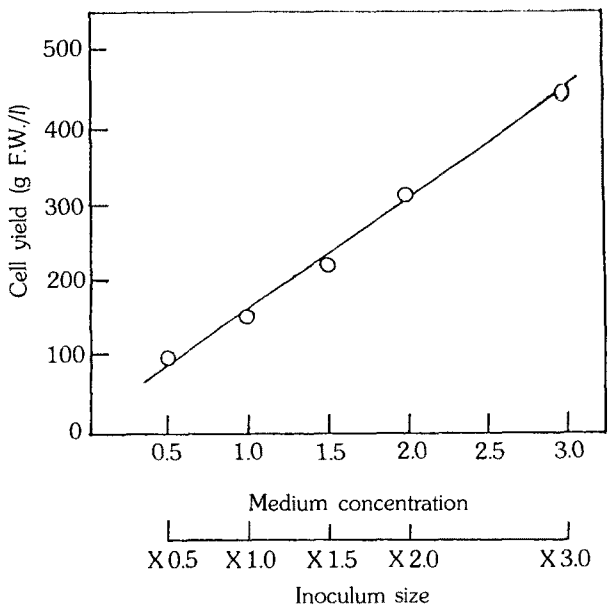

Fig. 6. Effects of medium concentration and inoculum size on cell yield.
Different amount of cell was inoculated in 100 ml of B_5 medium (X1 = 1.0 ml of cell). Inoculum density was increased proportionally to medium concentration.

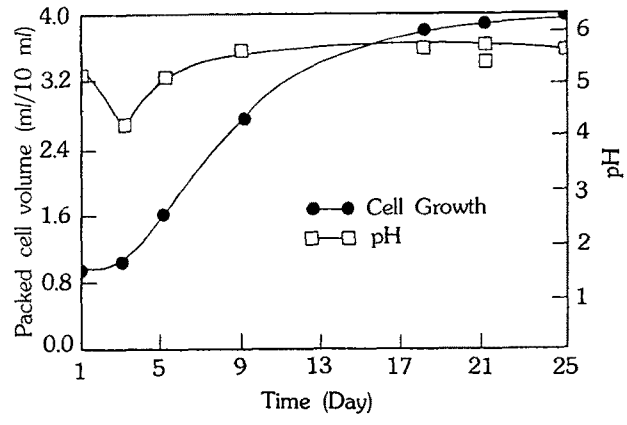

Fig. 7. Growth curve of *Gardenia jasminoides* Ellis and change of pH according to cell growth.
10 ml of cell suspension was inoculated in 100 ml of B_5 medium containing 10 μM IAA and 1.0 μM BA.

Immobilization of *Gardenia jasminoides* Cell on Alginate Bead

The immobilized cells tend to direct their metabolism toward product synthesis instead of cell growth, and thereby increased product yield (24, 26). Fig. 8 shows the morphology of cell immobilized in alginate bead where the cells were found to hold larger vacuoles containing yellow pigment compared to those in suspension

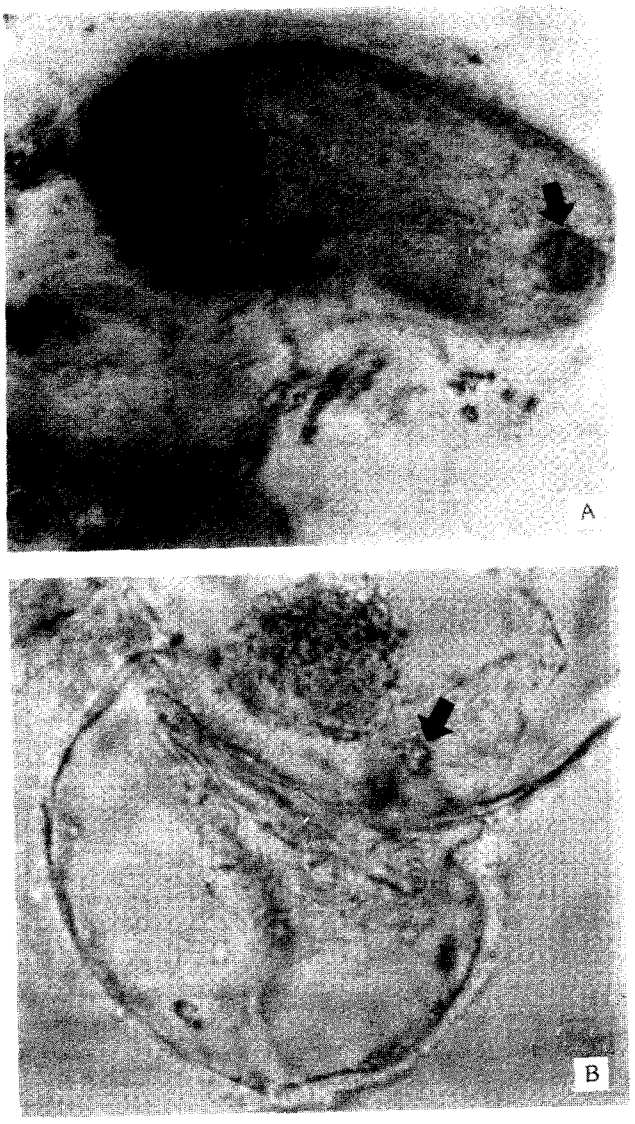

Fig. 8. Morphology of immobilized and suspension cultured cells (X 400).
A; immobilized cell in alginate bead, B; suspension cultured cell. Arrow indicates vacuoles containing yellow pigment.

cultures. After 14-day culture the alginate beads were dissolved in 0.05 M potassium phosphate buffer (pH 7.5) and the carotenoids were extracted with 80% acetone. The content of carotenoid was measured to be about 264.4 μg/g F.W. which was more than twice of that of suspension culture (112.08 μg/g F.W.).

Co-cultivation of *Gardenia jasminoides* Cell with Fungus and Bacterium

Many species of fungus and bacterium cause disease on plant. The components excreted from these microorganism tend to induce chemical protection mechanism of plant, which plays a decisive role in building up of

Table 5. Effects of bacterial and fungal treatments on carotenoid synthesis of gardenia cell

(Absorbance at 450 nm)

Microorganism / Sterilization	Tr* F	Tr A	Af** F	Af A	Agro*** F	Agro A
Dark	0.13	0.27	0.11	0.11	–	.12
Light	0.16	0.29	0.21	0.18	0.33	–

Tr*; *Trichoderma reesei*, Af**; *Aspergillus fumigatus*, Agro***; *Agrobacterium tumefaciens*, F; Filter sterilization, A; Autoclave, Control; 0.14 (Dark), 0.32 (Light)

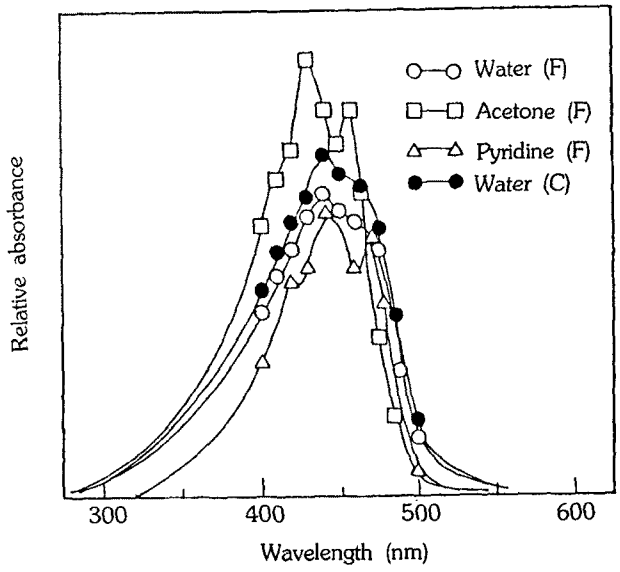

Fig. 9. Comparison of absorption spectra of extract of suspension cultured cell with that of dry fruit.
5.0 g (fresh weight) of 14-day suspension cultured cell was extracted with 20 ml water. 0.5 g of dry fruit was extracted with 100 ml of water, 80% acetone, and pyridine.

resistance of plant against pathogens and herbivores (12, 30). The fungi, *Trichoderma* and *Aspergillus* species, secret various carbohydrases, such as cellulase, α-amylase, and β-glucosidase.

Some chemicals originated from plant can be elicitated by co-cultivation of plant cells with plant pathogenic microorganism. The carotenoid formation in gardenia cell was not elicitated, when gardenia cell was co-cultivated with sterilized microbial cells, as shown in Table 5, and also color change was not occurred.

However, when non-sterilized *Trichoderma* cell was added to gardenia cell cultures, the colour of gardenia cell was changed from yellow into red. This phenomenon may be due to modification of chemical structure of α-crocin, especially gentiobiose bound to both ends of α-crocin, by the activity of secreted chemicals or enzymes

of living *Trichoderma*. The detail mechanism of colour change needs to be studied.

Identification and Quantification of Carotenoid

Spectrophotometric spectrum of the water extract from cultured gardenia cell was measured from the wavelength of 300 to 600 nm. This peak showed carotenoid specific trident type between 400 and 500 nm, which is very similar to spectral data of extract of dry fruit as shown in Fig. 9. and to standatd curve obtained by using crocetin as an authentic material.

The quantity of carotenoids of extracts was determined by measuring the absorbance at 540 nm and using β-carotene as a standard. The content of carotenoid in suspension cultured cells was 112.08 μg/ F.W. The effort to select better cell line has to be continued to achieve high yield of carotenoid production.

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