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Caffeic Acid Metabolites from *Eritrichium sericeum* Cell Cultures

Abstract

Eritrichium sericeum (Boraginaceae) callus and root cultures were established and analyzed for caffeic acid metabolite (CAM) production. Two substances, (–)-rabdosiin and rosmarinic acid, were identified as main CAMs produced by these cultures. The *E. sericeum* Er-1 root culture accumulated up to 1.5% and 4.5% DW of (–)-rabdosiin and rosmarinic acid, respectively. Rabdosiin in the *Lithospermum erythrorhizon* callus cultures was produced exclusively as the (+)-enantiomer while in both *Eritrichium* cultures it occurred as the (–)-enantiomer. The *E. sericeum* Er-1 culture accumulated 3-fold higher levels of CAMs than the *L. erythrorhizon* culture. A new compound, named eritrichin, was isolated from the cultured *E. sericeum* cells. The structure of this compound was established as (*2R*)-3-(3,4-dihydroxyphenyl)-2-[4-(3,4-dihydroxyphenyl)-6,7-dihydroxy-2-naphthoyloxy]propanoic acid on the basis of spectral data.

Abbreviations

CAM: caffeic acid metabolite
RA: rosmarinic acid
DW: dry weight
FW: fresh weight

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Introduction

Rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is a common metabolite accumulated in plants belonging to the families Boraginaceae and Lamiaceae [1]. RA exhibits a variety of pharmacological activities, e.g., antiviral, antibacterial and antioxidant [1]. Some recent data reveal that RA has the potential to inhibit the lymphocyte cell-specific kinase, whereby impairing T cell-restricted signalling and generating immunosuppression [2], [3]. RA was suggested to be beneficial in the treatment of rheumatoid arthritis [4].

More complex esters of caffeic acid, such as rabdosiin and lithospermic acids, have been found in Boraginaceae and Labiatae plants [5], [6]. Although the biogenesis of these caffeic acid derivatives has not been studied, the co-occurrence of rabdosiin and lithospermic acids with RA in some plants and plant cell cultures led Yamamoto et al. [7] and Agata et al. [8] to suggest that RA was a biosynthetic precursor of rabdosiin and lithospermic acids.

(–)-Rabdosiin was isolated for the first time from stems of *Rabdosia japonica* [5], later, two enantiomers, (+)-rabdosiin and (–)-rabdosiin, were found in *Macrotomia euchroma* roots [9].

Lithospermum erythrorhizon cell suspension cultures, which were initially established for shikonin production, synthesized four caffeic acid derivatives: rosmarinic acid, lithospermic acid B, a monoglucoside of lithospermic acid B and (+)-rabdosiin [7], [10].

A few years ago, rabdosiin was proposed as an active pharmacological agent demonstrating potent anti-HIV [11] and antiallergic activities [12]. More recently, RA and lithospermic acids were characterized as substances inhibiting HIV-1 integrase activity [13], [14]. Interestingly, the more caffeol groups the substance contains the higher is its activity: the antiallergic activity increased in the order caffeic acid – RA – rabdosiin [12]. The same tendency was shown for the anti-HIV activity of CAMs [13].

Taking into account these considerations, we initiated research on plant cell cultures possessing an increased ability to synthesize rabdosiin and related metabolites. *Eritrichium sericeum* (Boraginaceae) callus and root cultures were established and analyzed for caffeic acid metabolites production. Two substances, (–)-rabdosiin and rosmarinic acid, were identified as the predominant CAMs produced by these cultures.

Materials and Methods

Plant material
Plants and seeds of *Eritrichium sericeum* (Lehm.) A. DC. (Boraginaceae) and *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) were collected in the southern regions of Kamchatka and Primorye (Russian Far East), respectively, and identified by Dr. V. Yakubov (Botany Department of Institute of Biology and Soil Science). The voucher specimens of *E. sericeum* (VLA No 69 663) and *L. erythrorhizon* (VLA No 70 000) are deposited in the Botany Department of the Institute of Biology and Soil Science, Vladivostok, 690 022, Russia.

Cell cultures
The seeds of *E. sericeum* were sterilized and germinated *in vitro*, then seedlings were cut in to 2 – 3 mm explants and placed on solid $W_{B/NAA}$ medium [15] supplemented with 0.5 mg/L 6-benzyladenine and 2.0 mg/L α-naphthaleneacetic acid. Explants were incubated in the dark at 25 °C in 100-mL Erlenmeyer flasks, containing 30 mL of $W_{B/NAA}$ medium. Calluses, emerging on the explants, were excised and cultivated on the same medium at one-month intervals. The selection of fast-growing callus aggregates for 3 – 4 subcultures yielded the E-4 callus line. This line was subcultured with 30-day intervals on 30 mL of $W_{B/NAA}$ medium in 100-mL Erlenmeyer flasks, in which 0.6 – 0.7 g of fresh calli were inoculated.

The primary *E. sericeum* calluses spontaneously formed adventitious roots. An Er-1 root culture of *E. sericeum* was established by placing a root tip, isolated from the adventitious roots, into liquid W_{IBA} medium [15], supplemented with 1.0 mg/L indole-3-butyric acid. This culture was further subcultured with 28-day intervals in the dark at 25 °C in 250-mL Erlenmeyer flasks, containing 50 mL of liquid W_{IBA} medium, in an orbital shaker (100 rpm). The inoculum mass of this culture was 0.9 – 1.0 g.

The callus culture BK-39 was established from roots of wild-growing *L. erythrorhizon* plants [16]. The culture was deposited at the Russian Collection of Plant Cell Cultures (accession No 36) as a source of shikonin pigments. The calluses were cultivated in 100-mL Erlenmeyer flasks, containing 30 mL of the solid $W_{K/IAA}$ medium [16], supplemented with kinetin (2.0 mg/L), indole-3-acetic acid (0.2 mg/L) and $CuSO_4$ (0.25 mg/L), in the dark at 25 °C with 30-day subculture intervals. The inoculum mass of BK-39 calluses was 0.6 – 0.7 g.

General experimental procedures
Optical rotations $[\alpha]_D$ were measured with a Perkin-Elmer 343 polarimeter. CD spectra were recorded on a JASCO-J 500A spectropolarimeter. UV spectra were obtained on a spectrophotometer Cecil CE 7250. IR spectra were recorded on an IR-FT spectrophotometer Vector 22. ^1H- and ^{13}C-NMR spectra were determined on an Avance DPX-300 NMR instrument. Samples were run in deuterated chloroform with tetramethylsilane as the internal standard. The chemical shift values are reported in ppm (δ), and coupling constants (J) are given in Hz. FAB-MS were obtained using an AMD-604S spectrometer (direct sample inlet, ionizing energy 10 eV).

Extraction and isolation
Callus or tissue samples of *Eritrichium sericeum* were dried under a hot air flow (50 °C) and powdered. The sample (20 g) was extracted with hexane (200 mL × 2) and $CHCl_3$ (150 mL × 2) to remove pigments and fatty substances. The residue was macerated with EtOH (50 mL), containing 2 % HCl (v/v), for 6 hours and extracted with 50 % EtOH (200 mL × 2). The combined extracts were evaporated under reduced pressure to 120 mL and extracted with EtOAc (100 mL × 3). The vacuum-dried EtOAc extract (0.58 g) was dissolved with 20 % EtOH and separated by column chromatography on Toyopearl HW-40 (40 × 2 cm column). The column was eluted with H_2O-EtOH (containing 0.04 % HCOOH) with gradually increasing EtOH concentrations (20 – 70 %). Fractions were tested for phenols by TLC with $FeCl_3$ reagent and monitored at 280 nm. Phenolic fractions A (35 – 45 % EtOH), B (50 – 60 % EtOH) and C (60 – 70 % EtOH) were purified by silica gel (40 – 60 mesh, 5 g; $CHCl_3$-EtOH 10 : 1) to afford **2a** (45 mg), **3** (70 mg) and **4** (12 mg) (Fig. **1**). The same procedure was applied to isolate **2b** (39 mg) and **3** (80 mg) from the BK-39 callus culture of *L. erythrorhizon*.

(–)-*Rabdosiin* (**2a**): $[\alpha]_D^{20}$: –72° (*c* 0.5, MeOH); CD (3.0 × 10⁻³ M, MeOH): $[\Theta]_{255}$ + 26 000, $[\Theta]_{275}$ – 2650, $[\Theta]_{288}$ – 5815, $[\Theta]_{313}$ + 13 100, $[\Theta]_{350}$ – 24 480; $[\alpha]_D$, UV, IR, MS, ^1H- and ^{13}C-NMR data, see [5], [8], [9].

(+)-*Rabdosiin* (**2b**): $[\alpha]_D^{20}$: + 137° (*c* 0.65, MeOH); CD (3.0 × 10⁻³ M, MeOH): $[\Theta]_{252}$ – 26 535, $[\Theta]_{275}$ + 2600, $[\Theta]_{290}$ + 5930, $[\Theta]_{312}$ – 13 425, $[\Theta]_{350}$ + 24 975; $[\alpha]_D$, UV, IR, MS, ^1H- and ^{13}C-NMR data, see [9], [10].

Rosmarinic acid (**3**): $[\alpha]_D^{20}$: + 52° (*c* 0.5, MeOH); CD (1.61 × 10⁻³ M, MeOH): $[\Theta]_{211}$ + 1910, $[\Theta]_{228}$ – 6000, $[\Theta]_{273}$ – 623, $[\Theta]_{294}$ + 2560, $[\Theta]_{327}$ + 2085; $[\alpha]_D$, UV, IR, MS, ^1H- and ^{13}C-NMR data, see [10], [17].

Eritrichin (**4**) or (2*R*)-3-(3,4-dihydroxyphenyl)-2-[4-(3,4-dihydroxyphenyl)-6,7-dihydroxy-2-naphthoyloxy]propanoic acid:
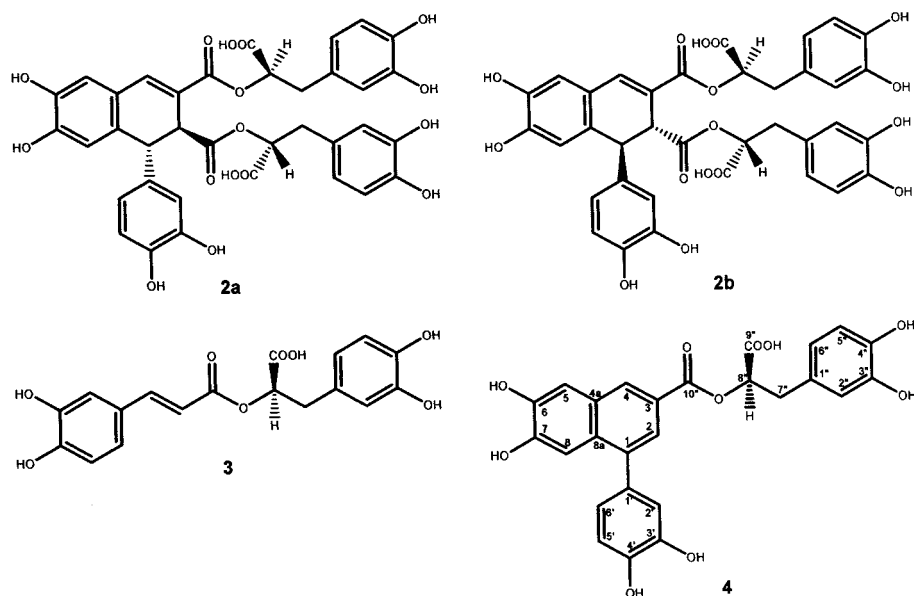
Fig. 1 Chemical structures of caffeic acid metabolites isolated from *E. sericeum* and *L. erythrorhizon* cell cultures: (−)-rabdosiin (**2a**), (+)-rabdosiin (**2b**), rosmarinic acid (**3**) and eritrichin (**4**).

light brown amorphous powder from EtOAc, $[\alpha]_D^{20}$: + 39° (*c* 0.4, MeOH); UV (MeOH): λ_{max} (log ε) = 253.4 (4.38), 285.4 (4.25), 344.2 nm (4.23); CD (MeOH): $[\Theta]_{211}$ + 2733, $[\Theta]_{226}$ − 2733, $[\Theta]_{248}$ + 8467, $[\Theta]_{275}$ + 4009, $[\Theta]_{300}$ + 1458, $[\Theta]_{311}$ + 1895, $[\Theta]_{355}$ − 583; IR (KBr): ν_{max} = 3414, 2924, 2854, 1698, 1609, 1524, 1458, 1383, 1254, 1118, 1040 cm⁻¹; HR-FAB-MS: *m/z* = 493.1150 [M + H]⁺ (calcd. for $C_{26}H_{21}O_{10}$: 493.1134); FAB-MS: *m/z* (%) = 493 [M + H]⁺ (20), 492 [M]⁺ (17), 475 (4), 391 (8), 369 (10), 357 (11), 312 (30), 295 (60), 277 (50), 241 (38), 207 (28), 185 (100), 181 (30), 149 (95), 115 (60); ¹H- and ¹³C-NMR data, see Table **1**.

Analytical HPLC

The dried and powdered plant or tissue culture sample (100 mg) was macerated with EtOH (1.5 mL), containing 2% HCl (v/v). The mixture was dried overnight at room temperature and extracted with 50% EtOH (5 mL) for 2 h at 40–45 °C. An ethanolic solution of caffeic acid (**1**) (0.050 mL, 1 mg/mL) was added to 0.450 mL of the extract as an internal standard. The sample solution was membrane-filtered (0.45 μm, Agilent) and 5 μL aliquots were used for analysis. The analytical HPLC was carried out using an Agilent Technologies 1100 Series HPLC system equipped with VWD detector (λ = 254 nm). Extracts and fractions were analyzed using a ZORBAX Eclipce® XDB-C_8 column (5 μm, 150×4.6 mm) thermostatted at 30 °C. The mobile phase consisted of 1% aqueous acetic acid (A) and acetonitrile containing 1% acetic acid (B). For the analyses the following three gradient steps were programmed: 0–25 min, 5–50% B; 25–30 min, 50–90% B; 30–35 min, 90–95% B. The flow rate was 1 mL/min. The data were analyzed with the ChemStation® program var. 09 (Agilent Technologies, Germany).

Result and Discussion

The E-4 callus and the Er-1 root culture of *E. sericeum* were established and analyzed for CAMs. The compounds **2a, 3** and **4** were isolated from these cultures as the main CAMs, and their structures were determined according to UV, MS, ¹H- and ¹³C-NMR data. The BK-39 callus strain of *L. erythrorhizon* produced **2b** and **3,** respectively.

Eritrichin (**4**) was obtained as a light brown amorphous powder. The UV spectrum indicated the characteristics of caffeic acid and a 1-arylnaphthalene lignan in the molecule. The high resolution FAB-MS, m/z = 493.1150 [M + H]⁺, was in agreement with the molecular formula $C_{26}H_{21}O_{10}$ (requires 493.1134). Signals of 10 aromatic protons at δ = 6.75–8.32 and 6 signals of hydroxy group protons at δ = 7.80–8.95 were observed in the ¹H-NMR spectrum of **4**. The signals of the hydroxy groups were distinguished by the appreciable temperature dependence of their resonance positions. The analysis of spin systems revealed the presence of three aromatic rings in structure **4.** Two of them were 1,3,4-trisubstituted benzene rings and the third represented a tetrasubstituted naphthalene ring. The resonances at δ = 7.70 and 8.32 showed the spin coupling (1.9 Hz) that specifies a *meta* arrangement of the corresponding protons. The signals of three aliphatic protons at δ = 3.17 (1H, dd, *J* = 14.3, 8.2 Hz), 3.24 (1H, dd, *J* = 14.3, 4.5 Hz) and 5.40 (1H, dd, *J* = 8.2, 4.5 Hz) form the classical ABX spin system. The protons, belonging to this system, are in a side chain between an oxygen atom and the aromatic ring of the caffeoyl group. The relative arrangements of the aromatic rings were derived from COSY-45 and NOE-difference experiments (Table **1**). The ¹³C-NMR spectrum of **4** contains 26 signals, 12 of which correspond to the protonated carbon atoms, two of which are aliphatic, and 10 have an aromatic character. The assignment of quatenary carbon atom signals was executed by means of HMBC experiments. Two signals of quaternary atoms at δ = 166.7 and 170.9 belong to carbon atoms of carbonyl groups located near an oxygen atom. The signal assignments of the protonated carbon atoms were achieved by HSQC experiments.

The determination of the absolute configuration of **4** was achieved by application of the circular dichroic (CD) exciton chirality method to rosmarinic acid (**3**). The $[\alpha]_D^{20}$ value (+ 52°) of **3** indicates that rosmarinic acid has the *R*-configuration [17]. Compound **3** showed a split CD curve with a positive first Cotton effect at 211 nm and a negative second Cotton effect at 228 nm. Eritrichin (**4**) also exhibited virtually the same split CD curve in this region. On the basis of these results, the absolute configuration of eritrichin was concluded to be *R*. All the above-mentioned

Table 1 ^{1}H- and ^{13}C-NMR spectral data (J in Hz) for compound **4** recorded in acetone-d_6 at 40 °C (300 MHz for ^{1}H and 75 MHz for ^{13}C)

Site	δ_H	NOE	δ_C	HMBC[a]
1	–		139.6	
2	7.70, d (1.7)	H-2′, H-6′	124.1	C-7, C-8, C-8a, C-10″
3	–		124.9	
4	8.33, bd (1.7)	H-5	128.9	C-1, C-3, C-4a
4a			129.9	
5	7.42, s	H-4	112.2	C-6, C-7, C-8, C-8a
6			147.6	
7			149.4	
8	7.34, d (0.6)	H-2′, H-6′	109.1	C-1, C-4a, C-5, C-6, C-7
8a			131.3	
1′			133.2	
2′	6.97, d (2.1)		117.6	C-1, C-3′, C-6, C-1′, C-6′
3′			145.5	
4′			145.6	
5′	6.98, d (8.0)		116.0	C-1′, C-2′, C-3′
6′	6.81, dd (8.0, 2.1)		122.0	C-1, C-2′, C-4′,
1″			129.1	
2″	6.93, d (2.0)		117.3	C-6″, C-5″
3″			145.7	
4″			144.7	
5″	6.77, d (8.0)		115.9	C-1″, C-3″
6″	6.75, dd (8.0, 2.0)		121.6	C-1″, C-2″,C-4″, C-7″
7″	3.17, dd (14.3, 8.2), 3.24, dd (14.3, 4.5)	H-2″, H-6″, H-8″	37.5	C-1″, C-9″, C-8″
8″	5.40, dd (8.2, 4.5)		74.1	C-1″, C-7″, C-9″, C-10″
9″			170.9	
10″			166.7	

[a] The HMBC experiment was optimized for 2.5 Hz couplings.

data on the compound **4** suggest that it is a new caffeic acid metabolite, (2*R*)-3-(3,4-dihydroxyphenyl)-2-[4-(3,4-dihydroxyphenyl)-6,7-dihydroxy-2-naphthoyloxy]propanoic acid.

Before analyzing the CAM content in our cultures, we examined whether determinations were possible in the dry roots and calluses. Yamamoto and co-workers [7], [10] reported that caffeic acid metabolites were partially decomposed by the lyophilization of cultured cells (*ca*. 30–40% of the extract from the fresh cells) and completely decomposed by drying in an oven at 50 °C for three days. We dried our cultures under a hot air flow for a short time (4–5 h) and analyzed the CAM content by HPLC according to the procedure described in Materials and Methods. The fresh material from the same tissue samples was analyzed according to Yamamoto et al. [7], [10]. The fresh and dry samples had the same composition of CAMs. The contents of rabdosiin and rosmarinic acid in dry calluses and roots were 5.5–10.0% less than those of the fresh samples. The storage of the fresh material in a freezer at –20 °C is not appropriate for analysis because of substantial decomposition of CAMs, probably during the procedure of freezing-thawing (data not shown). Therefore, taking into account these results, as well as technological considerations, we chose to use dry material for further analysis.

The quantification of the main components of the air-dried callus cultures of both Boraginaceae species was carried out using HPLC (Fig. **2**). The method allowed a good separation and quantification of the main caffeic acid oligomers (**2a**, **2b**, **3**, **4**) within 25 min. Caffeic acid (**1**) was absent in callus and tissue extracts and, thus, was used as an internal standard. It is interesting to note that the *E. sericeum* and *L. erythrorhizon* callus cultures synthesize enantiomeric rabdosiins (**2a**) and (**2b**), respectively, that was established by comparison of their CD spectra.

The *L. erythrorhizon* BK-39 culture was included in this investigation to compare our results with information from other studies. The BK-39 culture accumulated 0.82 and 1.10% DW (0.53 and 0.72 mg/g FW) of **2b** and **3**, respectively, comparable with the results on M18TOM culture of *L. erythrorhizon* obtained by Yamamoto et al. [10]. In contrast to the previous results reported for the M18TOM culture [6], [10], we could not detect lithospermic acid B in our culture. This difference might be explained by different culture conditions for the M18TOM and BK-39 cultures and the different nutrient media used. Indeed, BK-39 culture was adapted for the one-stage cultivation process, whereas M18TOM adapted for the two-stage process [10], [16]. However, this explanation seems to be unlikely because the biosynthetic capacity of both cultures for phenylpropanoid metabolites, such as **2b** and **3**, as well as shikonin derivatives is similar [10], [16]. The analysis of *L. erythrorhizon* plants, growing in the Primorye region of Russia, showed the absence of lithospermic acid B
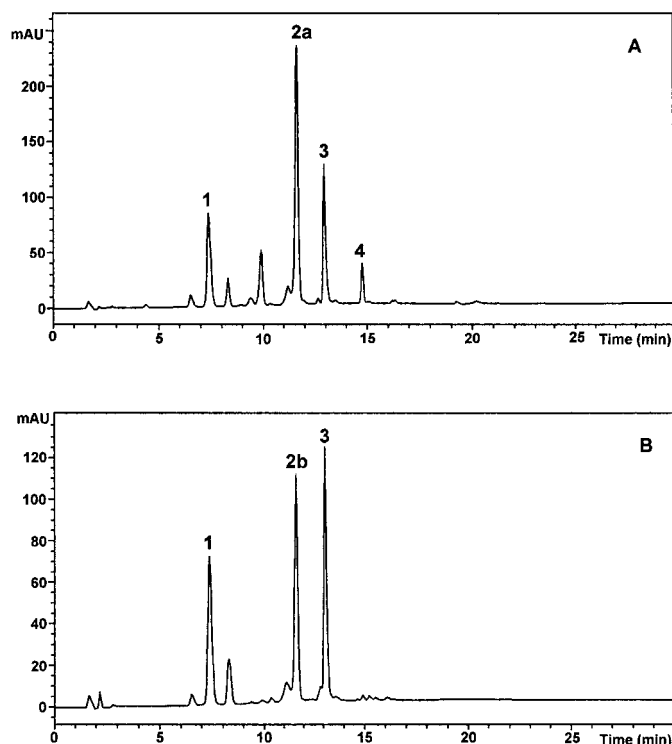
Fig. **2**   HPLC profiles of polyphenol fractions from *E. sericeum* root (**A**) and *L. erythrorhizon* callus (**B**) cultures. λ = 254 nm. The experimental conditions are given in Materials and Methods.

(data not shown). Therefore, the absence of lithospermic acid B in the BK-39 culture may be more likely explained by genetic differences of *L. erythrorhizon* plants growing in different populations.

The content of CAMs in the *E. sericeum* callus and root cultures, as well as natural plants, was determined by HPLC (Table **2**). The *E. sericeum* root culture accumulated 1.51 and 4.50 % DW (1.22 and 3.64 mg/g FW) of **2a** and **3**, respectively; the E4 callus culture accumulated approximately two times less of these sub-

stances (Table **2**). It is interesting to note that the Er-1 culture, being cultured without any elicitor treatments, produced much higher levels of **2a** and **3** than the corresponding natural roots.

We analyzed whether **2a** production by the Er-1 culture correlated with **3** synthesis. The correlation coefficient (r) between **2a** and **3** contents was calculated to be 0.18, indicating that the production of rosmarinic acid did not coincide with rabdosiin production. A similar result was obtained for the BK-39 culture (r = 0.19). These data suggest that both RA and rabdosiin production in Boraginaceae cell cultures is under individual regulatory control. Some evidence supporting this proposition was obtained in transformation experiments recently. We could show that the *Agrobacterium rhizogenes rolC* gene, being expressed in *E. sericeum* cells, predominantly affected RA but not rabdosiin production [18].

The biosynthesis of rosmarinic acid in cultured plant cells is well studied [19], [20], [21] while the biosynthetic route of rabdosiin formation is unknown. It is possible that rabdosiin is a dimerization product of rosmarinic acid. One can suppose also that eritrichin is a biosynthetic precursor of rabdosiin or, alternatively, it is a decomposition product of rabdosiin. The first possibility seems to be more plausible because eritrichin has not been found in the *L. erythrorhizon* calluses and plants (Table **2**). It is evident that the biosynthesis of plant CAMs represents an interesting model system, in which such important problems as coordination and hierarchy of key regulatory genes of secondary metabolism can be explored.

Conclusion

E. sericeum cell cultures emerge as a new and rich source of caffeic acid metabolites. Further investigation aimed at the increase of production parameters of *Eritrichium* cells, such as transgene technologies, elicitor and precursor treatments, are under progress in our laboratories.

Table **2**   Content of CAMs (% DW) in intact plants and 30-day callus and root cultures of *E. sericeum* and *L. erythrorhizon*[a]

Sample	Fresh biomass[b] (g/L)	Dry biomass (g/L)	% of dry biomass	Rabdosiin	Rosmarinic acid	Eritrichin	Total CAM	CAM production (mg/L)
E. sericeum								
Callus culture E-4	180 ± 30	9.2 ± 0.8	5.1	0.66 ± 0.22	2.04 ± 0.40	0.04 ± 0.005	2.74 ± 0.34	252
Root culture Er-1	155 ± 15	12.6 ± 0.9	8.1	1.51 ± 0.15	4.50 ± 0.79	0.10 ± 0.02	6.11 ± 0.84	770
Aerial parts				0.01 ± 0.007	tr[c]	tr	0.01 ± 0.007	
Underground parts				0.32 ± 0.12	0.07 ± 0.02	0.02 ± 0.005	0.41 ± 0.12	
L. erythrorhizon								
Callus culture BK-39	290 ± 30	18.9 ± 0.9	6.5	0.82 ± 0.08	1.10 ± 0.15	nd[d]	1.92 ± 0.12	363
Aerial parts				0.15 ± 0.03	0.28 ± 0.08	nd	0.43 ± 0.06	
Underground parts				0.07 ± 0.03	0.66 ± 0.19	nd	0.73 ± 0.17	

[a] Mean values ± s. e. The determinations of CAMs in callus and root cultures were repeated eight times (with three replicates each) during 2-year cultivation of the cultures. Determinations in intact plants were performed by using at least 5 individual plants.

[b] The inoculum concentrations for all cultures tested were 16 – 20 g/L.

[c] Trace amounts.

[d] Not detectable.

Acknowledgements

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