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PRODUCTION OF COUMARIN COMPOUNDS BY *HAPLOPHYLLUM PATAVINUM IN VIVO* AND *IN VITRO*

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Key Word Index—*Haplophyllum patavinum*; Rutaceae; tissue culture; suspension culture; coumarin compounds.

Abstract—Native plants, calli and suspension cultures of *Haplophyllum patavinum* were found to produce several coumarin compounds, eight of which were identified as umbelliferone, scopoletin, 7-isoprenyloxycoumarin, umbelliprenin, osthenol, columbianetin, angelicin and psoralen. Umbelliferone, angelicin and psoralen were found both in plant organs and in tissue cultures, scopoletin and umbelliprenin only *in vivo*, osthenol, 7-isoprenyloxycoumarin and columbianetin only *in vitro* conditions. The coumarin production *in vitro* was strongly affected by the cell strain. The co-occurrence of the linear and the angular furanocoumarins, psoralen and angelicin, had never been previously reported in the genus *Haplophyllum*.

INTRODUCTION

H. patavinum (L.) G. Don fil. (Rutaceae) is a perennial herb with a discontinuous distribution, namely a wide illyric range and a punctiform relict disjointed range on the Euganean Hills where it forms unstable endangered populations facing slow extinction [1].

No use of *H. patavinum* in folk medicine is reported and no phytochemical investigation had been previously undertaken. Nevertheless, this species is worthy of investigation since chemical constituents with antimicrobial, antimalarial [2] and insecticidal [3] activities were detected in a *Haplophyllum* species used in Iraqi and Saudi Arabian folk medicine [4]. Moreover, biologically active natural substances of potential pharmaceutical interest are known to occur in the genus *Haplophyllum*, namely coumarin compounds [5, 6], quinoline and other types of alkaloids [7–9], and lignans [10].

The scarcity of plant biomass available in the natural habitat has seriously restricted systematic chemical investigation of *H. patavinum*. *In vitro* culture of this species has been undertaken in our laboratory both to obtain additional plant material for chemical analysis [11, 12] and to set up non-conventional methods for plant propagation [13], the production of viable seeds being very scarce in the natural habitat.

In the present paper, the results of comparative analyses of the coumarins produced *in vivo* by native plants from the Euganean Hills sites, and *in vitro* by calli and suspension cultures, are given.

RESULTS AND DISCUSSION

The callogenesis of *H. patavinum* can be easily obtained from leaf explants, and two different cell strains were established after three years. Homogeneous suspensions were obtained only from strain A (friable white-yellow calli); the dark, hard calli (strain B) failed to produce suspensions. Light conditions are known to stimulate coumarin biosynthesis, at least in some species [14]. Cell suspensions were therefore kept both in the dark and under a 12 hr photoperiod.

Several coumarin compounds were isolated and eight of them, namely five simple coumarins [umbelliferone (**1**), scopoletin (**2**), 7-isoprenyloxycoumarin (**3**), umbelliprenin (**4**) and osthenol (**5**)] and three furanocoumarins [columbianetin (**6**), angelicin (**7**) and psoralen (**8**)] were identified from their spectral data and by comparison with authentic samples.

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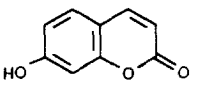

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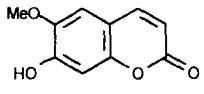

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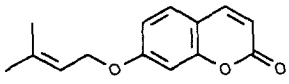

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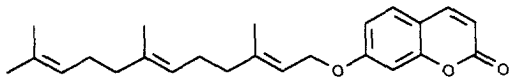

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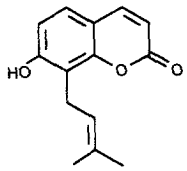

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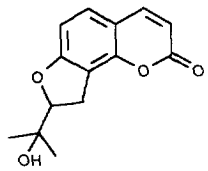

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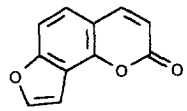

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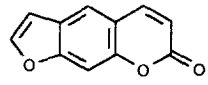

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The amounts of the coumarin compounds recovered from plants, calli and media of the two investigated cell strains and from cell suspensions are reported in Table 1.

Two hydroxycoumarins, i.e. umbelliferone and scopoletin, were found in all the plant parts of *H. patavinum*. From the aerial organs, two furanocoumarins, namely the linear compound psoralen and its angular isomer angelicin were also isolated. Umbelliferone had been previously reported in *H. hispanicum* [15] and in *H. patavinum* [12]; scopoletin is rather widespread in this genus [15–20]. The furanocoumarin psoralen had never been previously detected in the genus *Haplophyllum*, although marmesin, the immediate precursor of psoralen in the biogenetic pathway, occurs in *H. glabrinum* [21]. The only angular furanocoumarin hitherto reported in this genus is thesiolen from *H. thesioides* [22].

In vitro, the biogenetic potential is strictly dependent on the cell strain. The white-yellow friable calli (strain A) proved to be characterized by poor biogenetic potential and only umbelliferone was detected in both tissue and suspension cultures. Higher contents of umbelliferone were found *in vitro* (both in tissue and in suspension cultures) than in intact plants; considerable amounts were excreted into the medium. In the dark-yellow hard calli (strain B), both of the biosynthetic pathways leading from umbelliferone to angelicin (*via* C-8-prenylation) and to psoralen (*via* C-6-prenylation), were operative. Key intermediates in the pathway leading to the angular furanocoumarin angelicin, i.e. osthenol and columbianetin, were also found. No intermediate in the biogenesis of the linear furanocoumarin psoralen was detected, in spite of the fact that the accumulation of marmesin frequently occurs *in vitro* [23]. The 7-O-prenylation of coumarin was also observed *in vitro*, the 7-isoprenyloxycoumarin having been recovered from the strain B calli. Scopoletin, which occurs in all the plant parts *in vivo*, was not detected *in vitro*. *In vitro*, furanocoumarin biosynthesis also occurred in the dark, and psoralen and angelicin contents comparable to those occurring in the aerial parts of the plants were observed in callus; slightly higher amounts were recovered from the medium. Unfortunately, the cell line with strong coumarin biosynthesis (strain B) is quite unsuitable for suspension culture.

Since the exploitation of the natural sources of *H. patavinum* is unrealistic, the biologically active secondary metabolites can be obtained only by means of *in vitro* cultures.

Table 1. Coumarin content of *H. patavinum* (mg/100 g of dry wt)

	Native plants		Tissue cultures				Suspension cultures			
			Strain A dark condition		*Strain B* dark condition		*Strain A* dark condition		*Strain A* light condition	
	aerial parts	roots	callus	medium	callus	medium	cells	medium	cells	medium
Umbelliferone	**21.3** ± 0.8	**24.3** ± 1	**72.2** ± 2.7	**57.6** ± 2.2	**65.1** ± 2.3	**47.6** ± 1.7	**88.1** ± 2.6	**64.3** ± 1.2	**79.3** ± 2.4	**68.2** ± 2.1
Scopoletin	**15.9** ± 0.6	**20.5** ± 0.8	–	–	–	–	–	–	–	–
7-Isoprenyloxycoumarin	–	–	–	–	**9.4** ± 0.3	–	–	–	–	–
Umbelliprenin	**18.3** ± 0.7	–	–	–	–	–	–	–	–	–
Osthenol	–	–	–	–	**11.2** ± 0.4	**8.7** ± 0.3	–	–	–	–
Columbianetin	–	–	–	–	–	**10.3** ± 0.4	–	–	–	–
Angelicin	**5.2** ± 0.2	–	–	–	**5.6** ± 0.2	**7.7** ± 0.3	–	–	–	–
Psoralen	**6.4** ± 0.3	–	–	–	**6.3** ± 0.2	**9.3** ± 0.3	–	–	–	–

EXPERIMENTAL

Plant material

Native plants of *H. patavinum* from the Euganean Hills (a voucher is deposited at the Botanical Garden of the University of Padova, PAD 3703), tissue and cell cultures were analysed.

Tissue and cell cultures

Surface sterilized leaf explants from native plants were placed on B5 medium [24] containing sucrose (30 g/l) and supplemented with 2,4-D (2 mg/l), NAA (0.5 mg/l), IAA (0.5 mg/l) and K (0.2 mg/l) [13]. The pH of the medium was adjusted to 5.7 and the medium was solidified with agar (8 g/l). The induced callus tissues were grown in the dark at 25° and subcultured on fresh media every 4 weeks. After about 20 subcultures, two different cell strains were obtained, namely calli with white-yellow colour, good friability and very high growth rate (strain A), and calli with dark-yellow colour, hard consistency and good growth rate (strain B). Cell suspension cultures were initiated from well-established strain A calli and grown in 500 ml Erlenmeyer flasks containing 150 ml B5 liquid medium supplemented with sucrose (30 g/l) and 2 mg/l 2,4-D, 0.5 mg/l, 0.5 mg/l IAA and 0.2 mg/l K at pH 5.7. Cell suspensions were kept in the dark and also under a 12 hr photoperiod (1000 lux).

Reference compounds

Umbelliferone, scopoletin, angelicin and psoralen were purchased from Aldrich (Steinheim, Germany); 7-isoprenyloxycoumarin, umbelliprenin, osthenol and columbianetin were supplied by the Department of Pharmaceutical Sciences of the University of Padua.

Extraction, isolation and identification of coumarin compounds

Samples of dried plant material from native specimens grown in the natural habitat, frozen-dried callus tissues, solid media and frozen-dried cells from suspension cultures were extracted with MeOH in a Soxhlet extraction apparatus for 48 hr. The liquid media, obtained by centrifuging (5,000 rpm) the suspension cell cultures, were extracted with Et_2O (48 hr), the solvent was removed by distillation, and the residue dissolved in MeOH and analysed.

The methanolic solns were analysed by prep. and analytical TLC (silica gel, Merck) using $CHCl_3$ or EtOAc-cyclohexane in different ratios (3:1, 2:1, 1:1, 1:2; v/v) as eluents. Several compounds were isolated; the purity of the compounds was checked by HPLC (LiChrosorb RP-8, 7μ, Merck; AcCN/H_2O, 1:3; 1.5 ml min^{-1}). Eight coumarin compounds were identified as umbelliferone (**1**), scopoletin (**2**), 7-prenyloxycoumarin (**3**), umbelliprenin (**4**), osthenol (**5**), columbianetin (**6**), angelicin (**7**) and psoralen (**8**) by their UV and MS spectra, and by comparison with authentic samples.

Spectroscopy

All mass spectrometric measurements (EI, CAD-MIKE) were obtained on VG ZAB 2F instrument.

Quantitative determination of coumarin derivatives by HPLC

Plant material and freeze-dried cells were treated under the same conditions as described above, and the methanolic residue was dissolved in $CHCl_3$ for quantification by the method of external standard using the following conditions: silica gel column (LiChrosorb Si 60 7 μm, 250 × 4 mm, Merck), with a small precolumn (LiChrosorb Si 60), eluents $CHCl_3$-n-hexane (for coumarins 7:3; for furanocoumarins 3:7), flow rate 1 ml min^{-1}. Coumarin elution was routinely monitored at 300 nm.

Limits of detection range from 5 to 12 μg (R = 0.9999 – 0.9996). Quoted data are the average values of quantitative determination performed on three samples. Standard deviation was shown to be less than 4%.

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