Clonal Micropropagation of *Gentiana scabra* BUNGE var. *buergeri* MAXIM. and Examination of the Homogeneity Concerning the Gentiopicroside Content

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When the axillary buds of *Gentiana scabra* BUNGE var. *buergeri* MAXIM. were cultured on Murashige–Skoog (MS) medium supplemented with a combination of gibberellin A₃ (GA) and 6-benzylaminopurine (BAP) (1 mg/l each) at 25°C under 16 h light conditions for 60 d, the direct multiple shoot formation (18.5 shoots per axillary bud) occurred. The MS medium containing GA–BAP (2.5 mg/l each) mostly stimulated the multiple shoot formation having approximately 71 shoots per tissue in the second subculture of the regenerated shoot. The hormone free MS medium stimulated root formation. These clonally propagated plants were transplanted to vermiculite. Quantitative analysis of gentiopicroside contained in the root of plantlets was carried out by high performance liquid chromatography using *p*-anisic acid as an internal standard. Obviously, the variation of the clonally propagated plants was smaller than that of the cultivated plants bred by morphological selection. From this system, 4.7×10^8 clonally propagated plants can be theoretically obtained from an axillary bud in a year.

Keywords *Gentiana scabra* var. *buergeri*; axillary bud culture; clonal propagation; quantitative analysis; gentiopicroside

It is well-known that *Gentiana* spp. contain a bitter principle, gentiopicroside, and that these roots have been widely used as a stomachic. *Gentiana scabra* var. *buergeri* is a perennial herb of the family Gentianaceae indigenous to Japan. Some differences in the content of gentiopicroside depend on the strain as well as the growth place.^{1,2} However, since there has been no survey of the homogeneity of gentiopicroside content in wild strains and in the seasonal variation of gentiopicroside content in individuals, it is still obscure as to whether the difference in content of gentiopicroside depends on individuals or on their ages. Therefore, the breeding of a homogeneous strain with respect to gentiopicroside content is necessary. The homogeneous characteristics of secondary metabolites in the clonally propagated plants have been determined in several species, e.g. *Stevia rebaudiana*,³ *Bupleurum falcatum*,⁴ *Angelica acutiloba*,⁵ *Aconitum carmichaeli*,⁶ *Rehmannia glutinosa*⁷ and *Atractylodes* spp.⁸

In this investigation, the clonal micropropagation of *G. scabra* var. *buergeri* by axillary bud culture will be presented. Furthermore, in order to estimate the homogeneity of the populations, the quantitative analysis of gentiopicroside in the root of clonally propagated plantlets will also be investigated.

Materials and Methods

Plant Materials The cultivated *G. scabra* var. *buergeri* were collected from a field in Iizuka City, Fukuoka Prefecture in March, 1989. The roots of clonally propagated plants were collected from plantlets cultured on hormone free Murashige and Skoog (MS)⁹ medium for 3 months. These roots were stored at 0°C until analysis. The axillary buds for tissue culture were obtained from a cultivated strain in the herbal garden of the Faculty of Pharmaceutical Sciences, Kyushu University in May, 1987 and from cultivated plants in a field in Sakugi, Futamigun, Hiroshima Prefecture in July, 1987.

Tissue Culture Portions of a node (1 cm in length) having axillary buds were removed from one plant, sterilized with 3% sodium hypochlorite solution containing 0.1% Tween 80 for 10 min, then with 70% ethanol for 30 s and finally were thoroughly washed twice with sterilized water. The axillary buds were dissected from the nodes under a binocular microscope. Sections (1.0–2.0 mm in length) had two leaf primordia. The basal medium consisted of MS, and Gamborg B5 (B5)¹⁰ salts supplemented with indoleacetic acid (IAA), indolebutyric acid (IBA), 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and gib-

berellin A₃ (GA) in the concentrations and combinations indicated in the Tables. Culture conditions were indicated in individual tables. In the initiation stage, 4–5 axillary buds as indicated in Table I were individually cultured for 60 d. In the subculture of shoots propagated in the initial stage, 6–8 shoots, as indicated in Table II, were cultured. In the root forming media, various strengths of IAA, NAA or IBA were used at $25 \pm 1^\circ\text{C}$ under 16 h of light for 60 d.

Quantitative Analysis of Gentiopicroside Gentiopicroside was isolated from the roots of *G. scabra* var. *buergeri* and identified by field-desorption mass (FD-MS) spectrum, and proton nuclear magnetic resonance (¹H-NMR) and carbon-13 nuclear magnetic resonance (¹³C-NMR) spectra of its tetraacetate. These data were as follows. FD-MS *m/z*: 356 (M)⁺. ¹H-NMR (CD₃Cl) δ : 5.44 (d, *J*=2 Hz, C₁-H), 7.40 (d, *J*=1 Hz, C₃-H), 5.66 (ddd, *J*=7, 10, 17 Hz, C₆-H), 5.25 (bd, *J*=6 Hz, C₇-H), 5.57 (brs, C₈-H), 3.30 (d, *J*=7 Hz, C₉-H), 5.19 (brs, C₁₀-H), 5.20 (d, *J*=9 Hz, C₁-H), 4.86 (d, *J*=8 Hz, C₂-H), 4.96 (t, *J*=9 Hz, C₃-H), 5.08 (t, *J*=9 Hz, C₄-H), 3.74 (ddd, *J*=2, 5, 11 Hz, C₅-H), 4.16 (dd, *J*=2, 12 Hz, C₆-H), 4.28 (dd, *J*=4, 12 Hz, C₆-H), 2.1, 2.0, 1.9 (CH₃CO). ¹³C-NMR (CD₃Cl) δ : 116.9 (C-1), 147.7 (C-3), 125.2 (C-4), 132.7 (C-5), 96.1 (C-6), 69.4 (C-7), 96.5 (C-8), 45.1 (C-9), 118.7 (C-10), 104.4 (C-1'), 68.1 (C-2'), 70.4 (C-3'), 72.3 (C-4'), 72.3 (C-5'), 61.7 (C-6'), 163.0 (C=O), 170.6, 170.1, 169.4, 169.1 (CH₃CO), 20.7, 20.6, 20.5 (CH₃CO). Quantitative analysis was carried out by high performance liquid chromatography (HPLC) as in the following. The dried root powder (50 mg) was extracted 3 times with MeOH (5 ml) for 15 min under sonication. The solvent was removed *in vacuo*. The residue was dissolved in H₂O (0.5 ml) and injected into SEP-PAK C18 (waters), and washed with H₂O (15 ml), and then eluted with 50% MeOH (15 ml). The eluate was evaporated *in vacuo*. The residue was dissolved in the mobile phase, and an internal standard (*p*-anisic acid; 0.25 mg/ml) solution (2 ml) was added and injected into HPLC. Analyses were performed on a Gasukuro Kogyo Model 576 HPLC which was connected with an Inatosil ODS (4.6 × 250 mm, Toso) and equipped with a variable wave length detector (detection set at 265 nm). The mobile phase was the mixture of 0.01 M AcONa (pH 4.6)–MeOH (1:1). Gentiopicroside (2.10 mg) was dissolved in the mobile phase solution, diluted stepwise, and *p*-anisic acid solution (0.25 mg/ml) (1 ml) was added, and the 10 μ l portions were injected into HPLC twice. The calibration plots were obtained from the ratio of peak heights of *p*-anisic acid and gentiopicroside as indicated in Fig. 3. In this procedure, the recovery of gentiopicroside was found to be 96.7%.

Results and Discussion

The effect of GA–BAP on shoot formation from axillary buds is shown in Table I. All combinations of GA and BAP stimulated shoot formation, especially 1 mg/l GA and 1 mg/l BAP gave direct multiple shoot complexes having an average of 18.5 shoots per tip during a 60-day-culture. The addition of IAA and BAP to the medium gave shoot

TABLE I. Multiple Shoot Formation from Axillary Bud

Hormone (mg/l)		Shoot No. per culture	Multiple shoot forming culture	Fresh weight (mg)
GA	BAP			
0.1	0.1	8.8	4/5	224
0.1	0.5	8.0	3/5	282
0.1	1.0	11.8	4/5	388
0.5	0.1	9.0	3/4	346
0.5	0.5	6.5	3/4	332
0.5	1.0	8.0	3/4	225
1.0	0.1	13.3	3/4	456
1.0	1.0	18.5	4/4	509

Culture condition: MS medium, $25 \pm 1^\circ\text{C}$, 16 h light, 60 d.

TABLE II. Multiple Shoot Formation from Axillary Bud

Hormone (mg/l)		Shoot No. per culture	Hormone (mg/l)		Shoot No. per culture
IAA	BAP		NAA	BAP	
0.1	0.1	3.2	0.1	0.1	4.3
0.1	1.0	3.0	0.1	1.0	6.0
0.5	0.1	2.8	0.5	0.1	3.3 ^{a)}
0.5	1.0	2.8	0.5	1.0	9.5
1.0	0.1	2.8	1.0	0.1	1.3 ^{a)}
1.0	1.0	2.5	1.0	1.0	11.0

Culture condition: MS medium, $25 \pm 1^\circ\text{C}$, 16 h light, 60 d. a) Callus forming segment.

TABLE III. Multiple Shoot Formation from Regenerated Shoot

Hormone (mg/l)		Multiple shoot forming culture	Shoot No. per culture	Fresh weight (mg)
GA	BAP			
0.5	2.5	6/6	54.5	1128
0.5	5.0	7/7	38.6	973
1.0	2.5	8/8	63.8	1105
1.0	5.0	8/8	42.3	966
2.5	2.5	7/7	70.7	2097
2.5	5.0	6/6	44.0	1323

Culture condition: MS medium, $25 \pm 1^\circ\text{C}$, 16 h light, 60 d.

TABLE IV. Root Formation of Shoot Propagated

Hormone (mg/l)		Root No. per culture	Root forming culture	Fresh weight (mg)
NAA	0.1	4.3	4/6 ^{a)}	487
	1.0	9.1	6/7 ^{a)}	263
IAA	0.5	6.4	8/10	841
	1.0	4.2	7/10	315
IBA	0.5	3.4	4/8	470
	1.0	3.6	4/8	445
FH ^{b)}		10.7	19/20	895

Culture condition: MS medium, $25 \pm 1^\circ\text{C}$, 16 h light, 60 d. a) Callus formation. b) Hormone free medium.

Fig. 1. Multiple Shoot Formation from Regenerated Shoot

Fig. 2. Root Formation of Shoot Propagated

GA and BAP as shown in Table III, since the addition of 1 mg/l GA and 1 mg/l BAP stimulated the multiple shoot formation in the first stage. During a 60-day-culture the base of shoot growth manifested multiple complexes and finally produced multiple shoots. The medium containing GA-BAP (2.5 mg/l each) mostly stimulated the multiple shoot formation having approximately 71 shoots per culture as shown in Fig. 1. This multiplication rate was constantly obtained for several generations. On the other hand, abnormal shoot formation occasionally occurred during culture on the medium supplemented with concentrations of GA higher than 2.5 mg/l and BAP. Therefore, the medium containing GA-BAP (2.5 mg/l each) can be routinely used for the micropropagation system.

In order to determine the suitable basal medium and effective hormone for rooting, the MS media containing

formation, however, the multiplication ratio was low (Table II). The supplement of NAA and BAP was much better than the combination of IAA and BAP (Table II), although callus formation occurred at the base of a shoot.

In order to accelerate multiple shoot formation, in the second stage the shoots propagated were transferred to a new medium supplemented with higher concentrations of

Fig. 3. Calibration Curve of Gentiopicroside

$Y = 0.8966X - 0.0011$ ($r = 0.9999$). X : content ratio (gentiopicroside/*p*-anisic acid). Y : peak height ratio.

Fig. 4. Chromatogram of HPLC for Quantitative Analysis of Gentiopicroside and *p*-Anisic Acid

HPLC conditions: Column, Inertsil ODS (4.6×250 mm). Mobile phase, 0.1 M AcONa (pH 4.6): MeOH = 1:1. Flow rate, 0.5 ml/min. Detection, 265 nm. *a*) Internal standard.

IAA, NAA and IBA or without hormone were tested on shoots propagated at 25°C under 16 h of light for 60 d (Table IV). It was found that the MS medium supplemented with 0.5 mg/l IAA promoted root formation (80%). On the other hand, the addition of NAA to the medium stimulated callus formation. The hormone free medium also stimulated rooting. The ratio was 95% and the growth of plant was good as indicated by the fresh weight in Table IV and in Fig. 2. Therefore, this medium can be used as a root-forming medium. These clonally propagated plants were completely transplanted to vermiculite.

Quantitative analysis of gentiopicroside was carried out by HPLC using *p*-anisic acid as an internal standard. The calibration curve was quite linear (Fig. 3), showing that the recovery was complete. The chromatogram for quantitative

TABLE V. Gentiopicroside Contents of Clonally Propagated Plantlets (1) and Cultivated Strain (2) of *Gentiana scabra* var. *buergeri*

Material	N	% of dry weight	% of C.V. ^{a)}
1 ^{b)}	30	$8.07 \pm 0.91^c)$	11.3
2	25	11.72 ± 2.21	18.8

a) Coefficient of variation. *b*) Cultured on the hormone free MS medium for 90 d. *c*) Significantly different from the cultivated strain, $p < 0.01$.

analysis of gentiopicroside is also shown in Fig. 4.

Table V shows the content of gentiopicroside and the coefficient of variation in the roots of clonally propagated plantlets derived from a single original plant and of the cultivated strains which were sexually propagated from seeds. A homogeneous tendency was observed in the clonally propagated plants with respect to the gentiopicroside content when compared on the basis of the coefficient of variation although differences in the coefficient of variation were not statistically significant. The cultivated strain has been bred by sexual selections concerning flower color, flower shape, flower period and/or plant height. Therefore, the breeding of a homogeneous strain of *G. scabra* var. *buergeri* concerning the gentiopicroside content may be difficult by morphological selection.

Regeneration from the tip tissue culture of *G. scabra* var. *buergeri* has been previously presented.^{11,12)} Maruta *et al.*¹¹⁾ reported that the supplement of 0.1 mg/l NAA and 7.5 mg/l BAP to the MS medium mostly stimulated multiplication of tip tissue at a rate of 7 shoots per culture. The authors have succeeded in improving approximately 10 times the multiplication rate when shoots were cultured on a medium containing 2.5 mg/l GA and 2.5 mg/l BAP as already discussed. Significant differences in the multiplication rate may occur depending on the synergistic effect of GA and BAP supplemented in the medium. In this micro-propagation system, 4.7×10^8 clonally propagated plants can be theoretically gained from an axillary bud in a year. This procedure could be utilized to facilitate a large-scale cultivation of homogeneous plant of *G. scabra* var. *buergeri*.

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