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Production of asiaticoside and madecassoside in *Centella asiatica in vitro* and *in vivo*

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Abstract

The localization was determined of the triterpenoids, asiaticoside and madecassoside, in different organs of glasshouse-grown plants and cultured material, including transformed roots, of two phenotypes of *Centella asiatica* (L.) Urban of Malaysian origin. Methanolic extracts of asiaticoside and madecassoside were prepared for gradient HPLC analysis. The two phenotypes of *C. asiatica* exhibited differences in terpenoid content that were tissue specific and varied between glasshouse-grown plants and tissue culture-derived material. Terpenoid content was highest in leaves, with asiaticoside (0.79 ± 0.03 and 1.15 ± 0.10 % of dry mass) and madecassoside [0.97 ± 0.06 and 1.65 ± 0.01 %(d.m.)] in the fringed (F) and smooth leaf (S) phenotypes, respectively. Roots of the F-phenotype contained the lowest content of asiaticoside [0.12 ± 0.01 %(d.m.)], whereas petioles of S-phenotype plants contained the lowest content of asiaticoside [0.16 ± 0.01 %(d.m.)] and madecassoside [0.18 ± 0.14 %(d.m.)]. Transformed roots were induced using *Agrobacterium rhizogens* and their growth was maximal on Murashige and Skoog basal medium supplemented with 60 g dm^{-3} sucrose. However, asiaticoside and madecassoside were undetectable in transformed roots and undifferentiated callus.

Additional key words: *Agrobacterium* transformation, phenotypic variation, terpenoids.

Introduction

Centella asiatica (L.) Urban, a weakly aromatic, slender, creeping perennial plant that flourishes in wet areas of Malaysia and other Asian countries, has been used as a medicinal species since ancient times. Analytical studies have shown that *C. asiatica* contains triterpenoids, essential oils, amino acids and other compounds, such as vellarin. The terpenoids include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, centellose, and asiatic, brahmic, centellic and madecasic acids (Dutta and Basu 1968, Singh and Rastogi 1969). Asiaticoside is the most widely studied and its chemical structure is shown in Fig. 1.

Two morphologically distinct lines of *C. asiatica* exhibiting heavily fringed or smooth leaf margins

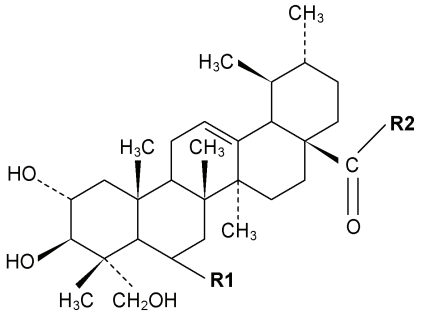

Fig. 1. Chemical structure of pentacyclic triterpenes. R1 = H (for asiaticoside) or OH (for madecassoside), R2 = glucose-glucose-rhamnose.

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Abbreviations: *ANOVA* - analysis of variance; BAP - 6-benzylaminopurine; d.m. - dry mass; 2,4-D - 2,4-dichlorophenoxyacetic acid; ELISA - enzyme-linked immunosorbent assay; f.m. - fresh mass; IBA - indolebutyric acid; MS - Murashige and Skoog (1962); LB - Luria broth; NAA - α-naphthaleneacetic acid; *npt*II - neomycin phosphotransferase gene; NPTII - neomycin phosphotransferase protein; SEM - standard error of the mean.
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(Fig. 2), are cultivated in Malaysia. This investigation evaluated the asiaticoside and madecassoside content in leaves, petioles and roots of these phenotypes. A comparison was made of the asiaticoside and madecassoside content of glasshouse-grown plants and those derived from callus. Further aims of this study were to *1*) initiate callus and determine the asiaticoside and madecassoside content, *2*) evaluate the transformation efficiency of *C. asiatica* by *A. rhizogenes* and subsequent growth of transformed roots, *3*) determine the effects of sucrose on growth of transformed roots, and *4*) determine the asiaticoside and madecassoside contents in transformed roots.

Materials and methods

Plant material: Two phenotypes of *Centella asiatica* (L.) Urban of Malaysian origin with heavily-fringed (designated F-line) and smooth leaf margins (designated S-line) were used. S-line plants had longer stolons than those of F-line specimens. Authenticity of *Centella* specimens was confirmed by Dr M. Watson (Herbarium, Royal Botanic Garden, Edinburgh, UK). Plants were maintained in the glasshouse in a 4:1:0.5 (by volume) mixture of *Levington M3* compost (*Fisons* plc., Ipswich, UK), *John Innes No. 3* compost and *Silvaperl*® graded *Perlite* (*William Sinclair Horticulture Ltd*., Lincoln, UK) in 9 cm diameter plastic pots. After 2 - 3 months, nodes from stolons were removed from a single plant of each phenotype and potted in the same compost-*Perlite* mixture to produce sub-clones. Plants were maintained in the glasshouse with maximum day/night temperatures of 22 ± 5/20 ± 2 ºC and natural daylight supplemented by a 16-h photoperiod (180 μmol m^{-2} s^{-1} provided by TLD 58W daylight fluorescent tubes; *Philips Lighting Ltd*., Croydon, UK) and 70 % relative humidity. After 12 weeks, sub-clones were transferred to the same potting mixture in 13 cm diameter pots and re-potted as required to ensure a supply of vigorously growing plants.

Establishment of shoot cultures: Cultured shoots of F- and S-lines were established from glasshouse-maintained plants. Stolons, each 1.0 cm in length with nodes and internodes, were transferred to 40 cm^3 aliquots of growth regulator-free Murashige and Skoog (MS)-based medium with 3.0 % sucrose and 0.8 % agar (*Sigma*, Poole, UK) at pH 5.8 (designated MS0.8 medium), in 175 cm^3 capacity screw-capped powder round glass jars (*Beatson Clark Co. Ltd*., Rotherham, UK). Prior to introduction into culture, stolon explants were surface-sterilised with 15 % *Domestos* bleach solution (*Lever Fabergé*, Kingston-upon-Thames, UK) for 25 - 30 min, followed by washing 3 times in sterile, reverse-osmosis water. Cultures were maintained at 25 ± 2 ºC with a 16-h photoperiod (25 μmol m^{-2} s^{-1}, daylight fluorescent tubes). Shoots were subcultured every 60 - 90 d by removing mature leaves and transferring individual nodes, or new leaf-rosettes, to 40 cm^3 aliquots of MS0.8 agar medium. These cultures were utilised for studies of the distribution and accumulation of asiaticoside and madecassoside in plants *in vitro*.

Micropropagation: S-line stolon explants, each with a node and single axillary bud, were placed horizontally on the surface of half-strength MS-basal agar medium containing 2.0 mg dm^{-3} of benzylaminopurine (BAP), 0.5 mg dm^{-3} of kinetin and 0.25 mg dm^{-3} of indolebutyric acid (IBA; Josekutty 1998), or semi-solidified MS0.8 medium. Stolon explants of the F-line were also placed on MS medium with BAP at 5 concentrations from 0 - 5.0 mg dm^{-3} with 0.5 mg dm^{-3} kinetin and 0.25 mg dm^{-3} IBA, pH 5.8. Cultures were maintained at 25 ± 2 ºC with a 16-h photoperiod, as for shoot cultures.

Transfer of plants to the glasshouse: Individual plants, each 3 - 5 cm in height with at least 2 leaves, were removed from MS0.8 medium, washed with tap water and transferred to compost in 7.5 cm diameter pots. Plants were covered with transparent polyethylene bags and maintained in the glasshouse. After 7 d, one corner of each polythene bag was removed to reduce the relative humidity; this was repeated at the 14th d and the bags were removed after 21 d. After 84 d, plants were transferred into 9.0 cm diameter pots of the same compost.

Plant regeneration from leaf and petiole explants: Petiole and lamina explants were cultured on MS-basal medium containing 2.0 mg dm^{-3} of BAP and 0.1 mg dm^{-3} of IBA, semi-solidified with 0.8 % agar (Banerjee *et al*. 1999). MS-based medium containing BAP (0 - 5 mg dm^{-3}) and NAA (0.1 mg dm^{-3}) were also evaluated. Cultures were maintained at 25 ± 2 ºC in a 16-h photoperiod as described earlier.

Initiation of callus and plant regeneration: Callus was initiated from surface sterilized petiole and lamina explants, 21 - 35 d after transfer of plants to the glasshouse. Petiole sections, each 1.0 cm in length and lamina explants (1.0 cm^2), were cultured on 20 cm^3 aliquots of MS medium with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.1 - 0.5 mg dm^{-3}), BAP (0.1 - 0.2 mg dm^{-3}), 3 % sucrose and semi-solidified with 0.8 % agar, pH 5.8 in 9 cm diameter Petri dishes. Petiole sections (10 per 9 cm dish) were placed horizontally and lamina explants (10 per dish) were placed with their abaxial surface in contact with the medium. Cultures were maintained at 25 ± 2 ºC as described earlier.

To induce shoot regeneration, 28- to 32-d-old proliferating, friable leaf- and petiole-derived calli (F-line; 50 - 60 mg f.m.), initiated on MS medium with 0.2 mg dm^{-3} 2,4-D and 0.2 mg dm^{-3} BAP, were transferred

to 9.0 cm diameter Petri dishes each containing 20 cm^3 medium. Cultures were maintained at 25 ± 2 ºC in a 16-h photoperiod.

Culture of bacteria: *Agrobacterium rhizogenes* strain R1601 carried pRiA4b with a *npt*II gene cointegrated into *Hind*III fragment 21 of the TL-DNA and pTVK291 *in trans* giving a supervirulent phenotype (Pythoud *et al*. 1987). Bacteria were grown on Luria broth (LB; Sambrook *et al*. 1989) supplemented with kanamycin sulphate (100 mg dm^{-3}) and ampicillin (100 mg dm^{-3}) in the dark at 28 ± 2 ºC. Suspensions were initiated by transferring one loopful of a 3 day-old culture from semi-solid agar (0.6 %) LB to 20 cm^3 of liquid LB with the appropriate antibiotics, in 100 cm^3 capacity Erlenmeyer flasks with shaking (125 r.p.m., 16-h dark, 28 ºC). Cultures were grown to a density corresponding to an absorbance of 1.1 - 1.6 at 600 nm (Curtis *et al*. 1994), prior to plant inoculation.

Inoculation of explants: Leaf explants, with petioles attached, and stem segments were excised from cultured shoots. Each was inoculated by 4 - 5 injections of bacterial suspension, using a sterile hypodermic needle (0.5 × 25 *Nr. 18*, *25GI*, *Microlance R3*®; *Becton Dickinson*, Dublin, Ireland) attached to a sterile 1.0 cm^3 syringe (*Plastipak*®, *Becton Dickinson*) containing an overnight culture of *A. rhizogenes* strain R1601. Explants were blotted dry on sterile filter papers (*Whatman No. 1*) and placed onto the surface of 20 cm^3 aliquots of MS0.8 medium in 9 cm diameter Petri dishes. Explants were co-cultivated with bacteria for 3 d with a 16 h photoperiod at 25 ± 2 ºC, followed by transfer to MS0.8 agar medium with 400 mg dm^{-3} cefotaxime (*Claforan*; *Roussel Laboratories*, Uxbridge, UK).

Roots which developed on explants at sites inoculated with the bacteria were excised 28 - 35 d after inoculation. Individual roots (1.5 - 2.0 cm in length) were transferred to the surface of 20 cm^3 aliquots of MS0.8 medium in 9 cm diameter Petri dishes supplemented with 400 mg dm^{-3} cefotaxime and 100 mg dm^{-3} kanamycin sulphate. Cultures were maintained under a 16-h photoperiod or in the dark at 25 ± 2 ºC. After 40 - 50 d, 0.5 mg of root f.m. was transferred to 50 cm^3 liquid MS0.8 medium in 250 cm^3 Erlenmeyer flasks on a horizontal shaker (90 - 100 r.p.m.) and cultured under a 16-h photoperiod. Roots were subcultured every 36 - 40 d. The concentrations of cefotaxime and kanamycin sulphate were reduced progressively at each subculture. Cefotaxime was removed during the third subculture, but kanamycin sulphate was maintained at 50 mg dm^{-3}.

The transgenic status of *A. rhizogenes*-induced roots was confirmed by their growth on MS0.8 medium containing kanamycin sulphate at 10, 25, 50, 75, 100, 150 or 200 mg dm^{-3} at 25 ± 2 ºC in a 16-h photoperiod and by ELISA for NPTII protein synthesis (Al-Forkan *et al*. 2004).

Effect of sucrose on the growth of transformed roots: The potential beneficial effect of sucrose concentration (0 - 100 g dm^{-3}) in MS-basal liquid medium was evaluated on growth of transformed roots. Approx. 50 mg f. m. of transformed roots were inoculated into 50 cm^3 liquid medium supplemented with sucrose in 250 cm^3 Erlenmeyer flasks. Flasks were placed on a horizontal shaker (90 - 100 r.p.m.) and maintained in the dark at 25 ± 2 ºC. All culture media were growth regulator free, containing 50 mg dm^{-3} of kanamycin with the pH adjusted to 5.8 before autoclaving. The final d.m. of transformed roots was recorded after 36 d of culture.

Preparation of plant material for extraction of terpenoids: Leaves, petioles and roots of glasshouse-grown plants were harvested and washed with tap water. Tissues were freeze-dried overnight (*Alpha 2-4 LD* instrument; *Christ*, Osterode am Harz, Germany) and either ground to a powder with a pestle in a mortar, or stored at -20 ºC. Liquid nitrogen was used to facilitate sample homogenization.

Extraction of terpenoids: Terpenoid extraction was based on the method of Inamdar *et al*. (1996) with modification. One g of powdered tissue was mixed with 10 cm^3 methanol:water (9:1, v/v) in a 100 cm^3 Erlenmeyer flask and covered with *Nescofilm* (*Bando Chemical Co*., Kobe, Japan). Samples were shaken at 90 - 100 rpm for 30 h at room temperature, filtered (*Whatman No. 1* paper) under reduced pressure and filtrates transferred to screw-capped 30 cm^3 capacity glass jars. Filtrates were evaporated to dryness. Residues were re-dissolved in either 0.5 or 1.0 cm^3 methanol:water (9:1, v/v). Samples were transferred into 1.5 cm^3 microfuge tubes and vortexed for 2 - 3 min, followed by centrifugation (1 min) at 5 800 *g* to remove particulate matter. Supernatants were filtered through bacteriological membranes (0.2 μm pore size) before HPLC.

HPLC analyses: These were performed using a *Series 1100* Chromatograph (*Hewlett Packard*, Houston, USA), consisting of a binary pump, autosampler, diode array detector and a data station. Chromatographic separation was performed using a *Novapak C18* column (5 μm diameter particle size; 3.9 × 150 mm; *Waters Associates Inc*., Milford, USA) with water-acetonitrile (HLPC grade; *Fisher Scientific*, Loughborough, UK) as the mobile phase. Separation was carried out with a flow rate of 1 cm^3 min^{-1}. The sample injection volume was 0.01 cm^3 at 25 ºC.

In preparation of standards for HPLC, 1.0 mg of asiaticoside and madecassoside were dissolved in 1.0 cm^3 of methanol (HPLC grade; *Fisher Scientific*) and analysed at 200, 210 and 230 nm. The wavelength at which the maximum peaks were detected was used for plant sample analysis; the contents of asiaticoside and madecassoside in plant extracts were determined by comparing peak areas of plant samples with those of standards. Three replicates of plant extracts from leaves, petioles and roots of each phenotype were analysed. The UV spectra of the peaks for the samples were compared

with those of the standards to confirm that the peaks eluted at the specified retention times were asiaticoside and madecassoside.

Statistical analyses: Means and SEM were used throughout; statistical significance between mean values was assessed using conventional *ANOVA* (Snedecor and Cochran 1989).

Results

Establishment of shoot cultures: Internode explants cultured on MS0.8 agar medium for up to 90 d readily produced leaves with extended petioles arising from a rosette-like base. Internode explants of the S-line produced leaves after 7 - 10 d with each explant giving a mean of 4.6 ± 0.6 (n = 12) rosettes. The S-line produced 2.6 ± 0.5 stolons per explant after 63 d of culture, with individual rosettes forming at the end of each stolon. Additionally, some primary stolons developed secondary stolons beyond the terminal leaf-rosette which formed leaf-rosettes. In contrast, F-line internode explants failed to produce stolons and differentiation was restricted to clusters of leaf-rosettes (2.5 ± 0.2).

Micropropagation: Stolon segments of the S-line, each with a single axillary bud, produced rosettes within 28 d of culture on the medium of Josekutty (1998). Rosettes proliferated by multiplication either through axillary buds or via primary and secondary stolons. The mean number (n = 6) of rosettes per explant on micropropagation medium after 70 d of culture was significantly ($P < 0.05$) higher (10.7 ± 1.2) than for explants on MS0.8 agar medium (2.6 ± 0.4). Similarly, explants cultured on Josekutty (1998) medium also produced a significantly ($P < 0.05$) higher mean (n = 5) number of primary stolons per explant (3.0 ± 0.6) compared with those cultured on MS0.8 medium (0.8 ± 0.3).

F-line stolon explants, each possessing a single axillary bud, formed yellow-green leaves when micropropagated. Culture of such explants on MS-basal medium with varying BAP concentrations (1.0 - 5.0 mg dm^{-3}), but with constant concentrations of both IBA (0.25 mg dm^{-3}) and kinetin (0.5 mg dm^{-3}) only marginally improved chlorophyll synthesis. However, for cultures on all media, the mean number of rosettes formed by 60 d was significantly ($P < 0.05$) higher than on MS0.8 medium (Table 1). The highest mean number of rosettes per explant (23.8 ± 2.1, n = 5) occurred on medium containing 5.0 mg dm^{-3} of BAP (Table 1). All explants produced primary stolons on BAP-containing media.

Regeneration of shoots from leaf and petiole explants: Shoot regeneration frequency from explants of F- and S-phenotypes cultured on Banerjee *et al*. (1999) medium and MS medium supplemented with NAA (0.1 mg dm^{-3}) and BAP (1.0 - 5.0 mg dm^{-3}) was highly variable,

Table 1. Mean number of leaf-rosettes and primary stolons per explant from the F-phenotype of *C. asiatica* after 60 d of culture on different media. Values are mean (± SEM) of 5 replicates. Control - MS-basal medium alone, Media 1-6 - MS-basal medium with 0.5 mg dm^{-3} kinetin and 0.25 mg dm^{-3} IBA, together with 0, 1.0, 2.0, 3.0, 4.0 or 5.0 mg dm^{-3} BAP, respectively.

	Control	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6
Number of leaf rosettes	2.0 ± 0.3	21.0 ± 3.8	7.0 ± 0.3	9.2 ± 0.7	14.2 ± 1.1	14.5 ± 1.0	23.8 ± 2.1
Number of primary stolons	0	3.8 ± 1.9	0	<1	<1	1.8 ± 0.2	2.7 ± 0.2

Table 2. Growth of *Agrobacterium rhizogenes* transformed roots of the *C. asiatica* S-phenotype after 40 d of culture on medium containing kanamycin. Values are mean ± SEM (n = 3 throughout). For callus, - = absent, +++ = prolific growth. D = root explants died.

Kanamycin concentration [mg dm^{-3}]	Transformed roots final d. m. [g]	survival [%]	callus formation	Non-transformed roots final d. m. [g]	survival [%]	callus formation
0	0.09 ± 0.01	100	-	<0.01	100	-
10	0.07 ± 0.01	100	-	<0.01	15 ± 3	-
25	0.09 ± 0.01	100	-	D	0	-
50	0.07 ± 0.05	95 ± 5	-	D	0	-
75	0.03 ± 0.01	100	+	D	0	-
100	0.04 ± 0.01	100	+	D	0	-
150	0.05 ± 0.01	100	++	D	0	-
200	0.02 ± 0.01	50 ± 6	+++	D	0	-

depending on the genotype and explant. For petiole explants of the F-line, the mean maximum regeneration frequency (42 %) was on Banerjee *et al*. (1999) medium with 2.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} IBA. However, for leaf explants of the same line, maximum regeneration frequency (30 %) occurred with 3.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA. Similarly, for petiole explants of the S-line, maximum regeneration frequency (44 %) occurred when the explants were cultured on Banerjee *et al*. (1999) medium. In contrast, maximum regeneration frequency (25 %) for leaf explants of the S-line occurred on medium containing 5.0 mg dm^{-3} BAP and 0.1 mg dm^{-3} NAA. Shoots were regenerated after 21 - 28 d of culture from leaf explants of both lines. In contrast, for petiole explants, shoot regeneration was initiated after 28 - 35 d, with compact, green calli being produced prior to shoot formation.

Callus induction: Supplementation of MS medium with 2,4-D at 0.1 - 0.5 mg dm^{-3} induced rhizogenesis in F-line petiole and leaf explants. However, further supplementation of these media with BAP at 0.2 mg dm^{-3} (Solet *et al*. 1993) induced the formation of friable callus from both types of explants, but petiole explants still exhibited limited rhizogenesis. Further culture of both petiole and leaf explants of the F-line on medium containing 0.5 mg dm^{-3} BAP, but with increasing concentrations of NAA (1.5 - 4.0 mg dm^{-3}), produced fast growing, compact green calli, with typically > 5 roots per explant.

Friable callus was not induced in S-line petiole and leaf explants when cultured on media containing low concentrations (0.1 - 0.5 mg dm^{-3}) of 2,4-D. Petiole and leaf explants cultured on the medium of Solet *et al*. (1993) containing 0.22 mg dm^{-3} 2,4-D and 0.22 mg dm^{-3} BAP, produced compact, green calli which, after 70 d, differentiated into thin chlorophyll-containing roots. Culture of S-line petiole and leaf explants on medium containing 0.1 - 0.5 mg dm^{-3} 2,4-D produced compact, light green callus. Addition of BAP at 0.1 - 0.6 mg dm^{-3} to medium containing 2.0 mg dm^{-3} 2,4-D also induced the formation of rapidly growing friable callus from both types of explants.

Plant regeneration from callus: Shoot buds were produced by petiole-derived callus of the F-phenotype after 21 - 28 d of culture, with subsequent shoot development after 35 - 56 d. Maximum regeneration occurred on medium containing 0.8 mg dm^{-3} BAP and low concentrations (0.01 - 0.02 mg dm^{-3}) of 2,4-D (67 % of callus producing shoots). In contrast, when medium containing 0.8 mg dm^{-3} BAP, together with higher concentrations (0.04 and 0.05 mg dm^{-3}) of 2,4-D was used, only 30 % of callus produced shoots. Regenerated shoots produced roots after 70 - 84 d of culture; such shoots developed normally when transferred to MS-basal medium lacking growth regulators. Regenerated plants from petiole-derived callus were established in the glasshouse and grew into phenotypically-normal plants. In contrast, leaf-derived callus failed to regenerate shoots.

Cytology of regenerants: The chromosome numbers of regenerated plants (one plant selected randomly for each medium) were constant at 2n=2x=18, irrespective of the regeneration medium. This was consistent with the somatic chromosome complement of glasshouse-grown material.

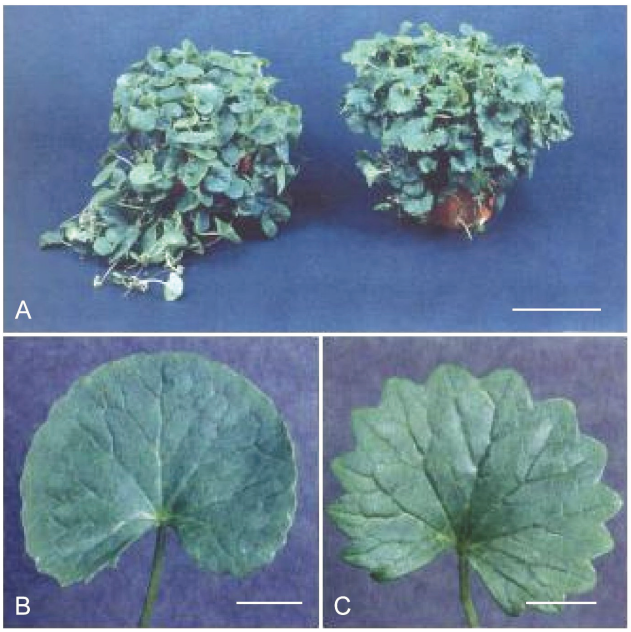

Fig. 2. Two phenotypes of Malaysian *C. asiatica* (*A*): smooth leaf margin phenotype (S-line) (*B*) and heavily fringed phenotype (F-line) (*C*). *Bars* = 7.25 cm (*A*) or 1.0 cm (*B*, *C*).

Initiation of transformed roots: The transformation frequency of *C. asiatica* with *A. rhizogenes* strain R1601 was variable and explant dependent. Stem segments gave the highest mean (n = 3) transformation frequency for both lines (84 ± 10 % and 75 ± 8 %, F- and S-lines, respectively). The mean transformation frequencies, for the F- and S-lines, decreased up to 5-fold ($P < 0.05$) for leaf explants.

Putatively transformed roots developed within 8 - 10 d from stem explants. Transformed roots exhibited two phenotypes. The first were thin and short, generally slow growing with limited lateral branching, cream in colour and produced some callus, whereas the second type grew rapidly with a high degree of lateral branching and constituted the majority of roots observed. When the latter were excised from their source explants and cultured on semi-solid MS-basal medium lacking growth regulators, but containing kanamycin and cefotaxime, they grew rapidly with further extensive lateral branching. Control (uninoculated) stem segments produced roots, but these roots became necrotic on MS-basal medium lacking growth regulators.

Confirmation of transformation of cultured roots: Transgenic roots survived when cultured in medium with all concentrations of kanamycin sulphate evaluated, although root growth was reduced on medium containing 75 - 200 mg dm^{-3} kanamycin sulphate, with less lateral branching and callus formation (Table 2). For example, with 200 mg dm^{-3} kanamycin, lateral roots failed to grow beyond 8 mm in length. Only 15 ± 3 % of control (non-transformed) roots survived at the lowest concentration (10 mg dm^{-3}) of kanamycin evaluated.

Influence of sucrose on growth of transformed roots: Growth of transformed roots correlated positively with the concentration of sucrose in the culture medium, with maximum growth occurring with 60 g dm^{-3} (Fig. 3). In contrast, with concentrations of sucrose higher than 60 g dm^{-3}, transformed root growth progressively decreased. Indeed, transformed roots cultured with sucrose concentrations of 70 - 100 g dm^{-3} were shorter with reduced lateral branching; there was a tendency for transformed roots to produce callus.

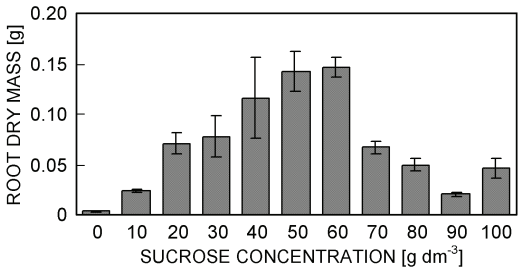

Fig. 3. Effect of sucrose on growth of transformed roots of *C. asiatica* S-phenotype measured by final dry mass. *Vertical bars* = SEM.

Detection of asiaticoside and madecassoside: UV spectra for leaf extracts of F and S phenotypes of glasshouse-grown plants (Fig. 4) showed peaks corresponding to those for asiaticoside and madecassoside standards with elution times for madecassoside and asiaticoside of 6.8 and 8.7 min, respectively.

Asiaticoside and madecassoside content: HPLC analysis of glasshouse-grown plant material revealed differences in asiaticoside and madecassoside content between the two lines of *C. asiatica*. In general, the S-line exhibited the highest content of both compounds in all plant organs, compared to the F-line (Fig. 5). The mean (n = 3) content of both compounds was maximal in leaves [0.79 ± 0.03 % and 1.15 ± 0.10 %(d.m.) asiaticoside and

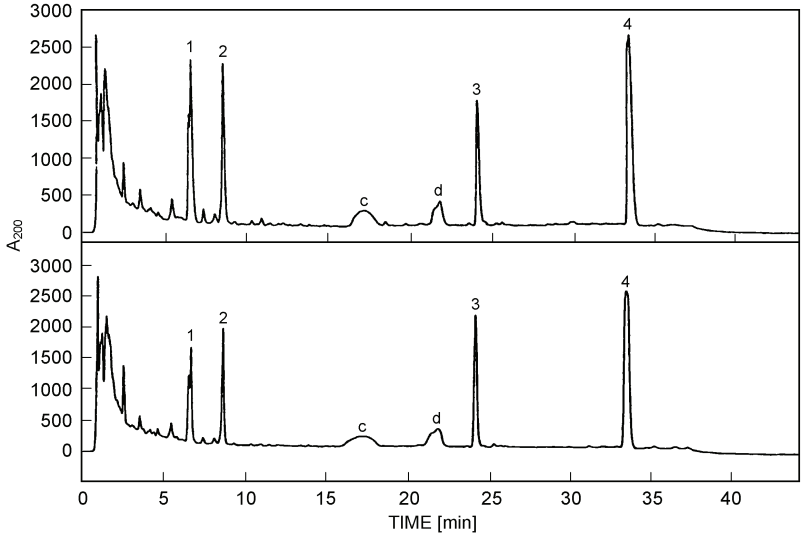

Fig. 4. HPLC profiles of leaf extracts (S phenotype, upper trace; F phenotype, lower trace) of *C. asiatica* glasshouse-grown plants (1 - madecassoside, 2 - asiaticoside; 3, 4 - unknown peaks at 23 - 25 min and 33 - 34 min, respectively; c, d - solvent interference).

0.97 ± 0.06 % and 1.65 ± 0.10 %(d.m.)] madecassoside in F- and S-lines, respectively). In contrast, roots of the F-line exhibited the lowest mean content of asiaticoside (0.12 ± 0.01 %) whereas for the S-line, petioles contained the lowest mean content of asiaticoside (0.16 ± 0.01 %) and madecassoside (0.17 ± 0.01 %).

HPLC analysis of regenerated plants, callus and transformed roots: HPLC analysis of leaf extracts taken from regenerated plants revealed peaks for asiaticoside and madecassoside that were identical to those of glasshouse-grown plants (Fig. 4). The mean content of asiaticoside and madecassoside in regenerated plants varied according to the regeneration medium. For example, the highest mean asiaticoside content (0.9 ± 0.1 %), was observed in leaf extracts from plants regenerated in the presence of 0.8 mg dm^{-3} BAP and 0.01 mg dm^{-3} 2,4-D. Plants regenerated on medium supplemented with 0.8 mg dm^{-3} BAP and 0.05 mg dm^{-3} 2,4-D contained the lowest mean asiaticoside content (0.5 ± 0.1 %). The mean madecassoside content followed a similar pattern. Asiaticoside and madecassoside were undetectable in callus extracts, in transformed roots or liquid medium used for their culture.

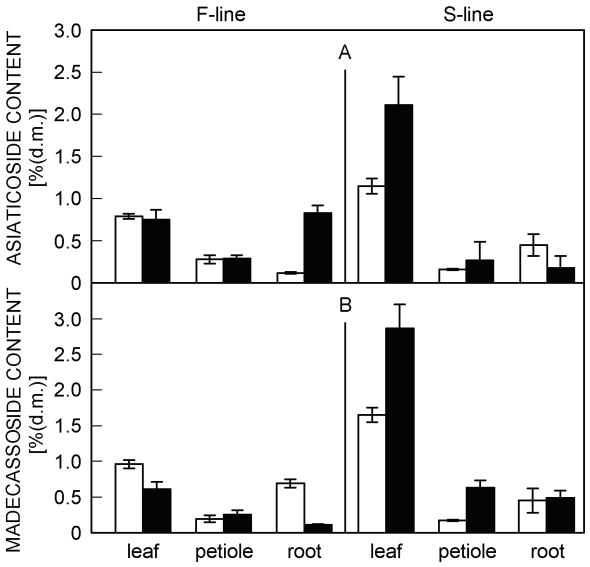

Fig. 5. Mean asiaticoside (*A*) and madecassoside (*B*) content in leaves, petioles and roots of *C. asiatica* F- and S-phenotypes. *Vertical bars* - SEM; *white columns* - glasshouse-grown plants, *dark columns* - *in vitro* grown plants.

Discussion

The two lines of *C. asiatica* of Malaysian origin were distinct morphologically and in their content of the triterpenoid, asiaticoside. Differences between varieties (chemotypes) in medicinal species are common, even if cultivated under identical conditions (Flück 1976). Variation in secondary metabolites has been observed in medicinal species with identical phenotypes (Soldati 2000), for example, in *Digitalis lanata,* which produces the cardiac glycoside, digoxin. The content of pharmacologically-active compounds in medicinal species is influenced both genetically and environmentally. Thus, the environmental conditions should be optimised to maximise synthesis of pharmacologically-active constituents. However, the limits of such biosynthesis will always be determined by the genetic composition of the plant and cannot exceed specific thresholds (Samuelsson 1992).

Variations of asiaticoside content in *C. asiatica* have been associated with plant origin. Thus, a *C. asiatica* population growing at high altitude contained more asiaticoside than plants from a lower altitude (Das and Mallick 1991). Plants collected from 609 m above sea level contained 0.11 % of asiaticoside per leaf d.m., whereas those from a lower altitude (*ca.* 5 m above sea level) contained almost half of this value. Interestingly, Gupta *et al*. (1999) also reported variable asiaticoside content in 5 lines of *C. asiatica* collected from a field trial in India, with the mean content varying from 0.42 - 1.17 %(d.m.). Similarly, Rouillard-Guellec *et al*. (1997) investigated the secondary products in *C. asiatica* from India and Madagascar, and reported that the latter produced more secondary compounds. The asiaticoside content in leaf extracts of the Malaysian S-line almost matched that in plants of Indian origin (Gupta *et al*. 1999). Indeed, identifying plant lines or races with high and stable content of active secondary metabolites is important for the commercial cultivation of plant species of medicinal relevance.

The distribution of asiaticoside and madecassoside throughout the plant was organ specific, with leaves of both lines, containing the higher content of these compounds. Related work with other medicinal plant

species has revealed a similar, tissue-specific pattern of secondary metabolite distribution. For example, the phenolic phenylphenalenone compounds, allophanyl-glucosides, in *Xiphidium caeruleum* were localised in distinct cells of the roots, apical meristem, cortex and epidermis (Opitz *et al*. 2003). In trees of *Ginkgo biloba*, the terpine compounds, ginkgolide and bilobalide, are present in young seedlings and accumulate in roots and leaves, but not in stems (Cartayrade *et al*. 1997). Carbon-labelling indicated that the enzymatic systems responsible for the biosynthesis of the ginkgo diterpine end-products were localised in roots, but not leaves. Thus, terpines are translocated from roots to leaves in a source-sink manner. It is likely that a similar translocation pathway occurs in *C. asiatica*, since both asiaticoside and madecassoside predominated in the leaves with less in roots. However, the precise biosynthetic pathway for asiaticoside and madecassoside is still unknown.

An interesting finding was the difference in distribution of asiaticoside and madecassoside between culture-derived plants and glasshouse-grown material. Roots of tissue culture-derived plants of the F-line, accumulated more of these compounds, compared with their glasshouse-grown counterparts. Conversely, for the S-line, the leaves of *in vitro* plants accumulated more of both compounds, compared with glasshouse-grown plants. In contrast, Baek (1997) noted that in *C. asiatica* of Korean origin, the asiaticoside content in micropropagated shoots was only 50 % of that of field-grown material. This difference re-emphasises the point that race and geographical location are important determinants of terpenoid distributions in *Centella*.

Generally, the present results agree with those of Massot *et al.* (2000) showing that micropropagated shoots of *Ruta graveolens* accumulated more of the secondary metabolites, furanocoumarins, compared with glasshouse-grown plants. Manipulation of secondary product formation in medicinal plants is possible by varying the culture conditions, including medium composition and pH, light/photoperiod and growth regulator type and concentration (Collin 2001). Indeed, the present finding that the asiaticoside and madecassoside content in leaves of *C. asiatica* varied according to culture medium composition, mainly different concentrations of the growth regulator, 2,4-D, further emphasises this point. It is unlikely that there were any influences of somaclonal variation, since the ploidy of regenerated plants was constant at 2n=2x=18, as reported by Booncong *et al.* (1995).

The inability to detect asiaticoside and madecassoside in callus is consistent with the observations of Kim *et al*. (2004) who failed to detect asiaticoside in undifferentiated cells of Korean *C. asiatica*. Plant secondary metabolites are normally synthesised by specialised cells, often at distinct stages of plant development, and certain compounds are not synthesised if cells remain undifferentiated (Kim *et al*. 2002). Interestingly, in contrast to the present observations and those of Kim *et al*. (2004), Nath and Buragohain (2005) reported that callus and suspension cultured cells of *C. asiatica* of Indian origin did, in fact, synthesise asiaticoside.

The present investigation represents the first assessment of the genetic transformation of *C. asiatica* with *A. rhizogenes*. Although *C. asiatica* was amenable to transformation, asiaticoside and madecassoside were not detectable in transformed roots, in contrast to some other plants. It is unlikely that this was due to the strain of *Agrobacterium* used in the present study, since strain R1601 was effective in transforming other medicinal plants, including *Centranthus ruber* (Granicher *et al*. 1995a) and *Isatis indigotica* (Xu *et al*. 2004) and, importantly, that transformed roots of these plants retained their normal biosynthetic capacity. Furthermore, Granicher *et al*. (1995b) also reported that strain R1601 was not only effective in transforming *Valeriana officinalis*, but such transformed roots synthesised a novel iridoid diester.

Secondary metabolite production may require interaction between roots and leaves, with metabolic precursors generated in roots passing to aerial parts of plants for bioconversion in the leaves (Giri *et al*. 2000). One explanation that transformed roots of *Centella* exhibited undetectable asiaticoside and madecassoside contents is that they were unable to interact with other plant tissues/organs leading to the biosynthesis of these triterpenoids. This possibility requires detailed investigation, perhaps through the use of root-shoot co-cultures, using transformed roots and shoot teratomas (Subroto *et al.* 1996, Mahagamasekera and Doran 1998).

This study has provided an important baseline for the culture and genetic transformation of *C. asiatica*. Future studies should elucidate the biosynthetic pathways for asiaticoside, madecassoside and related triterpenoids in this important Asian medicinal plant. An improved understanding of the biochemical pathways leading to triterpenoid synthesis will, in turn, be exploitable in manipulating and maximising product synthesis under tissue culture and, ultimately, bioreactor conditions.

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