Cellular localisation of the anti-cancer drug camptothecin in *Camptotheca acuminata* Decne (Nyssaceae)

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In *Camptotheca acuminata*, we studied the cellular sites of accumulation of the alkaloid camptothecin (CPT), in both plants grown in the field and those grown in a greenhouse, subjecting the latter to stress (i.e., draught, nutritional deficit, and pruning). Fresh sections of the leaf, stem, and root were analysed for the presence of CPT by examining the autofluorescence that the CPT molecule emits when exposed to UV radiation. In the plants grown in the field, CPT was observed only rarely. In the greenhouse plants, CPT had accumulated in crystalline form in the vacuole of specialised cells (i.e., segregator idioblasts), which were not morphologically distinguishable from the cells of the surrounding tissues. In the organs examined, the segregator idioblasts were localised in parenchymatic and epidermal tissues. CPT crystals were also detected in the glandular trichomes on both the stem and leaf.

Key words: *Camptotheca acuminata*, camptothecin, indole alkaloids, histochemistry, fluorescence analysis.

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Paper accepted on June 10, 2004

European Journal of Histochemistry
2004; vol. 48 issue 3:321-328

Camptothecin (CPT) is a monoterpene indole alkaloid produced by the Chinese tree *Camptotheca acuminata* Decne (Nyssaceae) and first isolated in 1966 by Wall and coworkers. CPT is known for its remarkable anti-cancer activity, which results from its ability to inhibit the eukaryotic DNA topoisomerase I (Kjeldsen *et al.*, 1992). It also inhibits retroviruses such as the human immunodeficiency virus (HIV) (Priel *et al.*, 1991), apparently as a result of the inhibition of Tat-mediated transcription (Li *et al.*, 1994).

Although much is known about the pharmacological effects of CPT, little is known about its biosynthetic pathway (Silvestrini *et al.*, 2002) or the cellular sites of biosynthesis and accumulation. Recent studies have shown that the biosynthesis and accumulation of alkaloids are tissue-specific and cell-type-specific (De Luca and St-Pierre 2000, Samanani *et al.*, 2002). One of the species that has been most commonly used in the study of alkaloid biosynthesis and accumulation is *Catharanthus roseus*, which produces more than 100 different terpenoid indole alkaloids (St-Pierre *et al.*, 1999), of which the most well known are vinblastine and vincristine. In 1976, Yoder and Mahlberg, using histochemical analysis, observed that the alkaloids in *C. roseus* had accumulated only in "specialized parenchymatic cells" and in laticifers. Immunocytochemical analyses for localising the enzymes involved in alkaloid biosynthesis in *C. roseus* have shown that the site of biosynthesis can differ from the site of accumulation. Moreover, the various stages of alkaloid pathways occur in different tissues. For example, the enzymes involved in the early stage of the vindoline pathway (i.e., tryptophan decarboxylase and strictosidine synthase) are only found in the epidermis of stems, immature leaves, and flowers, and in the apical meristem of the root tips, whereas the enzymes involved in the late stages (i.e., deacetylvindoline-4-hydroxylase and deacetylvindoline-4-O-acetyltransferase) are limited to the laticifer and idioblast cells

of the leaf, stem, and flower bud (De Luca and Laflamme, 2001). At the subcellular level, alkaloids actively accumulate in the vacuole, although their biosynthesis generally occurs in the cytoplasm or in some other cellular compartment (Wink, 1997).

In *C. acuminata*, chemical analyses have demonstrated that CPT accumulates in all organs of the plant. The highest levels of CPT are found in the first young leaves near the shoot apex, and as the leaves mature, the CPT concentration decreases rapidly. In the other vegetative organs, the correlation between the level of CPT and the degree of organ development is not clear (Lopez-Meyer *et al.*, 1994). CPT concentrations have also been observed to vary significantly by specific genotype, season, and plant age (Liu *et al.*, 1998) and depending on whether or not the plant is subjected to environmental stress, specifically, pruning (Liu *et al.*, 1999) and drought (Liu, 2000).

In the present study, we conducted a histological analysis of the vegetative organs of *C. acuminata* and investigated the cellular localisation of CPT in these organs through autofluorescence. The study was conducted both on plants grown in the field and those grown in a greenhouse, subjecting the latter to stress.

Materials and Methods

Plant material

Leaf, stem, and root samples were collected in the Summer of 2003 from three-year-old *C. acuminata* trees, ranging from 1.5 to 2 m in height, which had been grown from seed either in the field or in a greenhouse at the Botanical Garden of "La Sapienza" University (Rome, Italy). The greenhouse plants were grown in 10 L pots with commercial soil and were subjected to the following conditions of stress: beginning in March 2002, they were watered infrequently (i.e., every 15 days) and with scarce amounts of water; the soil was never fertilised; and in March 2003 they underwent drastic pruning. The plants grown in the field were instead subjected to the climatic conditions of the city of Rome, which are illustrated in the Bagnouls-Gaussen's diagram in Figure 1. In the Spring and Summer, they were watered every day.

Chemical analyses

Quantitative chemical analyses were conducted on leaves (i.e., the organ with the greatest CPT accumulation, according to Lopez-Meyer *et al.*, 1994) taken from *C. acuminata* plants. The samples were collected from ten individuals chosen at random (five grown in the field and five in the greenhouse). The samples were extracted following the protocol of Liu *et al.* (1999) and analysed with high-performance liquid chromatography (HPLC). A Waters 2487 Dual λ Absorbance Detector was used, coupled with a 5-μm Symmetry C_{18} 4.6 ×150 column (Waters) operated in the reversed-phase mode using an MeOH-H_2O-MeCN mobile phase. The linear gradient elution profile started with MeOH-H_2O-MeCN (5:55:40) and ended with MeOH-H_2O-MeCN (35:25:40) within 15 min. The flow rate was 1 ml/min, and all chromatograms were plotted at the absorption wavelength 245 nm by a UV detector (Waters). Injection volumes of both standard CPT (Acros Organics) and unknown samples were 50 μL throughout the analysis. CPT was identified by direct comparison of the UV spectrum and retention time of the standard. Standard curves for the quantitative analysis of CPT, run daily, were constructed by plotting the peak area ratios against amount ratios of CPT to the internal standard. The CPT concentrations in the unknown samples were calculated using the curves developed on the standard.

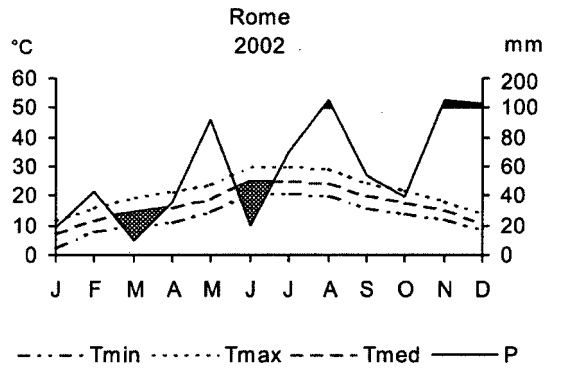

Figure 1. Bagnouls-Gaussen's diagram of the city of Rome: monthly temperature and precipitation in the year 2002.

Histologic and histochemical analyses

Leaf, stem, and root samples were embedded in agar (4%) and sectioned (~30 μm thickness) with a vibratome (TPI series 1000). Fresh sections were examined with a Zeiss microscope (Axioscop 2

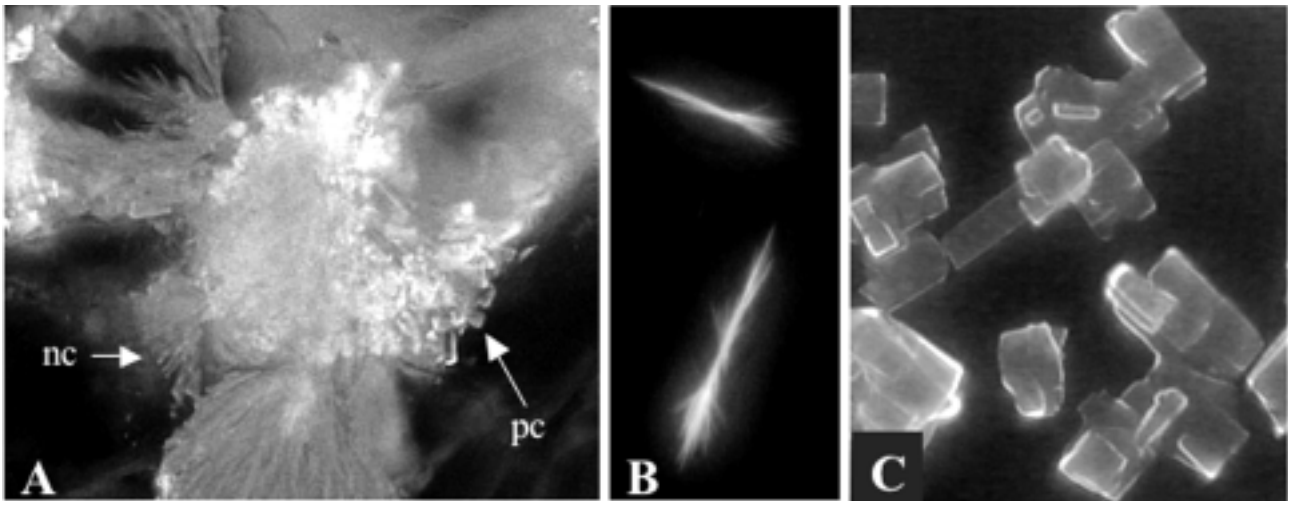

Figure 2. Micrographs showing CPT crystals under UV light. A: CPT crystals accumulated in the vacuole of medullar parenchymatic cells of the stem; needle-like crystals (nc) and prismatic crystals (pc); x 800. B: autenthic CPT, needle-like crystals; x 1000. C: autenthic CPT, prismatic crystals x1000.

Plus) using a Zeiss UV-filter combination (BP 365 nm, LP 397 nm) for CPT detection; Dey and Warner (1997) demonstrated that the CPT molecule emits visible light ($\lambda_{max\ flu}$ ~430 nm) when exposed to UV radiation ($\lambda_{max\ abs}$ ~370 nm). For each type of organ, approximately 50 samples were analysed; 15 sections were taken from each of these samples, for a total of 750 sections per organ. Authentic CPT crystals were observed with UV radiation and compared with the crystals present in the plant samples.

Some sections were observed in brightfield after having been stained using reagents specific for alkaloids: the Jeffrey (Johansen, 1940), Dittmar (Furr and Mahlberg, 1981), Wagner (Furr and Mahlberg, 1981), and Dragendorf (Merck Index, 1940) reagents. Other sections were also stained with 1% toluidine blue.

Results

CPT detection

The chemical analyses carried out on leaf samples revealed that the concentration of CPT was on average 11 times higher in the plants grown in the greenhouse and subjected to environmental stress (0.262 mg/g DW), compared to those grown in the field (0.024 mg/g DW).

The histochemical analysis performed on fresh sections of vegetative organs using specific reagents for alkaloids did not reveal CPT, whereas the non-stained fresh sections examined under the microscope using UV light showed autoflorescent CPT in the tissues. CPT had accumulated in crystalline form in specialised cells (i.e., segregator idioblasts), which, in terms of shape, size, and colour, were not distinguishable from the cells of the surrounding tissues. The CPT crystals in the segregator idioblasts (Figure 2A) were characterised by a blue fluorescence similar to that shown by the authentic CPT (Figure 2B and 2C). CPT crystals were rarely observed in the plants grown in the field; thus the results presented in this paper refer only to the plants that had been grown in the greenhouse and subjected to stress.

Leaf

The leaves of *C. acuminata* are simple, elliptical, and dorsoventral, and they reach a maximum length of 25 cm. The abaxial and adaxial epidermis are composed of thin-walled cells arranged in a single layer, with the stomata confined to the adaxial surface. The mesophyll is arranged in a single layer of palisade parenchyma and a multilayered spongy parenchyma (Figure 3A). The midrib has several vascular bundles arranged in a ring (Figure 3B).

The leaf surface has three types of trichomes: unicellular non-glandular, unicellular glandular, and multicellular glandular (also referred to as "capitate trichomes"). The unicellular non-glandular trichomes are present on both the adaxial and abaxial side; they are pointed, erect, and thick-walled (Figure 3B). The unicellular glandular trichomes are present only on the abaxial side; they are bow-

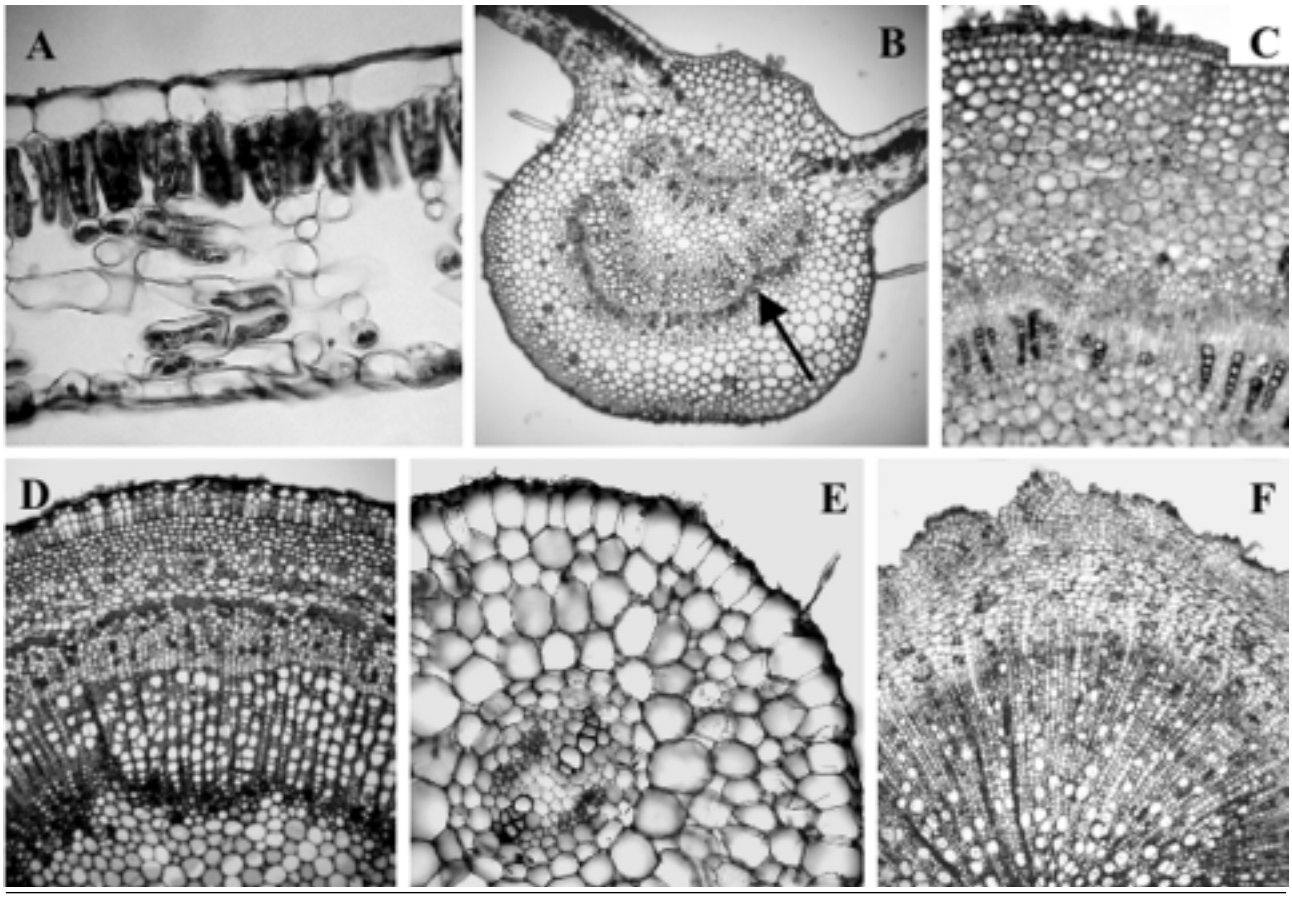

Figure 3. Cross-section of leaf, stem, and root of *Camptotheca acuminata* Decne (all sections stained with toluidine blue). A: Section of the lamina of the young leaf (1.5 cm in length); x420. B: Section of the young leaf showing the midrib with vascular bundles arranged in a ring (arrow); unicellular non glandular trichomes are also visible; x110. C. Section of the young stem in the primary body; x32. D. Section of the stem in the secondary body; x19. E: Section of the root in the primary body showing a vascular cylinder with 2 arches; x55. F. Section of the root showing secondary body; x20.

shaped, protruding from the epidermis and bending back onto it (Figure 4C). The multicellular glandular trichomes are present only on the adaxial side; they consist of one basal cell, one stalk cell with a thick cutinised lateral wall, and a single-celled round head with a large subcuticular space (Figure 4D). In young leaves, both the glandular and non-glandular trichomes are numerous; although as the leaf grows, the trichome density decreases.

Autofluorescent CPT crystals were never present in the leaf primordium. In the first three leaves beginning from the shoot apex (1-2 cm length), blue fluorescent crystals were observed both in the lamina and in the midrib (Figures 4A, B). CPT accumulation was observed in the abaxial epidermis (Figure 4A), specifically, in some unicellular (Figure 4C) and multicellular (Figure 4D) glandular trichomes. CPT was also observed in the cells of both the palisade parenchyma and the spongy parenchyma. In the midrib, CPT accumulation was localised in the abaxial parenchyma (Figure 4B).

In more developed leaves, the sites of CPT accumulation were the same as those described for young leaves; nonetheless, as the dimensions of the leaves increased, the segregator idoblasts, both in the epidermal and parenchymatic tissues, were less numerous and had fewer autofluorescent crystals.

Autofluorescence was also observed, though rarely, in the lumen of the xylematic vessels of the midrib.

Stem

The primary body of the stem is limited to the first internode. It is externally delimited by a single layer of epidermal cells with an outer periclinal wall thickened by the presence of a cuticle (Figure 3C). Unicellular non-glandular trichomes and unicellular glandular trichomes are present, and their

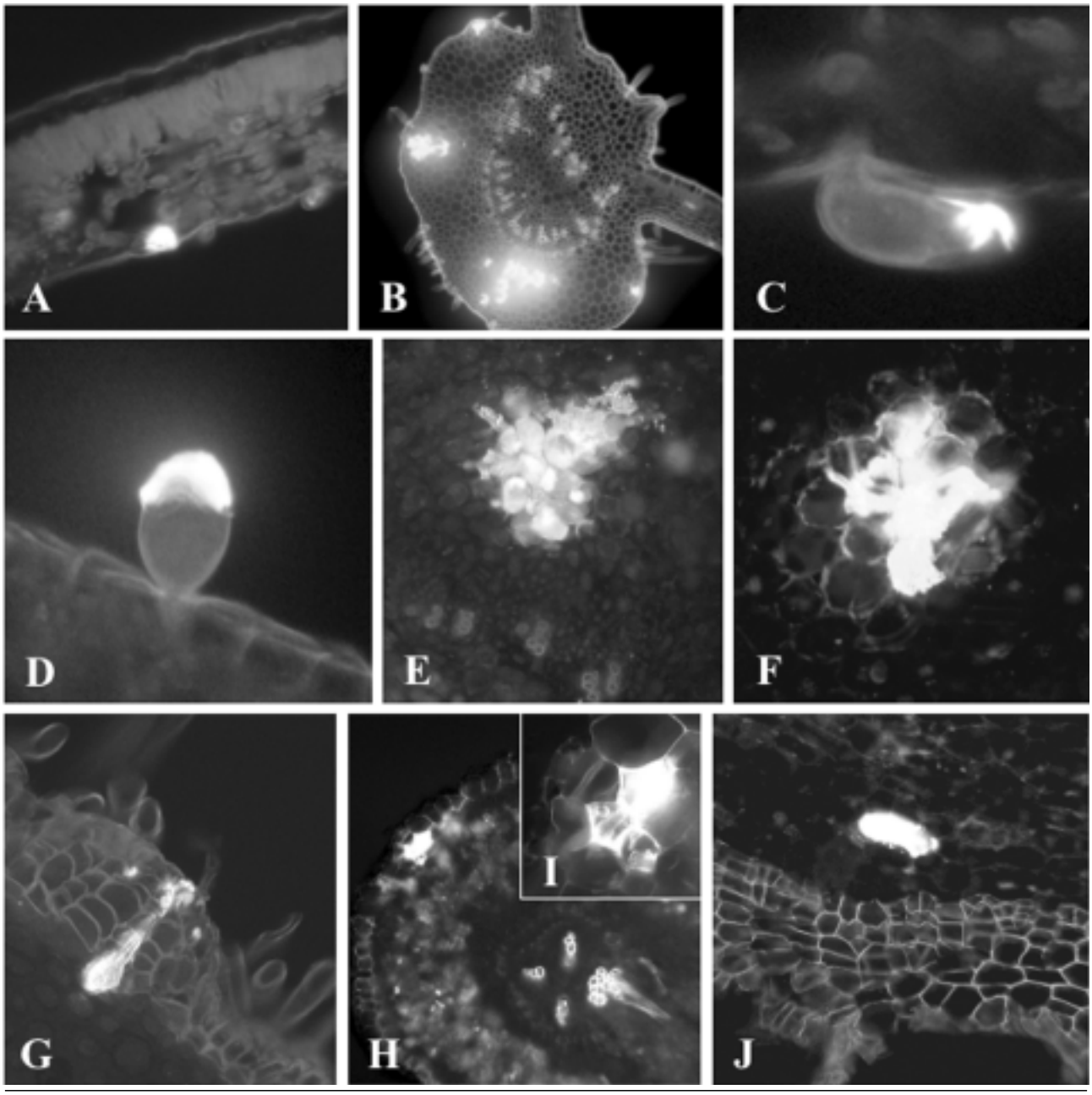

Figure 4. Micrographs showing CPT localisation under UV light. A: Section of the young leaf showing autofluorescent CPT crystals in the abaxial epidermis; x270. B. CPT accumulation in the abaxial parenchyma of the midrib; x100. C. CPT crystals in a unicellular glandular trichome on the abaxial side of the lamina; x750. D. CPT crystals in a multicellular glandular trichome on the adaxial side of the lamina; x600. E. Group of cells in the stem cortex filled with CPT crystals; x180. F. Group of cells in the stem pith filled with CPT crystals; x170. G. Initial secondary body of the stem showing CPT crystals in the periderm; x150. 4H. Section of the young root showing CPT crystals in the parenchymatic cells underlying the epidermis; the fluorescence signal in the central cylinder is due to lignin in the cell wall of xylematic elements; x45. I. Detail of photo shown in Figure 3H; x150. J. Root in the secondary body showing CPT accumulation in parenchymatic cells associated with the phloem; x85.

structure is like that described for the leaf. The cortex consists of 5 or 6 layers of angular collenchyma, followed by parenchymatic tissue. Many cells contain calcium-oxalate crystals. In the innermost cortical layers, isolated strands of fibres are present. A starch sheath surrounds the central cylinder. The xylem consists of a continuous cylinder with narrow medullar rays (Figure 3C).

Groups of 5-10 cells filled with large autofluorescent CPT crystals were observed in the inner cortical parenchyma (Figure 4E) and in the pith (Figure 4F). In the stem, as in the leaf, single small autofluorescent CPT crystals were observed in the unicellular glandular trichomes and, again as in the

leaf, autofluorescence was observed, though rarely, in the lumen of the xylematic vessels of the midrib.

The secondary body of the stem is characterised by a multilayered periderm, some collenchyma layers, an inner cortical zone formed by cells rich in starch, a discontinuous sheath constituted by groups of sclerenchymatic fibres, and a central cylinder (Figure 3D).

Cells containing autofluorescent crystals of CPT were detected in the periderm. The crystals, which had a needle-like shape, often spilled out from the vacuole after being cut (Figure 4G).

Root

The primary body of the root is composed of a monolayered epidermis consisting of cells with suberized yellow-brown coloured walls, a wide cortex internally delimited by the endodermis, with Casparian strips, and an actinostelic vascular cylinder with 2-6 arches (Figure 3E).

Autofluorescent CPT crystals were observed in parenchymatic cells underlying the epidermis; those cells with crystals were in groups of 2-5 cells (Figures 4H, I).

In the secondary body of the root (Figure 3F), CPT accumulation was observed in parenchymatic cells associated with the phloem (Figure 4J).

CPT accumulation was observed in more than 90% of the histological sections of all of the organs examined (leaf, stem and root). The preferential location was in the parenchymatic tissues (palisade and spongy in the leaf; cortical and medullar in the stem; cortical and phloematic in the root). In both the leaf and the primary stem, only 0.1-1% of the unicellular and multicellular glandular trichomes showed CPT accumulation. Autofluorescence in the lumen of the xylematic vessels of the leaf and stem, attributable to the presence of CPT, was only observed in some of the sections obtained from samples collected in early February 2002.

Discussion

The results of this study indicate that environmental stress induces the biosynthesis and accumulation of CPT, confirming the findings of previous studies, which have shown that pruning and drought can result in increased CPT accumulation (Liu, 2000) and that soil fertilisation, which leads to the growth of both slow-growing and fast-growing *C. acuminata* plants, can significantly decrease the CPT concentration in the leaf (Liu *et al.*, 1999). It has been hypothesised that abiotic stress makes the plant more vulnerable to predators, inducing a defence response in the form of secondary-metabolite production (Hoft *et al.*, 1996). Moreover, it is possible that the increase in the CPT concentration is attributable to the activation of "stress enzymes" in response to draught (Liu, 2000).

The results of this study also indicate that CPT accumulation occurs in all of the vegetative organs (i.e., leaf, stem, and root), with the exception of leaf primordia. CPT accumulation was observed in segregator idioblasts localised in the parenchymatic and epidermal tissues. The segregator idioblasts, observed in brightfield, were not distinguishable from the surrounding cells in terms of their shape, colour, or size, and it is perhaps for this reason that the cellular localisation of CPT had never been described in *C. acuminata*. In other species, the idioblasts that are specialised in alkaloid accumulation can be distinguished from the surrounding parenchymatic cells because they are larger, as reported for *Catharathus roseus* (De Luca and St-Pierre, 2000), or because they differ in colour, as observed in *Sanguinaria canadensis*, in which they are coloured an intense red by sanguinarine (Newmann and Mueller, 1972).

In the segregator idioblasts, the observed CPT crystals were located in the vacuole. It has been hypothesised that alkaloid vacuolar segregation is necessary to avoid cytotoxic effects on the cells (Wink, 1993). In fact, CPT, by interfering with DNA topoisomerase I, inhibits RNA synthesis (Kjeldsen *et al.*, 1992).

In addition to the segregator idioblasts, we observed CPT crystals in the glandular trichomes of both the leaf and the stem. This is consistent with the findings of Li *et al.* (2002), who showed that the CPT concentration was higher in young leaves with a high trichome density for all of the species examined (i.e., *Camptotheca lowreyana*, *C. yumarensis* and *C. acuminata*). It has also been reported that glandular trichomes represent one of the main sites of the biosynthesis and/or accumulation of some of the secondary metabolites that provide chemical defence against herbivores and pathogens (Bisio *et al.*, 1999).

In this study, CPT was also observed in the lumen of the xylematic vessels, though rarely, suggesting

that CPT, or a soluble derivative, is transported via the xylem to various parts of the plant. Chemical analysis (Lopez-Meyer *et al.*, 1994) has shown that 10-hydrossicamptothecin, which is more water-soluble than CPT, is present in all plant organs, yet in relatively low concentrations. It is probable that CPT, through hydroxylation, transforms into 10-hydrossicamptothecin so that it can be transported to various parts of the plant, where it would be reconverted into CPT. The transport of alkaloids via the xylem has also been observed in other species, specifically, *Atropa belladonna* (De Luca and St-Pierre, 2000), *Catharanthus roseus*, *Papaver somniferum*, and *Nicotiana tabacum* (St- Pierre *et al.*, 1999).

The accumulation of alkaloids in the form of crystals is not common, although it has been observed for the alkaloids sanguinarine and berberine in the species *Chelidonium majus* and *Coptis japonica*, respectively (Renaudin and Guerin, 1990).

Acknowledgments

This research was supported by funds (contribution no. C26F038227, year 2002) from the University "La Sapienza", Rome, Italy.

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