Atti Soc. Tosc. Sci. Nat, Mem., Serie B, 106 (1999)
pagg. 93-98, figg. 8, tabb. 2

S. BOTTEGA (*), F. GARBARI (*), A. M. PAGNI (*)

SECRETORY STRUCTURES IN *HYPERICUM ELODES* L. (*HYPERICACEAE*). I. PRELIMINARY OBSERVATIONS

Abstract - The secretory structures of *Hypericum elodes*, a diploid (2n = 16) Atlantic chorotype, in Italy known only for the San Rossore estate (Pisa, Tuscany), were identified, localized and studied from an anatomical and histochemical point of view. Their functional role was also hypothesized.

Key-words - *Hypericum elodes*, secretory structures, anatomy, histochemistry

Riassunto - Strutture secernenti in *Hypericum elodes* L. (*Hypericaceae*). I. Osservazioni preliminari. Sono state studiate le strutture secernenti di *Hypericum elodes*, una specie che in Italia, nell'unica stazione nota (Tenuta di San Rossore presso Pisa), rappresenta un relitto floristico diploide (2n = 16) di tipo atlantico, minacciato di estinzione.
Sono state identificate tasche ghiandolari (stipitate e non), canali secretori di tre tipi, idatodi passivi, emergenze. Di tutte queste strutture é stata definita la localizzazione nei vari organi vegetativi (foglia, fusto, radice, rizoma) e fiorali(sepali, stami, pistillo) e ne sono state identificate, con test istochimici appropriati, le principali componenti secrete delle quali é stato ipotizzato il ruolo funzionale.

Parole chiave - *Hypericum elodes*; strutture secernenti; anatomia; istochimica.

INTRODUCTION

Hypericum L. includes 134 species (Robson, 1981). According to Pignatti (1982), 27 of these are present in Italy. In some of them, the morphological variability of the populations is very pronounced. Their precise taxonomic and geographic circumscription is not always possible. This is particularly true for the group *H. perforatum* and other related units. Many research centers in various parts of the world are carrying out studies on these plants. This is due to the pharmacological properties of the secondary metabolites that they produce (Bombardelli & Morazzoni, 1995; Chatterjee *et al.*, 1998; Erdelmeier, 1998; Meruelo *et al.*, 1988; Nahrstedt & Butterweck, 1997). *H. elodes* is one of the few species that does not have any apparent systematic, taxonomic or nomenclator problems. In Italy, it is currently represented by only one small population (less than 300 individual specimens), found in the «Bosco del Palazzetto» on the San Rossore estate near Pisa. It is threatened by extinction (Conti *et al.*, 1997). It is considered one of the most important Atlantic relics of Italian flora (Corti, 1955). A wide-ranging study of this plant seems justified also for this reason. This introductory paper shall be limited to a preliminary study of the morphoanatomical, karyological and histochemical aspects.

MATERIAL AND METHODS

Material

Vegetative and floral structures of the plant were utilized. Some of the plants had been cultivated in pots (H.B.P. n. 437/98; exsiccata in PI) at the Botanical Gardens in Pisa. They had been removed from the «Bosco del Palazzetto» (San Rossore estate); others were taken directly *in situ*. During the collection, appropriate precautions had been taken so as not to create any negative effects on the structure of the population. Several individual specimens were transferred to the Botanical Gardens of Padua, where they were propagated. They flower and bear fruit regularly, thus contributing efficaciously to their *ex situ* preservation.

Methods

Light microscopy was carried out on: a) hand cut cross-sections (40-50 μm thick) and longitudinal-sections of leaves, stems, rhizomes, roots, sepals and petals; b) sections (25 μm) of leaves, stems, roots and flowers at 14-18°C (Leitz 1720 Cryostat); c) cross-sections (3 μm thick) of leaves, stems, roots, and flowers, after fixation in FAA (Leica 2500 Microtome); d) squashed root tips treated in a 3% aqueous solution of colchicine, fixed in Carnoy, subjected to hydrolysis in HCl and dyed with leucobasic fuchsin (Schiff Reaction); e) leaves *in toto* fixed on absorbent paper, dampened with distilled water.

The following histochemical staining procedures were carried out: Alkanna tincture (Faure, 1914) for lipids; Nile blue (Cain, 1947) and NADI reagent (David & Carde, 1964) for essential oils; Ruthenium red (Jensen, 1962) and Delafield haematoxylin (Faure, 1914) for polysaccharides other than cellulose; Comassie brilliant blue R250 (Fisher, 1968) and Millon's reagent (Faure, 1914) for proteins; Wagner's reagent (Furr & Mahelberg, 1981), Dittmar's reagent (Furr & Mahelberg, 1981) and iodine-iodide solution (Johansen, 1940) for alkaloids; potassium bichromate (Faure, 1914) as generic stain for tannins; nitrous reaction (Reeve, 1951) for catechol tannins; concentrated H_2SO_4 (Geissmann & Griffin, 1971) for sesquiterpene lactones; $SbCl_3$ (Hardman & Sofowora, 1972) for terpenes containing steroids.

(*) Department of Botanical Sciences, Via L. Ghini, 5, 56126 Pisa.

Scanning electron microscopy (S.E.M.) was carried out on young and adult leaves. These were fixed in glutaraldehyde phosphate (2% with buffer solution, pH 7.4), dehydrated with increasing concentrations of alcohol and acetone, dried to the «critical point», metalized and examined at 15KV using a Cambridge Stereoscan 90.

Fluorescence microscopy was done using hand cut cross-sections (40-50 μm thick) of leaf, stem, rhizome, root, sepal and pistil. Flavonoids were detected by induction of fluorescence with the fluorochrome aluminum chloride (Guerin *et al.*, 1971). A Leica DM LB fluorescence microscope with Group A filters (BP 340-380, dichroic mirror 450, LP 430 arrest filter) was used.

Results

Morphology of the secretory structures

The following structures were identified:

a) Glandular pockets. Glandular pockets or traslucent glands or pale glands (Robson, 1981) were formed by a sub-epidermic cavity delimited by a layer of very thin walled secretory cells and by a more external layer of flattened but thicker walled cells, filled with granular cytoplasm (Fig. 1). In the young leaves, the immature stages of the pockets, characterized by the absence of a lumen and by the presence of a group of 4-5 cells with large nuclei and dense cytoplasm, were noted (Fig. 2). The intermediate stages of development showed structures with an internal cavity that was already formed. Cell residue was within the lumen; there were also turgid cells with dense cytoplasm around the central space.

b) Three types of secretory canals. Type A was delimited by 4 polygon-shaped cells, the lumen was not very large in diameter (Fig. 3).

Type B had a large lumen. It was delimited by two layers of cells. The more internal layer consisted of thin-walled flattened cells. During the primary stages of ontogenesis, these were round-like in shape. They had dense cytoplasm and partially occupied the internal cavity, too. The outer layer was made up of flattened cells, each with a thick wall, which conferred a certain rigidity to the structure. This layer was in continuity with the epidermis (Fig. 4).

Type C consisted of a large cavity delimited by a layer of polygon-shaped secretory cells: the cells had thin walls and dense cytoplasm. They were flattened in those canals at more advanced stages of development. There was also an irregular layer of cells that was not clearly distinguishable from the surrounding parenchymatic cells (Fig. 5).

c) Hydathodes. Hydathodes were identified on the epidermic surface as a consequence of the presence of a mass of achlorophyllous cells and stomata of larger dimensions than those commonly distributed on the leaf lamina (Fig. 6). The hydathodes were often protected by papilla-shaped coverings.

d) Glandular emergences. Glandular emergences or stipitate glands (Robson, 1981) were made up of a peduncle and a colorless head or a fuchsia head; the fuchsia head is more frequent in the flowers sampled *in situ* at San Rossore. The peduncle was made up of many parallel layers with rather elongated cells that were in continuity with the epidermic cells. The head consisted of an external layer of elongated cells, like those of the peduncle, and a mass of internal rounded secretory cells (Fig. 7). A thin-walled flattened, often sheathed cell was found in the external layer, in a distal position. This may have been an emission path for secretions.

e) Stipitate glandular pockets. Stipitate glandular pockets were made up of a short peduncle characterized by several parallel rows of cells in continuity with the epidermis, and a head made up of layers: a layer of epidermic cells and a layer of secretory cells with dense cytoplasm. The latter delimited a spherical cavity in which the secretion had been shifted (Fig. 8). Very thin cellular walls were present within the cavity. They appeared to be the residue of degenerated cells.

Localization of the secretory structures

The glandular pockets were present within the thickness of the leaf, in the vicinity of the lower surface. They were diffused in the spaces delimited by the nervations, but never in correspondence with them.

Type-A canals were located in the leaf, in correspondence with the nervations. They were in the parenchyma zone, closest to the lower surface. In the stem and in the rhizome, they were found both in the central cylinder, in the periliberian zone, and in the cortical cylinder, in the parenchyma below the epidermis. The

Fig. 1 - *Hypericum elodes* L.: glandular pocket in a leaf cross-section showing two distinct layers of cells around the lumen; the cells of the internal layer have very thin walls, those of the external have thick walls and dense cytoplasm (Toluidine blue).

Fig. 2 - *H. elodes* L.: early stage of an unopened pocket in a young leaf formed by few polygon-shaped cells with dense cytoplasm and large nucleus (Toluidine blue).

Fig. 3 - *H. elodes* L.: secretory canal (type A) showing four cells around a small lumen in a vascular bundle of leaf cross-section (Toluidine blue).

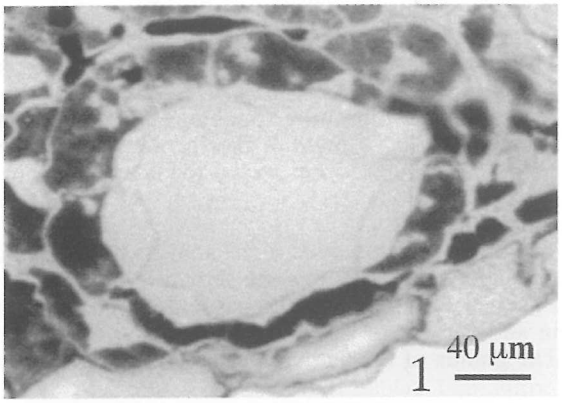
Fig. 4 - *H. elodes* L.: secretory canal (type B) in sepal cross-section. The large lumen is rounded by a layer of epithelial cells with thin walls. A layer of flattened, thick walled cells is visible (Toluidine blue).

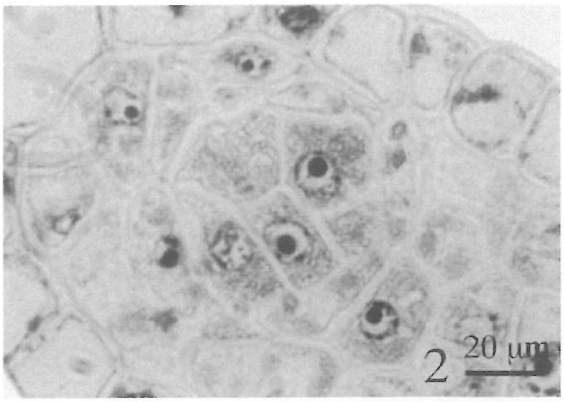
Fig. 5 - *H. elodes* L.: secretory canal (type C) in pistil cross-section. A layer of polygon-shaped secretory cells with dense cytoplasm surrounds the large lumen (Toluidine blue).

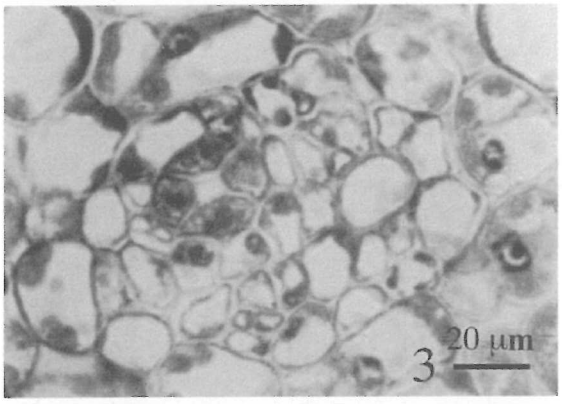
Fig. 6 - *H. elodes* L.: hydathode over the leaf surface close to the apical margin. Some stomata are visible (Wagner's reagent).

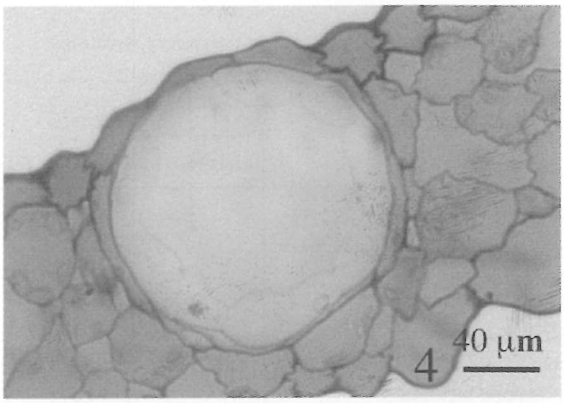
Fig. 7 - *H. elodes* L.: glandular emergence in sepal cross-section. The head is formed by a cluster of secretory cells surrounded by epidermal cells (Toluidine blue).

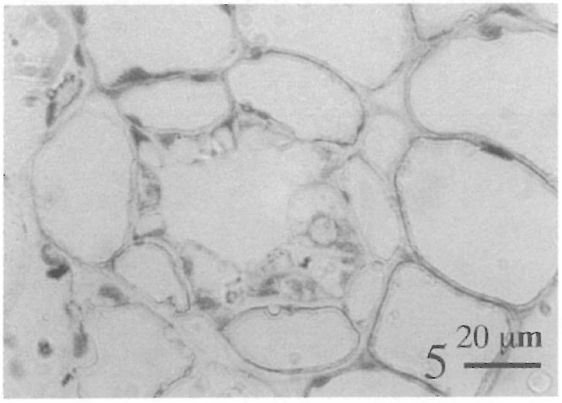
Fig. 8 - *H. elodes* L.: anther section with a stipitate glandular pocket formed by a pluriseriate stalk and a secretory head (Toluidine blue).

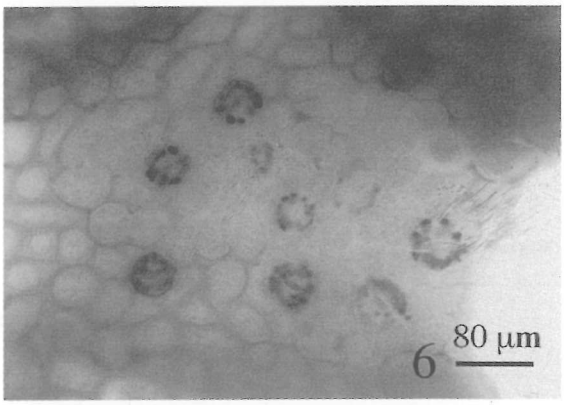


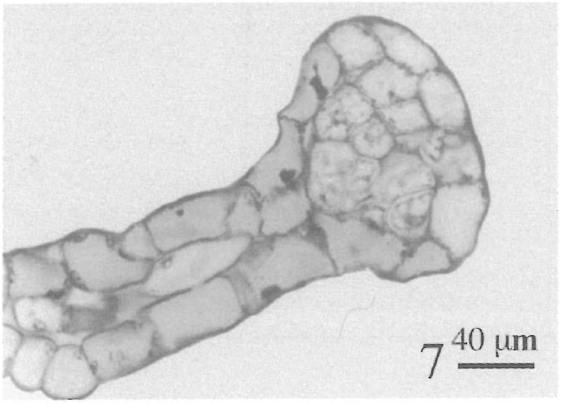


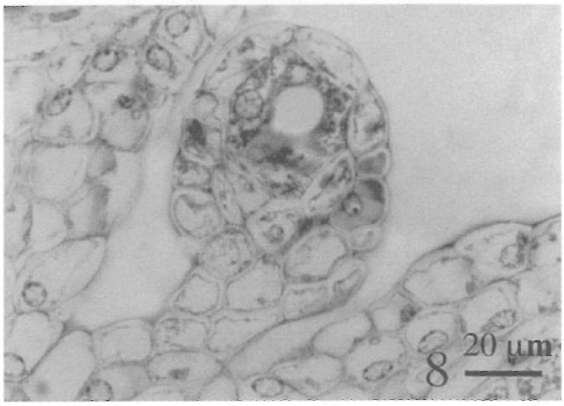












Figs. 1-8

canals of the cortical parenchyma had very reduced lumen, and were delimited by very flat cells.
In the root, the canals were arranged in the pericycle-liberian zone.

Type-B canals were distributed sparsely within the thickness of the sepals: they were far away from the nervations.
Type-C canals were found in the thick part of the carpel leaf, at the levels of the ovary and the styles. They considerably reduced the dimensions of their lumen by proceeding in the direction of the distal zones, until they disappeared in the vicinity of the stigmas.
Hydathodes were present at the tip of the leaf. They were found in a small depression that was very close to the ending of the primary nervation.
The glandular emergences were located on the margin of the sepals.
The stipitate glandular pocket was typical of the anthers. It was inserted at the level of the connective tissue, close to the insertion of the filament.

Histochemistry
The results of histochemical analyses carried out on the vegetative structures are shown in Table 1.
Results of the reproductive structures are summarized in Table 2.
The glands of the anthers produced lipidic substances,

Tab. 1 - Vegetative parts: histochemistry of secretory structures

	Leaves		Stem	Rhizome	Root
	Glandular pockets	Canals A	canals A	canals A	canals A
Lipids	+	+	+	+	+
Carbohydrates other than cellulose	-	-	-	-	-
Essential oils	+	+	+	-	-
Resins	+	+	+	+	+
Alkaloids	+	+	+	+	+
Proteins	-	-	-	-	-
Sesquiterpene lactones	-	-	-	-	-
Tannins	-	-	-	-	-
Cathecolic tannins	-	-	-	-	-
Steroids	+	-	-	-	-
Flavonoids	-	+	+	+	+

+: positive; -: negative

Tab. 2 - Reproductive parts: histochemistry of secretory structures

	Sepals		Pistils
	Emergences	Canals B	Canals C
Lipids	+	+	+
Carbohydrates other than cellulose	-	-	-
Essential oils	-	-	-
Resins	+	+	+
Alkaloids	-	+	+
Proteins	-	-	-
Sesquiterpene lactones	-	-	-
Tannins	-	-	-
Cathecolic tannins	-	-	-
Steroids	-	-	-
Flavonoids	++	-	-

+: positive; ++: strongly positive;
-: negative

essential oils, alkaloids and proteins. Further studies will enable us to give a better characterization of the secretion.

Karyological investigations

The karyological investigations were aimed at confirming the few data found in the literature relative to the chromosome number of *Hypericum elodes* L. All of the investigated samples were diploids (2n = 16).

DISCUSSION

Secretory structures are present in *H. elodes*. Glandular pockets and secretory canals have already been described, within the genus, by several authors (Weill, 1903; Metcalfe & Chalk, 1950; Curtis & Lersten, 1990). Other structures, such as the glandular emergences and the stipitate glandular pockets, have hardly been studied, or not at all.

The glandular pockets produced lipids, oleoresins, alkaloids and steroids, besides the essential oils that have already been reported in the literature (Robson, 1981). The presence of these substances should guarantee a good system of defense for the leaves against phytophagous insects. The leaves were the only organ in which the pockets were distributed. They are scarcely cutinized and, therefore, easily liable to deterioration.

Types B and C secretory canals have been phylogenetically correlated to the glandular pockets due to their morpho-anatomical resemblance (Robson, 1981). The canals secretion differed from that of the pockets only in the absence of essential oil and steroids.

The large quantity of alkaloids and oleoresins guarantees protection against predators even at the level of the floral structures.

The composition of the secretion in type-A canals differed in relation to their distribution in the plant. Those located in the hypogean organs did not contain any of the essential oils that abound in the leaves and stem. The secretion of the rhizome and that of the root corresponded to that found in types B and C canals in which flavonoids were not observed. The presence of flavonoids in the hypogean organs could be linked to their function against fungus attacks (Harborne, 1993). These attacks are particularly notable in humid environments, typical of *H. elodes*.

At the level of the reproductive structures, flavonoids were abundant only in the fuchsia-coloured glandular emergences that also contained lipids and oleoresins. The colour of these emergences may be due not only to the occurrence of hypericin as hypothesized by Robson (1981) but also to anthocyanin. The presence of anthocyanin is most probably linked to adverse environmental conditions or to particular plant/insect relationships. This could explain the greater quantity of colored emergences in the flowers of San Rossore when compared to those in the Botanical Gardens.

The stipitate glandular pockets also act as deterrents against predators. They therefore defend the reproductive structures. Alkaloids are produced by the pockets which carry out a precise and very specialized action at the level of the stamens. These structures are precociously well-developed and differentiated.

They could be fundamental, together with the canals of the sepals and the glandular emergences, in protecting the flowers in bud.

The hydathodes observed at the tip of the leaves may be considered epithems/hydathodes (Corsi, 1999) that passively expel the excess water in the tissues, to the outside. The peculiar ecology of the Italian population, localized in a single habitat that is seasonally subject to floodings, may confirm this hypothesis.

Further analyses will provide a better characterization of these structures from a morpho-functional point of view.

The karyological studies showed that this species is diploid (2n = 16). This result confirms the one reported by Al-Bermani *et al.*, (1993). It is not in agreement with that of Robson (1966), whose results showed the basic chromosome number as being n = 16. Robson has stated that his results can no longer be verified and sustained, due to the deterioration of the material that originally led him to this conclusion.

ACKNOWLEDGEMENTS

This research was carried out within the framework of the project «The Italian species of *Hypericum* as a potential pharmacobotanic resource», that was co-funded by the University of Pisa and M.U.R.S.T. (Person in charge: A.M. Pagni), and by the INTERREG II Project (Person in charge: F. Garbari).

Special thanks to the «Ente Parco Regionale Migliarino, San Rossore and Massaciuccoli» for having given us permission to collect the material used in this study.

REFERENCES

AL-BERMANI A.-K. K. A., AL-SHAMMARY K. I. A., BALEY J. P., GORNALL R. J. (1993). Contributions to a cytological catalogue of the British and Irish flora, 3. *Watsonia*, **19**: 267-278.

BOMBARDELLI E., MORAZZONI P. (1995). Hypericum perforatum. *Fitoterapia*, **66** (1): 43-68.

CAIN A.J. (1947). The use of Nile blue in the examination of lipids. *Q. J. Microsc. Sci.*, **88**: 383-392.

CHATTERJEE S.S., NÖLDNER M., KOCH E., ERDELMEIER C. (1998). Antidepressant activity of Hypericum perforatum and Hyperforin: the neglected possibility. *Pharmacopsychiat.*, **31** (Suppl.): 7-15.

CONTI F., MANZI A., PEDROTTI F. (1997). Liste Rosse Regionali delle piante d'Italia. Università di Camerino 139 pp.

CORSI G. (1999). Hydatodes in Italian taxa of the genus Urtica. Plant Biosystems (in press).

CORTI R. (1955). Ricerche sulla vegetazione dell'Etruria. X: Aspetti geobotanici della Selva Costiera. La Selva Pisana a S. Rossore e l'importanza di questa formazione relitta per la storia della vegetazione mediterranea. *Giorn. Bot. Ital.*, **62**: 75-262.

CURTIS J.D., LERSTEN N.R. (1990). Internal secretory structures in Hypericum (Clusiaceae): H. perforatum L. and H. balearicum L. *New Phytol.*, **114**: 571-580.

DAVID R., CARDE J.P. (1964). Coloration différentielle des pseudophylles de Pin maritime au moyen du réactif de Nadi. C. *R Acad. Sci. Paris, Ser. D.*, **258**: 1338-1340.

ERDELMEIER C.A.J. (1998). Hyperforin, possibly the major non-nitrogenous secondary metabolite of Hypericum perforatum L. *Pharmacopsychiat.*, **31** (Suppl.): 2-6

FAURE G. (1914). Manuale di micrografia vegetale. Ist. Naz. Medico Farmacol. Editore, Roma.

FISHER D.B. (1968). Protein staining of ribboned epon sections for light microscopy. *Histochemie*, **16**: 92-96.

FURR Y., MAHLBERG P.G. (1981). Istochemical analysis of laticifers and glandular trichomes in Cannabis sativa. *J. Nat. (Lloydia)*, **44**: 153-159.

GEISSMANN T.A., GRIFFIN T.S. (1971). Sesquiterpene lactones: acid-catalysed color reactions as an aid in structure determination. *Phytochemistry*, **10**: 2475-2485.

GUERIN H.P., DELAVEAU P.G., PARIS R.R. (1971). Localisations histochimiques II: procedes simples de localisation de pigments flavoniques. Application a quelques Phanerogames. *Bull. Soc. Bot. Fr.*, **118** (1-2): 29-36.

HARBORNE J.B. (1993). Introduction to ecological biochemistry. Fourth Edition. Academic Press: 264-275.

HARDMAN R., SOFOWORA E.A. (1972). Antimony trichloride as a test reagent for steroids, especially diosgenin and yamogenin, in plant tissues. *Stain Technol.*, **47** (4): 205-208.

JENSEN W.A. (1962). Botanical histochemistry principles and practice. W. H. Freeman and Co. San Francisco and London.

JOHANSEN D.A. (1940). Plant microtechnique. Mc Graw-Hill, New York-London.

METCALFE C.R., CHALK L. (1950). Anatomy of Dicotyledons. Leaves, stem, and wood in relation to taxonomy with notes on economic uses. Clarendon Press: 165-169. Oxford.

MERUELO D., LAVIE G., LAVIE D. (1998). Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: Aromatic polycyclic diones hypericin and pseudohypericin. *Proc. Natl. Acad. Sci.*, USA **85**: 5230-5234.

NAHRSTEDT A., BUTTERWECK V. (1997). Biologically active and other chemical costituents of the herb of Hypericum perforatum L. *Pharmacopsychiat.*, **30** (Suppl.): 129-134.

PIGNATTI S. (1982). Flora d'Italia **1**: 343-351.

REEVE R.M. (1951). Histochemical tests for polyphenoles in plants tissues. *Stain Technol.*, **26**: 91-96.

ROBSON N.K.B. (1981). Studies in the genus Hypericum L. (Guttiferae) 2. Characters of the genus. *Bull. Br. Mus. Nat. Hist. (Bot.)*, **8** (2): 55-226.

ROBSON N.K.B. (1996). Studies in the genus Hypericum L. (Guttiferae) 6. Sections 20. Myriandra to 28. Elodes. *Bull. Nat. Hist. Mus. Lond. (Bot.)*, **26** (2): 75-217.

WEILL G. (1903). Recherches histologiques sur la famille des Hypéricacées. Maison d'éditions A. Jaonin et Cie. Paris.

(ms. pres. il 20 luglio 1999; ult. bozze il 15 dicembre 1999)