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## **Histological Localization of Coumarins in Different Organs of *Smyrniun perfoliatum* (Apiaceae)**

By

Ferruccio POLI\*), Bruno TIRILLINI \*\*), Barbara TOSI \*\*\*),  
Gianni SACCHETTI \*\*\* and Alessandro BRUNI \*\*\*)

With 9 figures

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### **Summary**

POLI F., TIRILLINI B., TOSI B., SACCHETTI G. & BRUNI A. 1995. Histological localization of coumarins in different organs of *Smyrniun perfoliatum* (Apiaceae) – *Phyton* (Horn, Austria) 35 (2): 209–217, 9 figures. – English with German summary.

The surfaces and the internal secretory structures of various organs of *Smyrniun perfoliatum* L. were examined to explore the presence of coumarins by UV-induced autofluorescence. The fresh sections of each organ were then dipped in methanol and the extracts were analysed by HPLC (High Performance Liquid Chromatography) and MS (Mass Spectrometry). 4-methyl-umbelliferone and xanthotoxol were mainly identified and quantified. The greatest quantities of coumarins were found in thin roots and fruits.

### **Zusammenfassung**

POLI F., TIRILLINI B., TOSI B., SACCHETTI G. & BRUNI A. 1995. Histologische Lokalisation von Cumarinen in verschiedenen Organen von *Smyrniun perfoliatum* (Apiaceae) – *Phyton* (Horn, Austria) 35 (2): 209–217, 9 Abbildungen. – Englisch mit deutscher Zusammenfassung.

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\*) F. POLI, Institute of Botany and Botanical Garden, University of Cagliari, Viale S. Ignazio 13, 09123 Cagliari, Italy.

\*\*) B. TIRILLINI, Institute of Botany and Botanical Garden, University of Urbino, Via Bramante 28, 61029 Urbino, Italy.

\*\*\*) B. Tosi, G. Sacchetti and A. Bruni, Department of Biology, Section of Botany, University of Ferrara, Corso Porta Mare 2, 44100 Ferrara, Italy.

Die Oberflächen und innenliegenden Strukturen zur Sekretion wurden an verschiedenen Organen von *Smyrniium perfoliatum* L. zur Darstellung von Cumarinen durch UV-Fluoreszenz untersucht. Frische Schnitte von jedem Organ wurden in Methanol getaucht und die Extrakte mittels HPLC und Massenspektrometrie analysiert. 4-Methyl-Umbelliferon und Xanthotoxol wurden hauptsächlich nachgewiesen und quantifiziert. Die größte Menge an Cumerinen wurden in dünnen Wurzeln und Früchten gefunden.

## Introduction

Coumarins are naturally occurring bioactive compounds identified in several plant species belonging to *Apiaceae*, *Rutaceae* and *Fabaceae* families (MURRAY & al. 1982). In recent years, some *Apiaceae* plants were object of a through study about the presence and localization of coumarins. In fact, on the fruit and seed surfaces of *Angelica archangelica* (ZOBEL & BROWN 1991) and *Psoralea bituminosa* (ZOBEL & al. 1991, ZOBEL & MARCH 1993) as well as in the internal fruit tissues, in the seed covers and in the embryo tissues, different coumarin compounds were detected (CAPPELLETTI & al. 1984, INNOCENTI & al. 1984). The presence of coumarins on surfaces of external organs can be related to their protective role against phytopathogenic bacteria and fungi (FOWLKS & al. 1958, TOWERS 1987), while their internal localization can be due to their germination-inhibiting action (BASKIN & al. 1967, FRIEDMAN & al. 1982). Coumarins were also found in different tissues of the leaf and the stem, with low concentrations in the epidermis and greater contents in the parenchyma cells (e.g. *Daphne mezereum*, ZOBEL & BROWN 1988).

The object of this histological study was *Smyrniium perfoliatum* L., *Apiaceae*. The majority of plants of the tribe *Smyrnieae* produces several simple coumarins and linear furanocoumarins (NIELSEN 1971). Some of these plants are eaten as vegetables and some fruits of the genus *Smyrniium* are employed to treat scurvy (FRENCH 1971). Indications for the use of *Smyrniium perfoliatum* against hardnesses and other skin diseases were also described (HARTWELL 1980).

The present work was carried out employing UV-induced auto-fluorescence to locate coumarins occurring in different organs of *Smyrniium perfoliatum*. Correlations between the localization of these important chemicals and their possible functions as secondary metabolites were also discussed.

## Materials and Methods

### Plant Material and Microscopical Observations

Plants of *Smyrniium perfoliatum* were cultivated in the Botanical Garden of the University of Urbino; a sample was deposited in the *Herbarium Universitatis Ferrariensis* of the Institute of Botany, University of Ferrara (S.P. 1093).

The organs of various plants were collected in May-June, 1994, when the plants presented fruits.

Sections of leaves, stem, fruits, thin and tuberous roots were hand-cut, water-mounted and observed immediately with a Zeiss Axiophot equipped with UV-excitation filter BP 436/10 FT 460 LP 470 (set filter 06) or BP 365/12 FT 395 LP 337 (set filter 01).

The autofluorescence observed was compared with that of pure crystals of 4-methyl-umbelliferone, xanthotoxin (Roth, Karlsruhe, Germany), scoparone (Aldrich Chemie, Steinheim, Germany), fraxidin, xanthotoxol (Extrasynthese, Genay, France) with both set filters 01 and 06.

The same sections were dipped in methanol for 20" and immediately observed by UV-microscopy in the same solvent. At least 30 sections from each different organ were investigated.

#### Extraction and Analytical Procedures

For qualitative and quantitative analysis, 100 sections were extracted in methanol for 2 h.

The methanolic extracts of the sections were concentrated and subjected to preparative TLC (Thin Layer Chromatography) on Silica gel for a preliminary separation of the coumarins. The compound mixtures were then scraped from the plates and analyzed by HPLC and MS. For TLC, the Silica gel 60 was used; the solvents were toluene : ethyl acetate : acetic acid (50 : 45 : 5) or chloroform : methanol (80 : 20). For HPLC, a C8 spherisorb column (0.8 × 30 cm) (Jasco) was employed; the solvents were water : methanol (68 : 32) with detection set at 310 nm. For HPLC analysis three repetitions were performed.

To evaluate the occurring of other coumarin compounds, besides the chemicals separated by HPLC, it was applied EIMS (Electronic Impact Mass Spectrometry) to the fractions. For EIMS (70 eV), QMD 1000 quadrupole mass spectrometer (Carlo Erba Instruments) was used equipped with a direct insertion probe, operating at 100°C (TIRILLINI & STOPPINI 1995).

#### Results

In water-mounted sections, with an UV-light excitation at 365 nm, a blue-violet autofluorescence was observed in the external epidermis of the pericarp of *S. perfoliatum* (Fig. 1). At UV-light excitation of 436 nm the autofluorescence was yellow-green (data not shown). In the mesocarp, numerous secretory structures (vittae) were observed on both the dorsal and commissural surfaces. These structures showed the same autofluorescence colours at the different UV-light excitation levels (Fig. 1). In particular, this autofluorescence was caused by the secretion found in the vittae. The embryo also showed a weak blue fluorescence over the entire structure (Fig. 1). In the leaves some fluorescent zones were noted between the parenchymatic cells. The blue fluorescent compounds seem to be localized in secretory structures near the main vein (Fig. 2) and in the mesophyll. In the stem, secretory structures were observed in the cortical zones in front of the vascular bundles. The secretion showed a blue-violet autofluorescence



if excited at 365nm (Fig. 3). In the parenchymatic cells of the cortical zone of the thin and tuberous roots two fluorescent zones were evident under the exoderm (Fig. 4). Seriate hand-cut sections of the stem, thin and tuberous roots showed that these secretory structures were several mm long ducts (canals) and were filled by fluorescent secretions. The same sections were then dipped in methanol and immediately observed with UV-light microscopy. In the fruit, the strong autofluorescence in the pericarp was decreased by the treatment with methanol, while, in the vittae, the fluorescence had completely disappeared (Fig. 5). Furthermore, the fluorescence in the secretory ducts of leaves, stems and roots decreased with the methanolic treatment (Figs. 6–8).

Table 1

Concentrations of the main coumarins occurring in the different organs of *Smyrniium perfoliatum* L. detected by HPLC analysis. Each value is expressed in  $\mu\text{g/g}$  fresh weight (f.w.)  $\pm$  standard deviation (s.d.).

| Samples        | Xanthotoxol<br>$\mu\text{g/g f. w.} \pm \text{s. d.}$ | 4-methyl-umbelliferone<br>$\mu\text{g/g f. w.} \pm \text{s. d.}$ |
|----------------|---|--|
| THIN ROOTS     | $8.1 \pm 1.6$   | $7.3 \pm 1.5$  |
| TUBEROUS ROOTS | $0.9 \pm 0.2$   | $0.8 \pm 0.2$  |
| STEM           | $1.4 \pm 0.3$   | $1.2 \pm 0.2$  |
| LEAVES         | $0.2 \pm 0.1$   | $0.1 \pm 0.1$  |
| FRUITS         | $6.9 \pm 1.4$   | $6.1 \pm 1.2$  |

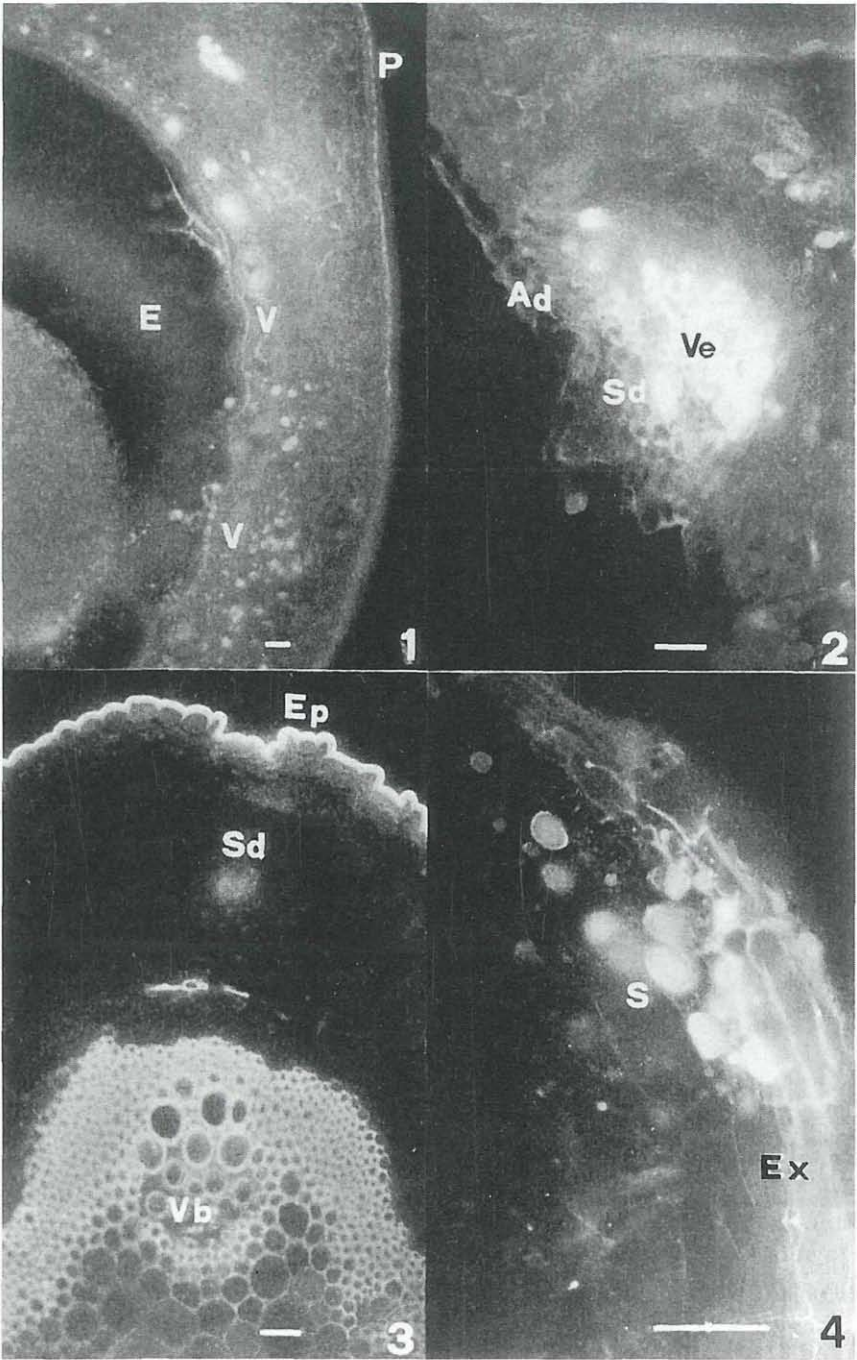
The extracts, obtained by washing with methanol the same sections used in the histological investigation, were analyzed by HPLC to identify the autofluorescent compounds. In Fig. 9, it is reported the HPLC chromatogram referring to the extract of the thin roots in which 4-methyl-umbelliferone and xanthotoxol were detected and quantified. Table 1 reports the content of the two most represented coumarins in different or-

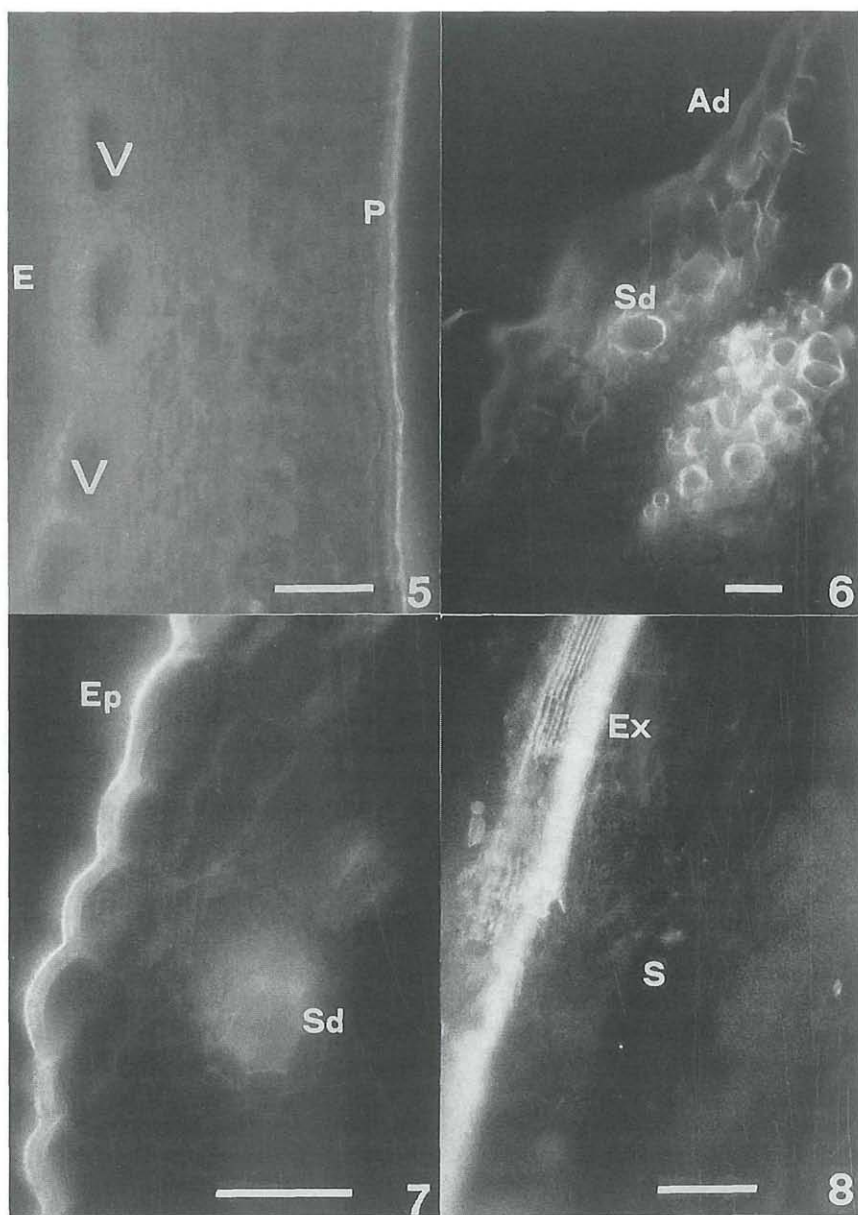
Fig. 1. Fresh cross-section of the fruit of *Smyrniium perfoliatum*. Note the bright blue-violet autofluorescence in the pericarp (P), in the vittae (V) of the mesocarp and over the entire structure of the embryo (E). Scale bar = 50  $\mu\text{m}$ .

Fig. 2. Fresh cross-section of a leaf at the level of a main vein (Ve). The secretory duct (Sd) is localized above the vascular bundle. (Ad = Adaxial surface). Scale bar = 50  $\mu\text{m}$ .

Fig. 3. Fresh cross-section of the stem. Note that the secretory duct (Sd) is in front of the vascular bundle (Vb). Note the blue-violet autofluorescence of the secretion (Ep = Epidermis). Scale bar = 50  $\mu\text{m}$ .

Fig. 4. Fresh cross-section of thin root showing secretory structures (S) filled of fluorescent secretion in the cortical zone, beneath the exoderm (Ex). Scale bar = 50  $\mu\text{m}$ .





Figs. 5-8. Fresh cross-sections of the fruit, leaf, stem and root respectively, treated with methanol for 20s. The autofluorescence of the secretory structures is decreased. Scale bar = 50  $\mu$ m. (P = Pericarp; V = Vittae; E = Embryo; Sd = Secretory duct; Ad = Adaxial surface; Ep = Epidermis; S = Secretory structures; Ex = Exoderm).

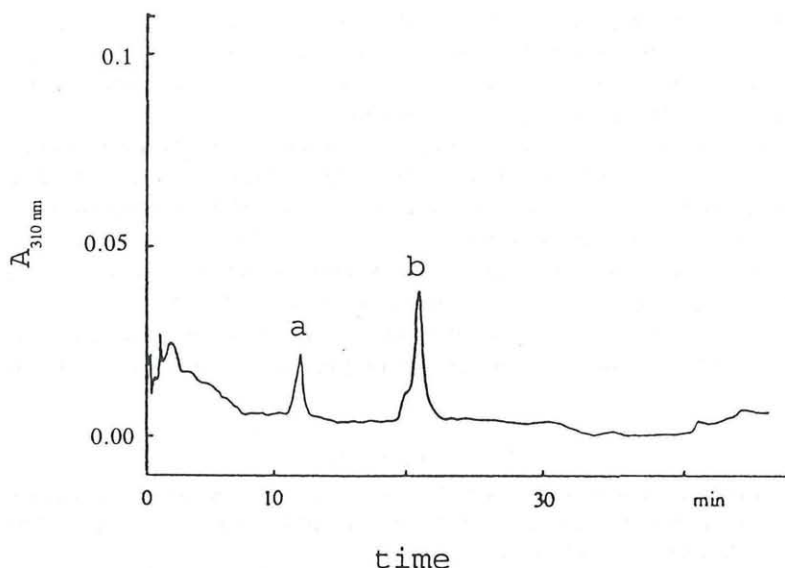


Fig. 9. Separation of the main coumarins occurring in the thin roots by HPLC. a: xanthotoxol (retention time = 12.06); b: 4-methyl-umbelliferone (retention time = 20.58).  $A_{310\text{ nm}}$  = Absorbance at 310 nm.

gans of *S. perfoliatum*. It can be noted that xanthotoxol and 4-methyl-umbelliferone showed the highest concentrations in thin roots and in fruits. In tuberous roots, stems and leaves the coumarins concentrations were about 8–10 fold lower. Afterwards, the analysis of the samples dipped in methanol by EIMS confirmed the presence of xanthotoxol and 4-methyl-umbelliferone but also revealed xanthotoxin, scoparone and fraxidin. Scoparone, fraxidin and xanthotoxin were not quantified by HPLC because of their very low concentrations.

### Discussion

Comparing the HPLC and MS analysis with the UV histological data, the observed autofluorescence in the different organs could be due to 4-methyl-umbelliferone and partially to the presence of scoparone and fraxidin. In fact, crystals and alcohol saturated solutions of 4-methyl-umbelliferone, scoparone and fraxidin gave a blue-violet autofluorescence similar to the histological specimens when irradiated with an UV-light excitation at 365 nm. The weak yellow-blue autofluorescence of the secretion could be due to the presence of xanthotoxin detected by EIMS in spite of its very low concentration. In fact, pure crystals and saturated solutions of xanthotoxin gave a yellow autofluorescence with an UV-light excitation at 365 nm.



The UV histological observation did not allow the non-fluorescent coumarins (e.g. xanthotoxol) to be located, but it can be supposed that these compounds belong to the coumarin pool and have a distribution similar to that of the other fluorescent coumarins.

In accord with BASKIN & al. 1967, the highest concentration of coumarins in the roots could be related to the role that these compounds play against microbial attack, but even more to their function of inhibiting the germination of neighbouring plants. Therefore, the location of coumarins on the surface of the aerial parts can be justified by their defence role against fungi (TOWERS 1987) or bacteria (FOWLKS & al. 1958).

These kinds of studies are not conclusive, but allow us to identify and partially localize biologically active compounds, like coumarins, in fresh samples.

#### Acknowledgement

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## Recensiones

SCHÜTT P., SCHUCK H. J., AAS G., LANG U. M. (Hrsg.) 1994. **Enzyklopädie der Holzgewächse. Handbuch und Atlas der Dendrologie.** Ca. 400 Seiten, Loseblattwerk im Leinenbuchordner. – sfr 298,–/DM 298,–/öS 2325,–. – ecomed Verlagsgesellschaft.

Nach langer Ankündigung des Verlages ist nun der erste Teil einer mehrbändigen „Enzyklopädie der Holzgewächse“ erschienen. Das Gesamtwerk ist in die Abschnitte I. Allgemeines, II. Systematik der Holzgewächse und III. Monographien der Baum- und Straucharten unterteilt. Da das Gesamtwerk als Loseblattsammlung konzipiert ist, können auch künftig erscheinende Ergänzungen und insbesondere neue Einzelmonographien entsprechend der pflanzen-systematischen Stellung der jeweils behandelten Art eingeordnet werden. Jährlich sind etwa 20 bis 30 Einzelmonographien geplant, so daß im Laufe der Zeit ein umfassendes Nachschlagewerk auf dem Gebiet der Dendrologie entstehen wird.

Der erste Abschnitt der Enzyklopädie informiert über den Aufbau des Gesamtwerkes und enthält neben dem Autoren- auch ein Stichwortverzeichnis der behandelten Baum- und Straucharten. Im zweiten Teil wird die Systematik der Holzgewächse dargestellt. Familienspezifische Merkmale von Bäumen und Sträuchern sowie Beiträge zur Blütenmorphologie, Baupläne von Holzgewächsen, Holz-anatomie u.a. werden noch ergänzt.

Bisher liegen Einzelmonographien von 19 vorwiegend nordamerikanischen Nadel- und Laubbaumarten der temperierten Klimazone, von 17 europäischen Straucharten und 15 tropischen Baum- und Straucharten vor. In diesen Monographien werden Morphologie, Verbreitung, Vermehrung, Ökologie, Genetik, Pathologie, Wuchs- und Ertragsleistungen behandelt. Eine Vielzahl großformatiger Farbfotos und eigens für dieses Werk angefertigte Zeichnungen charakterisieren die behandelten Holzarten.

Diese Enzyklopädie wird sicher bei Botanikern, Dendrologen, Forstwissenschaftlern und Ökologen zu einem Standardwerk gehören. Es bleibt zu hoffen, daß es die Herausgeber und der Verlag verstehen werden, kontinuierlich die angekündigten Ergänzungen zu veröffentlichen.

T. GEBUREK

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Autor(en)/Author(s): Poli Ferruccio, Tirillini Bruno, Tosi Barbara, Sacchetti Gianni, Bruni Alessandro

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