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High Rabdosiin and Rosmarinic Acid Production in *Eritrichium sericeum* Callus Cultures and the Effect of the Calli on Masugi-Nephritis in Rats

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During an investigation of plant cell cultures that might be useful in the treatment of renal disorders, we established a vigorously-growing E-4 callus culture of *Eritrichium sericeum* that produced large amounts of caffeic acid metabolites, (−)-rabdosiin (1.8% dry wt) and rosmarinic acid (4.6% dry wt). Elicitation of the calli by methyl jasmonate induced a 38% increase in total polyphenol production. The most efficient method of eliciting (−)-rabdosiin biosynthesis was through the treatment of E-4 calli with cuprum glycerate, which induced an increase in (−)-rabdosiin production of as much as 4.1% dry wt. Oral administration of E-4 callus biomass (100 mg/kg/d for 30 d) to rats with induced Masugi-nephritis caused an increase in diuresis and lowered creatinine excretion and proteinuria levels as compared with Masugi-nephritis untreated rats. While all of the Masugi-nephritis untreated rats began to suffer, near a quarter of the E-4 treated rats remained in good health. This result indicates that the E-4 culture has the potential to alleviate the symptoms associated with nephritis.

Key words: *Eritrichium sericeum*; rabdosiin; rosmarinic acid; methyl jasmonate; experimental glomerulonephritis

Rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, exhibits a variety of pharmacological activities, *e.g.*, antiviral, antibacterial, and antioxidant.[1)] Recent data have revealed that RA has the potential to inhibit lymphocyte cell-specific kinase, thereby impairing T cell-restricted signaling and generating immunosuppression.[2,3)] It has been suggested that RA is beneficial in the treatment of rheumatoid arthritis.[4)] More complex metabolites 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, co-occurrence of rabdosiin and lithospermic acids with RA in some plants and plant cell cultures led to the hypothesis that RA is a biosynthetic precursor of rabdosiin and lithospermic acids.[7,8)] (−)-Rabdosiin was isolated for the first time from stems of *Rabdosia japonica*,[5)] and later two enantiomers, (+)-rabdosiin and (−)-rabdosiin, were found in *Macrotomia euchroma* roots.[9)] *Lithospermum erythrorhizon* cell suspension cultures have been found to synthesize four caffeic acid derivatives: rosmarinic acid, lithospermic acid B, a monoglucoside of lithospermic acid B, and (+)-rabdosiin.[7,10)]

A few years ago, it was proposed that rabdosiin is an active pharmacological agent with potent anti-HIV[11)] and antiallergic activities.[12)] More recently, RA and lithospermic acids were characterized as substances inhibiting the activity of HIV-1 integrase.[13,14)] The more caffeol groups a substance contains, the higher its activity: anti-allergic activity increased in the order caffeic acid (one group)–RA (two groups)–rabdosiin (four groups),[12)] and the same tendency was evident in the anti-HIV activity of caffeic acid metabolites (CAMs).[13)]

There is a need to develop new, improved, effective drugs and food additives for prophylaxis and for the treatment of renal disorders associated with nephritis. Plant polyphenols have been studied extensively in this respect. A decoction of *Perilla frutescens* was shown to

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Abbreviations: CAM, caffeic acid metabolite; RA, rosmarinic acid; MeJA, methyl jasmonate; SNS, specific nephrotoxic serum

have a suppressive effect on the progression of glomerulonephritis in an animal model of spontaneous IgA nephropathy.[15] The main active substance was identified as rosmarinic acid. *In vitro* studies revealed that RA inhibits cytokine-induced murine mesangial cell proliferation, suggesting that this substance may be useful in preventing the progression of mesangioproliferative glomerulonephritis.[16] This result was later confirmed by *in vivo* experiments, using the experimental model of ATS-induced glomerulonephritis in rats.[17] Again, as in other cases, the dimer of caffeic acid showed a much more pronounced effect than monomeric caffeic acid. Hence, it is not surprising that a tetramer of caffeic acid, lithospermic acid B, has a strong protective effect on rats with induced diabetic nephropathy.[18] Lithospermic acid B decreased the activity of PKC, thus reducing the activity of MAPK cascades and the TGF-β1 signaling pathway and inhibiting the synthesis and secretion of extracellular matrix proteins. It has been proposed that this mechanism is efficient in reducing urinary protein levels both in laboratory animals with induced diabetic nephropathy and in humans (diabetic patients).[18] A administration of lithospermic acid B to diabetic patients proved an efficient way to treat and reduce the severity of kidney diseases, and ultimately renal failure. Moreover, the treatment did not result in significant adverse side-effects and was especially effective in those cases where clinical management strategies were difficult to implement.[18] Taking these data into account, we initiated research into plant cell cultures that may possess an increased ability to synthesize RA and more complex CAMs. *Eritrichium sericeum* (Boraginaceae) callus and root cultures were established and analyzed for CAM production. Two substances, (−)-rabdosiin and rosmarinic acid, were identified as the predominant CAMs produced by these cultures.[19] The content of rabdosiin was 3.4% dry wt in root cultures, representing the highest yield reported for biotechnological or natural rabdosiin sources,[20] but *E. sericeum* callus cultures synthesized much smaller quantities of CAMs than *E. sericeum* root cultures,[17] which restricted their practical usage. Since callus (suspension) cultures have several practical advantages over root cultures (besides hairy roots), it is of considerable interest to obtain a high-producing *E. sericeum* callus culture. In the present investigation, we report the establishment of a high rabdosiin-producing *E. sericeum* callus culture and show that this culture has the potential to alleviate symptoms associated with nephritis.

Materials and Methods

Plant material and cell cultures. Plants and seeds of *Eritrichium sericeum* (Lehm.) A. DC. (Boraginaceae) were collected in the southern regions of Kamchatka (Russian Far East) and identified at the Botany Department of the Institute of Biology and Soil Science (voucher specimens VLA no. 69663, Far-Eastern Regional Herbarium, Institute of Biology and Soil Science). The E-4 primary callus culture was established as described previously,[19] using *in vitro* plantlets obtained from germinated seeds. The E-4 callus line was cultivated in the dark at 25 °C with 30-d intervals, using 40 ml of $W_{B/NAA}$ medium.[21] $W_{B/NAA}$ medium is a modification of MS medium in which the content of ammonium nitrate is reduced to 400 mg/l.[22] This medium was supplemented with the following components (mg/l): thiamine HCl (0.2), nicotinic acid (0.5), pyridoxine HCl (0.5), meso-inositol (100), peptone (100), sucrose (25,000), agar (6,000), 6-benzyladenine (0.5), and α-naphthaleneacetic acid (2.0). For callus cultivation, we used 100-ml Erlenmeyer flasks, in which 0.6–0.7 g of fresh calli were inoculated. The transgenic Es-rolC calli[18] were cultivated under the same conditions as the E-4 calli.

Chemicals. Reagents for tissue culture and MeJA were obtained from Sigma (St. Louis, MO) the others were from ICN Pharmaceuticals.

Effector and inhibitor treatments. Sterile solutions of methyl jasmonate (MeJA) and piroxicam were added to autoclaved media aseptically in desired concentrations as described previously.[23] Equal volumes of appropriate solvents were added to the control flasks. Piroxicam was used at final concentrations of 1 µM, 10 µM and 100 µM. DL-phenylalanine, a CAM precursor, was dissolved in hot water and added to the culture medium aseptically in final concentrations of 50 µM and 100 µM. Sucrose was tested at 20–50 g per liter concentrations. The ammonium/nitrate ratios were tested using a basic concentration of KNO_3 (1,900 mg per liter) and varying concentrations of NH_4NO_3 (mg per liter: 0, 200, 400, 600, 800 and 1,650). To prepare the cuprum-glycerate complex, 100 mg of $CuSO_4 \times 5\ H_2O$ was dissolved in 50 ml of H_2O, and 20 ml of glycerine was added. The solution was mixed with 20 ml of 5% (w/v) aqueous KOH and brought up to a final volume of 100 ml with water.

Analysis of CAMs. Rabdosiin and rosmarinic acid were isolated from callus cultures and analyzed by ^1H, ^{13}C NMR, FAB-MS, UV, IR, and CD methods.[19] Quantitative HPLC determinations of rabdosiin and rosmarinic acid were performed using caffeic acid as an internal standard.[19]

Induction of Masugi-nephritis and E-4 biomass administration. Sprague-Dawley rats (weighing approximately 200–250 g) were housed individually in sterile microisolator cages. The rats were fed standard rat chow, and water was available *ad libitum*. The animals were maintained in temperature (25 °C) and humidity (40%) controlled rooms under a 12-h light/12 h dark cycle. They were assigned randomly to three experimental groups: group I, intact rats (n = 10), group II,

untreated Masugi-nephritis (n = 14); and group III, E-4-treated Masugi-nephritis (n = 13).

Masugi-nephritis was induced in the rats by a single intravenous injection of a specific nephrotoxic serum (rabbit anti-rat glomerular basement membrane serum, 50 µl/100 g body wt). Development of the disease was confirmed by the appearance of proteinuria at day 4. Urine samples were centrifuged (3,000 rpm for 10 min) and the supernatant was analyzed for creatinine and protein levels by standard techniques.[24)]

Following induction of nephritis, the animals were treated with a E-4 callus suspension with a stomach pump once daily (100 mg fine-powdered E-4 calli in 2% starch suspension/kg body weight) for 30 d.

Statistical analysis. In statistical evaluation, one-way analysis of variance (ANOVA) followed by a multiple comparison procedure according to Fisher's protected least significant difference (PLSD) *post-hoc* test was employed for inter-group comparison and Dunnett's *post-hoc* test was employed for data analysis within each group. A difference of $P < 0.05$ was considered significant.

Results

Establishment of a highly productive cell line

The *E. sericeum* primary callus culture consisted of yellow-white, light-brown, and dark-brown aggregates with different growth intensities. Two substances, (−)-rabdosiin (**1**) and rosmarinic acid (**2**), were identified as the main CAMs produced by these calli (Fig. 1). More deeply pigmented aggregates showed slower growth but a higher ability to produce rabdosiin and RA, and *vise versa*. Therefore, the selection strategy was aimed at selecting vigorously-growing light-brown cell aggregates. More than 20 subcultures were necessary to obtain actively-growing homogenous calli (E-4 line) with an increased ability to produce polyphenol (Table 1). This simple method was found to be most effective in establishing a high-productive cell culture of *E. sericeum*: polyphenol content was increased by the selection from 2.7% to 6.3% and was stable during prolonged periods (more than 2 years) of E-4 calli cultivation. Other commonly used methods, such as manipulation of sucrose content and the ammonium/nitrogen ratio, precursor feeding, and the use of piroxicam, an inductor of shikimate-derived secondary metabolism,[23)] were inefficient or decreased the growth of the calli (data not shown). Subsequent investigations revealed that further increases in polyphenol production (more than 6–7% of dry wt) in E-4 calli is a difficult problem. Genetic transformation of *E. sericeum* cells with a powerful inductor of plant secondary metabolism, the *rolC* gene, was ineffective.[20)] Increases in rabdosiin production by direct gene-engineering methods are not yet possible because the enzymatic steps between RA and rabdosiin is not known. However, two inductors, methyl jasmonate and cuprum ions, were found to be useful in inducing CAM production.

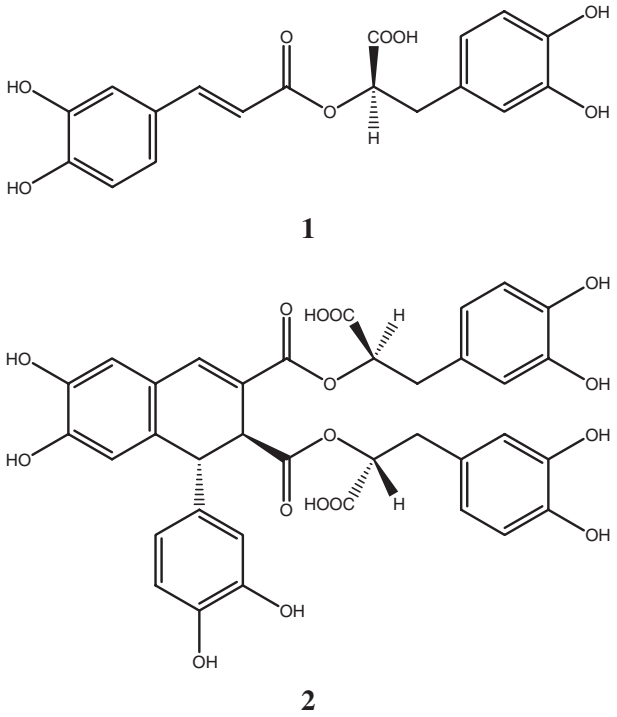

Fig. 1. Structures of Caffeic Acid Metabolites Isolated from *E. sericeum* Calli.
1, rosmarinic acid; **2**, (−)-rabdosiin.

Elicitation of polyphenol production by MeJA and cuprum glycerate

MeJA is presently the best-known inductor of plant secondary metabolism. *E. sericeum* root cultures have been shown to be highly sensitive to MeJA treatment: doses higher than 5 µM caused strong inhibition of root growth.[20)] We tested the effect of MeJA on the E-4 callus culture and found that the inductor, in concentrations of 1.0 µM and 5.0 µM, stimulated both rabdosiin and RA biosynthesis, causing a 20–38% increase in total polyphenol production (Table 2).

We were interested not only in increasing the accumulation of total polyphenols in *E. sericeum* cultivated cells, but also in increasing the relative proportion of rabdosiin. To change the rabdosiin/RA ratio to increase rabdosiin accumulation, we resorted to elicitation of E-4 by cuprum ions. Cuprum sulfate was dissolved in glycerin, and the resulting complex of Cu^{+2}-glycerate was added to the nutrient medium at a relatively high dose, 0.25 mg/l. The complex gradually released cuprum ions, ensuring continuous elicitation of calli without a negative effect on callus growth (at this dose, cuprum sulfate itself caused a toxic effect). The total content of polyphenols in the Cu^{+2}-glycerate-elicited calli was similar to that in the non-elicited calli, but the rabdosiin proportion increased substantially (Table 2). It is interesting to note that it has been

Table 1. Gradual Increases in Growth and Biosynthetic Parameters[a] of *E. sericeum* Calluses during Long-Term Cultivation

Sample	Fresh biomass[b] (g/l)	Dry biomass (g/l)	Rabdosiin (% dry wt)	Rosmarinic acid (% dry wt)	Total polyphenols (% dry wt)	Polyphenol production (mg/l)
Primary callus	90 ± 11	7.2 ± 0.6	0.62 ± 0.05	0.84 ± 0.22	1.45	104
E-4 callus, 10th subculture	165 ± 23	8.9 ± 0.7	0.33 ± 0.05	1.02 ± 0.12	1.35	120
E-4 callus, 20th subculture	180 ± 30	9.2 ± 1.0	0.66 ± 0.22	2.04 ± 0.40	2.70	249
E-4 callus, 30th subculture	310 ± 28	14.1 ± 1.2	1.83 ± 0.32	4.32 ± 0.33	6.15	867
E-4 callus, 40th subculture	311 ± 21	14.5 ± 1.1	1.80 ± 0.26	4.55 ± 0.45	6.35	920
E. sericeum stems			0.01 ± 0.007	tr[c]	0.01	
E. sericeum roots			0.32 ± 0.12	0.07 ± 0.02	0.39	

[a]Mean values ± SE representing three independent determinations. CAM determinations in intact plants were performed by using five individual plants.
[b]The inoculum concentrations for all cultures were 16–20 g/l.
[c]Trace amounts.

Table 2. Effect of Methyl Jasmonate and Cu^{+2} on E-4 Callus Growth and Polyphenol Content

Treatment	Fresh biomass[a], g	Polyphenol content, % dry wt		
		Rabdosiin	RA	Total (% of the control)
MeJA, μM				
0	7.38 ± 0.32	1.07	4.63	5.70
0.5	7.33 ± 0.28	1.37	4.53	5.90 (104)
1	7.24 ± 0.19	1.47	5.39	6.86 (120)
5	5.39 ± 0.48	2.56	5.30	7.86 (138)
$CuSO_4$, mg/l				
0.025[b]	7.67 ± 0.48	2.50	3.50	6.00
0.25[c]	7.02 ± 0.28	4.11	2.30	6.41 (107)

[a]Mean values ± SE based on 10 replicate samples obtained in a single experiment.
[b]$CuSO_4 \times 5\ H_2O$, standard $W_{B/NAA}$ medium.
[c]$CuSO_4 \times 5\ H_2O$ used as the glycerate complex (see "Materials and Methods").
For polyphenol determination, the contents of 10 replicate flasks were sampled and the average value is reported. The deviations of any flask from the average value was less than 10%.

proposed that both RA and rabdosiin production in Boraginaceae cell cultures are under individual regulatory controls.[19] This proposition is supported by data indicating that *rolC* gene expression in *E. sericeum* transformed cells predominantly affected RA, but not rabdosiin biosynthesis.[20] Apparently, MeJA activates both rabdosiin and RA biosynthetic pathways, whereas cuprum ions stimulate the conversion of RA to rabdosiin.

Time course of polyphenol accumulation

As shown in Fig. 2, the growth of E-4 calli corresponded to a typical sigmoid curve with the highest value of biomass accumulation at 6th week of cultivation. During the first five weeks of cultivation, rabdosiin and RA gradually accumulated in the calli, but with culture maturation, the RA content declined while the rabdosiin content was nearly constant. Thus, 5-week-cultivated calli accumulated maximal levels of polyphenols, and hence were used in further experiments.

Stability of rabdosiin and RA during drying

Yamamoto and co-workers[7,10] reported that caffeic acid metabolites were partially decomposed by the lyophilization of cultured *Lithospermum erythrorhizon* cells (about 30–40% of the extract from fresh cells), and were completely decomposed by drying in an oven at 50 °C for 3 d.

Considering the technological aspect of using cell biomass containing polyphenols, we tested a milder regime for callus drying. We dried our cultures under a hot air flow for a short time (4–5 h) and analyzed CAM content by HPLC according to the procedure described in "Materials and Methods." Fresh material from the same tissue samples was analyzed according to Yamamoto *et al.*[7] It was established that the fresh and dry samples had the same composition of CAMs (Table 3). The content of CAMs in dry calli was 5.4–9.6% less than that of fresh samples (Table 3). In contrast, storage of the fresh material in a freezer at −20 °C caused substantial decomposition of CAMs, most likely due to freeze-thaw cycles (data not shown). Therefore,

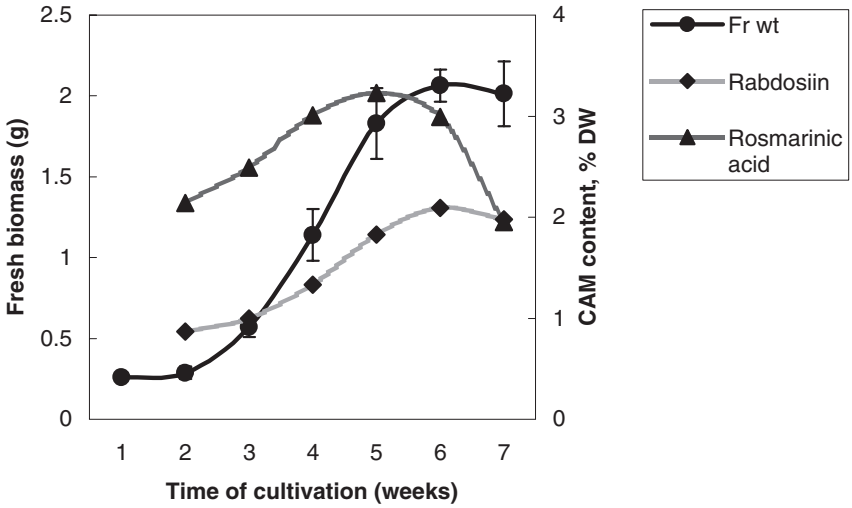

Fig. 2. Growth of E-4 Callus Culture and CAM Content during Prolonged Subculture on $W_{B/NAA}$ Medium.
The data were obtained in a single experiment, and were averaged from 10 independent calluses. Initial callus weight was approximately 200 mg. The calli were grown for 49 d and harvested for polyphenol determination.

Table 3. Changes in Polyphenol Content during *E. sericeum* Callus Drying[a]

Tissue	Polyphenol content, % dry wt			Loss of polyphenols, %
	Rabdosiin	RA	Total	
E-4 fresh callus	1.8	7.6	9.4	—
E-4 dry callus	1.9	6.6	8.5	9.6
Es-rolC fresh callus	1.95	1.75	3.7	—
Es-rolC dry callus	2.4	1.1	3.5	5.4

[a]Calli were grown for 6 weeks, weighted, and divided on two equal parts. The CAM content in fresh calli was analysed immediately by HPLC, and the second portion was dried under hot air (45 °C) for 5 h and analysed by HPLC.

taking into account these results, as well as technological considerations, we chose to use dry material for further experiments. Aqueous extracts (*e.g.*, methanol, ethanol, water) of the E-4 dry calli were unstable and the content of CAMs decreased rapidly in these extracts during several days. Since CAMs did not undergo decomposition in dry calli for at least several months, we attempted to assess baseline anti-nephritis activity using dry callus biomass. We used a standardized dry fine-powdered calli containing rabdosiin 1.5 g and RA 4.5 g in 100 g of dry biomass. For this, the calli of the 42–44th subcultures were grown for 5 weeks without any elicitor treatment. After drying, they were combined, powdered, and analyzed by HPLC.

Effect of dry E-4 callus biomass on diuresis, creatinine, and protein excretion in Masugi-nephritis rats

Masugi's nephritis, a rat model of experimental glomerulonephritis induced by anti-glomerular basement membrane serum, is a relevant model closely resembling human lobular glomerulonephritis.[25] In our experiments, injection of a specific nephrotoxic serum into rats induced an acute form of renal pathology. High values of proteinuria and increased rates of diuresis and creatinine excretion were observed in the Masugi-nephritis rats (Table 4). The effective dose of E-4 calli, which was determined by analyzing gradually-decreasing doses of E-4 callus biomass starting from an initial dose of 500 mg/kg animal body wt, was found to be 100 mg/kg. Administration of E-4 calli at this dose for 30 d caused an increase in diuresis, with a mean value of 38% (4.5 ml/d in group II *vs.* 6.2 ml/d in group III), lowered creatinine excretion on 29% (27.3 μmoles/d in group II *vs.* 21.2 μmoles/d in group III), and decreased protein excretion at 36% (53.4 mg/d in group II *vs.* 39.2 mg/d in group III). All of these changes are statistically significant (ANOVA followed by Fisher's test, Table 4). The dynamics of proteinuria were different as between group II and III. In group II, a persistent increase in the protein level was observed through the entire experiment, while the animals in group III began to decrease in protein level at 30 and 35 d (Table 4), although E-4 administration finished at day 30.

The most promising effect of the E-4 calli was that nearly a quarter of the rats in group III (E-4-treated rats) did not show symptoms of glomerulonephritis (Table 5), and that at the end of the experiment, they did not significantly differ from the rats of group I in behavior or body mass (data not shown). In contrast, all the rats began to suffer in the untreated group (group II). The number of animals with profound nephrogenic symptoms was about 2-fold less in group III as compared with

Table 4. Effect of E-4 Calli (Dry Powdered Biomass, 100 mg/kg/d, Orally Administrated) on Rats with Induced Masugi-Nephritis

Groups of rats[a]	Days after beginning of experiment								ANOVA followed by Fisher's PLSD test
	0	5	10	15	20	25	30	35	
					Diuresis, ml/d				
I	2.9 ± 0.2	2.7 ± 0.3	2.8 ± 0.2	2.8 ± 0.3	3.1 ± 0.3	2.7 ± 0.3	2.7 ± 0.2	2.8 ± 0.2	I *vs.* II, $P < 0.01$
II	3.0 ± 0.2	3.3 ± 0.3	4.5 ± 0.6	5.3 ± 0.8	5.0 ± 0.9	4.7 ± 0.9	5.3 ± 0.9	5.2 ± 1.0	I *vs.* III, $P < 0.001$
III	2.6 ± 0.2	6.8 ± 1.4	7.6 ± 2.1	6.5 ± 1.1	6.8 ± 1.2	6.7 ± 1.3	7.0 ± 1.7	6.4 ± 1.6	II *vs.* III, $P < 0.01$
					Creatinine excretion, μmoles/d				
I	14.2 ± 1.3	14.1 ± 1.7	13.3 ± 2.2	12.1 ± 2.0	13.2 ± 2.4	14.2 ± 2.7	13.1 ± 2.0	14.0 ± 2.9	I *vs.* II, $P < 0.0001$
II	14.3 ± 1.5	30.4 ± 5.5	24.4 ± 3.7	32.7 ± 6.1	28.1 ± 5.8	31.4 ± 7.5	27.1 ± 6.1	29.6 ± 6.5	I *vs.* III, $P < 0.01$
III	13.1 ± 1.6	24.3 ± 4.1	25.8 ± 6.1	20.6 ± 4.1	25.4 ± 7.4	21.1 ± 6.5	20.0 ± 4.7	18.2 ± 4.6	II *vs.* III, $P < 0.05$
					Urinary protein excretion, mg/d				
I	2.3 ± 0.2	1.9 ± 0.1	2.7 ± 0.3	2.4 ± 0.2	2.4 ± 0.3	2.0 ± 0.2	2.4 ± 0.2	2.2 ± 0.3	I *vs.* II, $P < 0.0001$
II	2.4 ± 0.2	23.9 ± 4.0	46.0 ± 6.0	64.4 ± 6.6*	86.6 ± 13.9*	71.4 ± 12.0*	68.0 ± 11.2*	64.7 ± 9.2*	I *vs.* III, $P < 0.0001$
III	2.8 ± 0.5	24.1 ± 6.7	30.1 ± 9.5	63.0 ± 13.1*	67.1 ± 16.2*	50.4 ± 13.0*	42.8 ± 11.0	40.3 ± 12.1	II *vs.* III, $P < 0.05$

[a]I, Group I, intact rats; group II, Masugi-nephritis, and group III, Masugi-nephritis + E-4 calli.
*$P < 0.05$ as compared to control (day 0) within each group, ANOVA followed by Dunnett's *post-hoc* test.

Table 5. Administration of E-4 Dry Powdered Calli (Once Daily for 30 D) Attenuates Symptoms of Induced Glomerulonephritis in Rats

Treatment (number of animals)	Severity of injury[a] (urinary protein excretion, mg/d)			
	No symptoms (< 10)	Slight symptoms (10–30)	Moderate symptoms (31–100)	Heavy symptoms (> 100)
Group I	100	0	0	0
Group II, 2% starch suspension (n = 14)	0	28.5	28.5	43
Group III, 2% starch suspension + E-4 dry powdered calli, 100 mg/kg (n = 13)	23	16	38	23

[a]The data are presented as percentage of total number of animals.

group II (Table 5). Hence administration of E-4 callus biomass alleviated and in some cases prevented symptoms associated with glomerulonephritis.

Discussion

Here we report the establishment of the highly productive and well-growing E-4 callus culture of *E. sericeum*, which produces high amounts of RA and rabdosiin (Fig. 1) during long-term cultivation (Table 1). The content of rabdosiin in the E-4 calli increased up to 4.1% dry wt with the use of cuprum glycerate (Table 2). This value represents, to our knowledge, the highest yield of rabdosiin yet reported for natural or biotechnological sources.

The biomass of the E-4 calli, administered orally to Masugi-nephritis rats, alleviated symptoms associated with glomerulonephritis (Table 4, 5). It is unknown whether this effect was caused by rabdosiin and RA or other yet unknown substances present in the calli. However, data from others suggest that these metabolites of caffeic acid might be responsible for the observed pharmacological effects. Initially, crude extracts of *Perilla frutescens* and *Salvia miltiorrhizae* were found to have a positive effect in different experimental models of nephritis.[15,18] In both cases, the active substances have been determined to be polyphenols: RA in the case of *P. frutescens* and lithospermate B in the case of *S. miltiorrhizae*.[15–18] A detailed investigation of the phenolic compounds in *E. sericeum* callus and root cultures revealed that RA and rabdosiin are the predominant CAMs synthesized by these cultures.[19] Additionally, the cultures contained trace amounts of eritrichin, a new substance identified as a trimer of caffeic acid that most likely represents a rabdosiin biosynthetic precursor.[19] No other polyphenols were detected in the cultures.[19]

In our experiments, the daily dose of caffeic acid metabolites used was as low as 6.0 mg (1.5 mg of rabdosiin and 4.5 mg of RA in 100 mg of dry biomass). This value was less than that used in the treatment of rats with the rabdosiin analog, a salt of lithospermic acid B (20 mg/kg/d, administered by intramuscular injections for 8 weeks), during induced diabetic nephropathy.[18] Likewise, a higher dose of RA (100 mg/kg/d for 8 d, administered orally) was used for the treatment of mesangioproliferative glomerulonephritis in rats.[17] It appears likely that rabdosiin would have a profound effect on nephritis, considering that rabdosiin and

lithospermic acid B are both tetramers of caffeic acid, *i.e.*, closely related substances.

While the bioavailability and metabolism of RA have been studied in an animal model,[26] little is known about the bioavailability of rabdosiin. Theoretically, rabdosiin, which contains more caffeol groups than RA and has a higher molecular weight, should be less bioavailable and thus less bioefficient. However, considering the strong effect of lithospermate B on renal functions,[18] one can speculate that rabdosiin possesses high enough bioavailability. In animals, it might be hydrolyzed into active metabolites, ensuring a durable action. Hence, combined introduction of RA and rabdosiin might have favorable effects in the treatment of nephropathy. This proposition should be investigated in future experiments to test not only the impact of each individual substance on the observed pharmacological effects but also to determine the most efficient combination of RA and rabdosiin for the treatment of renal disorders. Such a possibility exists, because we can now manipulate the proportion of RA and rabdosiin in callus cultures by the addition of elicitors (Table 2).

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