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ORIGINAL RESEARCH

Histochemical Localization of Essential Oils and Bioactive Substances in the Seed Coat of the Halophyte *Crithmum maritimum* L. (Apiaceae)

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Abstract Despite seeds and fruits of several halophytes being rich in essential oils and other bioactive substances, the histochemical characterization of these compounds has not received much attention. The aims of the present investigation were to localize the essential oils and the bioactive substances in the seed coat of the oilseed halophyte *Crithmum maritimum* L. Fruits were collected from the rocky coasts of Tabarka (NW of Tunisia, 36°57′ 12″ N, 08°45′18″ E). *C. maritimum* L. seed is shown to be surrounded by two envelopes: The first structure is a secretory envelope, consisting in about 20 canals. The second layer represents the endocarp. As revealed by fluorescence and light microscopy, the essential oils, *O*-dihydroxyphenols and flavonoids, known as bioactive compounds, were accumulated in the canals. The endocarp layer accumulated polysaccharides, *O*-dihydroxyphenols, and flavonoids. As a whole, these findings highlight the histochemical features and confirm the valuable quality of *C. maritimum* L. seeds.

Keywords Bioactive substances · *Crithmum maritimum* L. · Fluorescence microscopy · Histochemistry

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Introduction

Halophytes are naturally adapted to conditions with moderate to high soil salinity (Flowers and Colmer 2008). These plants accumulate high amount of biologically active substances that may play a role in their successful adaptation to such hostile environmental conditions (Ksouri et al. 2007, 2008). Halophyte species show many applications in folk medicine (Meot-Duros et al. 2008). For example, *Crithmum maritimum* L., *Eryngium maritimum* L., and *Cakile maritima* have been cited for their diuretic, antiscorbutic, digestive, and purgative properties (Davy et al. 2006; Meot-Duros et al. 2008). In *C. maritimum* L., the whole parts of the plant contain furanocoumarin, a substance with high antimicrobial activity and high antiradical activity (Ozçelik et al. 2004; Meot-Duros et al. 2008). This species also accumulates carotenoids, flavonoids, coumarins, tannins, and high amounts of vitamin C (Glowniak et al. 2006). Recently, Meot-duros and Magné (2009) reported that leaves of *C. maritimum* L are rich in chlorogenic acid.

The plant secretory structures are the site of synthesis and/or accumulation of biologically active substances (Ciccarelli et al. 2001). The seeds and fruits of Apiaceae contain coumarins, furanocoumarins, and flavonoids (Zobel and March 1993). Furthermore, these species produce volatile oils in specific secretory tissues (Corsi and Biasci 1998). These substances show antibacterial, antifungal, antitumoral, and spasmolytic activities (Ozçelik et al. 2004). For instance, the fruit of *C. maritimum* L. contains high amounts of essential oils and other bioactive substances that showed high antibacterial activity (Glowniak et al. 2006). Yet, the structural characterization and localization of these compounds remains largely unclear, as this aspect has not received much attention.

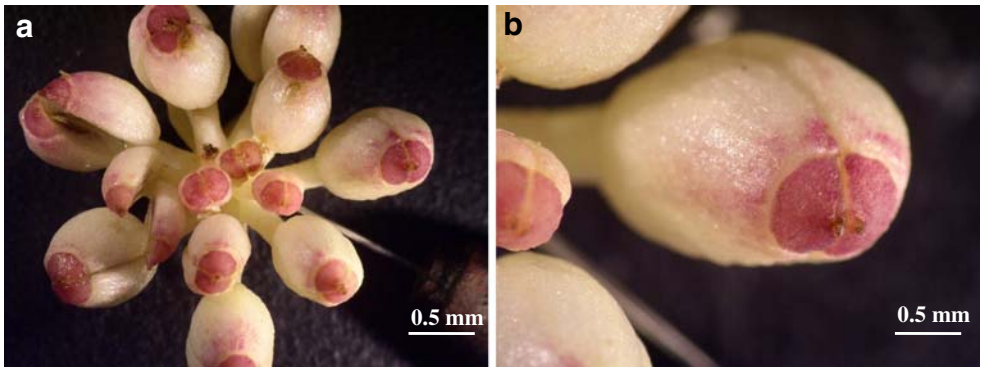

Fig. 1 Stereomicroscope micrograph of *C. maritimum* L. fruit. **a** Inflorescence. **b** A detailed view of mericarps. Note the high accumulation of anthocyanins

In a preliminary investigation, we showed that mature fruit of *C. maritimum* L. is composed of a spongy coat surrounding the seed. At full seed maturation, this spongy coat is easy to remove. However, a secretory layer and the endocarp layer remain firmly attached to endosperm and represent the seed envelope. Here, we focus on the secretory structure of the seed envelope of *C. maritimum* L. and the localization of the accumulated bioactive substances within this tissue.

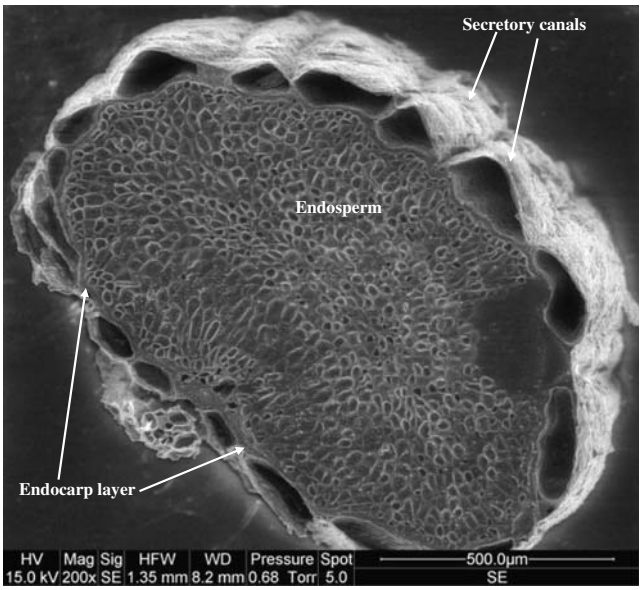

Fig. 2 SEM micrograph of a cross-section of *C. maritimum* L. seed, showing the secretory layer and the endocarp layer that surround the endosperm tissue

Materials and Methods

Fully ripened mericarps (fruits) of *C. maritimum* L. were collected in December 2007 from the rocky coasts of Tabarka (NW of Tunisia, 36°57′12″ N, 08°45′18″E). They were then dry-stored under laboratory conditions until experiments took place, in April 2008. After the spongy coats were removed, seeds were used for the microscopic study. Immature fruits were collected and immediately photographed with a stereomicroscope (type Leica).

Observations of free-hand sections of seeds were carried out by scanning electron microscope (SEM; type FEI Quanta 200). For light microscopy, free hand sections were stained with a 0.5% toluidine blue (TBO) solution, which stains acidic carbohydrates blue and phenolic compounds green (Egerton-Warburton 1998). $FeCl_3$ solution (1%) was used for *O*-dihydroxyphenols that stained dark brown or dark blue (Guerin et al. 1971). Stained sections were then observed under light microscope (type Olympus DX41).

For fluorescence microscopy, free-hand sections were prepared and stained with a 0.1% neutral red solution for lipids (Kirk 1970) or with a 0.1% $AlCl_3$ solution for flavonoids (Hienrich et al. 2002). A 0.5% TBO solution

Table 1 Summary of histochemistry of the *C. maritimum* L. seed envelope

Staining procedure	Target compounds	Coloration	Secretory canals	Endocarp
TBO	Polyphenols	Green	+++	+
	Polysaccharide	Blue	−	++
$FeCl_3$	*O*-dihydroxyphenols	Dark brown	+	+++
		Dark blue	+	++

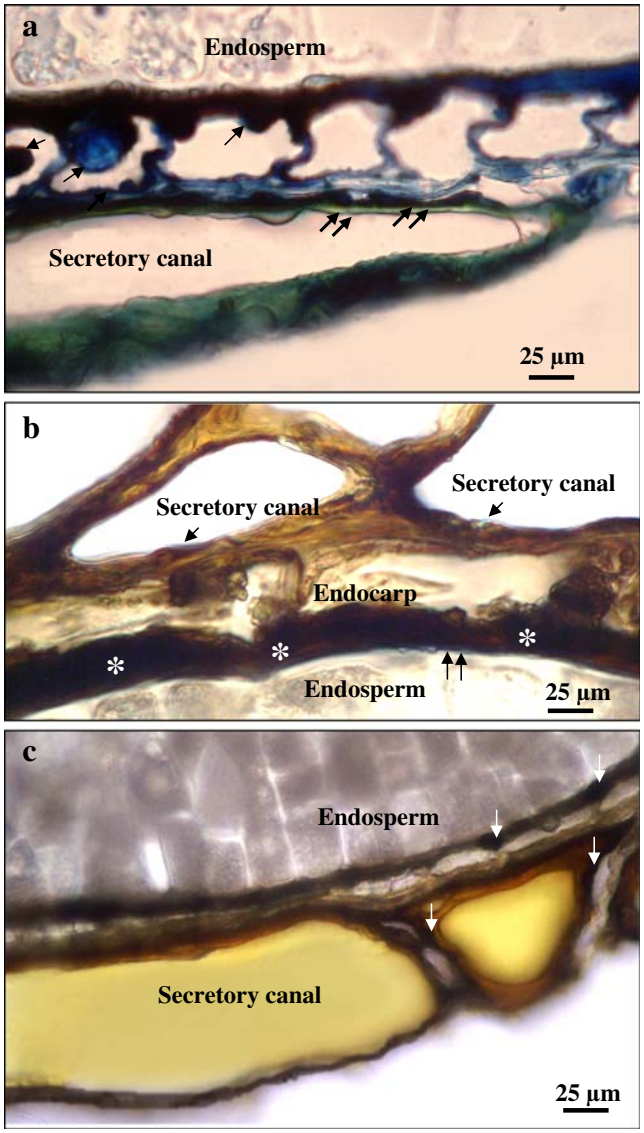

Fig. 3 Light microscope micrographs: **a** TBO-stained section showing a green coloration in the inner surface of the secretory layer (*double arrows*), a blue coloration of the endocarp layer, and a dark blue coloration at the interface between the endosperm and the endocarp. Note the presence of blue and black bodies (*arrows*). **b** $FeCl_3$-stained section showing the accumulation of the *O*-dihydroxyphenols in the endocarp (*asterisk*) and in the inner surface of secretory canals that appear as dark blue (*arrows*) at the edge of endosperm and the endocarp (*double arrows*). **c** $FeCl_3$-stained section showing the accumulation of the *O*-dihydroxyphenols in the endocarp and in the periphery of secretory canals that appear as dark blue deposit (*arrows*)

was also used for fluorescence microscopy; although this is not a fluorescent dye, it is used to quench the autofluorescence of carbohydrates and phenolic compounds, i.e., only the fluorescence of lipid was detected. Free-hand sections without staining were also prepared for autofluorescence. Sections were mounted in water on glass slides and observed under fluorescence microscopy (type Eurostar II, YG O301-0101-2) with blue light excitation. This microscope is equipped with a LED light source for epifluorescence microscopy in the range of 460–480 nm.

Results and Discussion

Stereomicroscope observation showed that *C. maritimum* L. pre-immature fruit is a schizocarp divided into two mericarps and highly accumulating anthocyanins, as revealed by the pink–red coloration (Fig. 1a, b). Each mericarp contained a single seed characterized by a brown coloration at full maturation. SEM observation of transversal sections of *C. maritimum* L. seed showed that the latter was surrounded by an external envelope composed of ca. 20 secretory canals and an endocarp layer enclosing the endosperm. Both layers constitute the seed envelope (Fig. 2). The number of the secretory canals is highly variable in the Apiaceae fruits, establishing for example at six in *Carum capticum* (Gersbach and Reddy 2002). In the Apiaceae, these structures are not only associated with essential oil production and accumulation (Sarafis et al. 1990) but also appear to be sites of synthesis of biologically active substances including coumarins, furanocoumarins, and flavonoids (Zobel and March 1993).

Staining with TBO revealed a green coloration in the inner surface of the secretory layer and a dark blue coloration in the endocarp layer, typical of polyphenols and polysaccharides, respectively (Table 1 and Fig. 3a). In the endocarp layer, black and blue spherical bodies were detected after staining with TBO (Fig. 3a). Hence, polyphenols and polysaccharides may be accumulated in the endocarp layer of *C. maritimum* L. fruit, suggesting the presence of specific glands or secretory cells in this envelope (Fig. 3a). $FeCl_3$-stained sections showed the presence of *O*-dihydroxyphenols in the endocarp layer and in the secretory layer, which was characterized by either

Table 2 Summary of the fluorescence of the *C. maritimum* L. seeds envelope

Staining procedure	Target compounds	Fluorescence	Secretory canals	Endocarp
Autofluorescence	Essentials oils	Yellow	+++	+
	Lignin	Blue	–	–
	Coumarin	Yellow	+	+
Neutral red	Lipids	Green or yellow	+++	++
$AlCl_3$	Flavonoides	Yellow	++++	++++

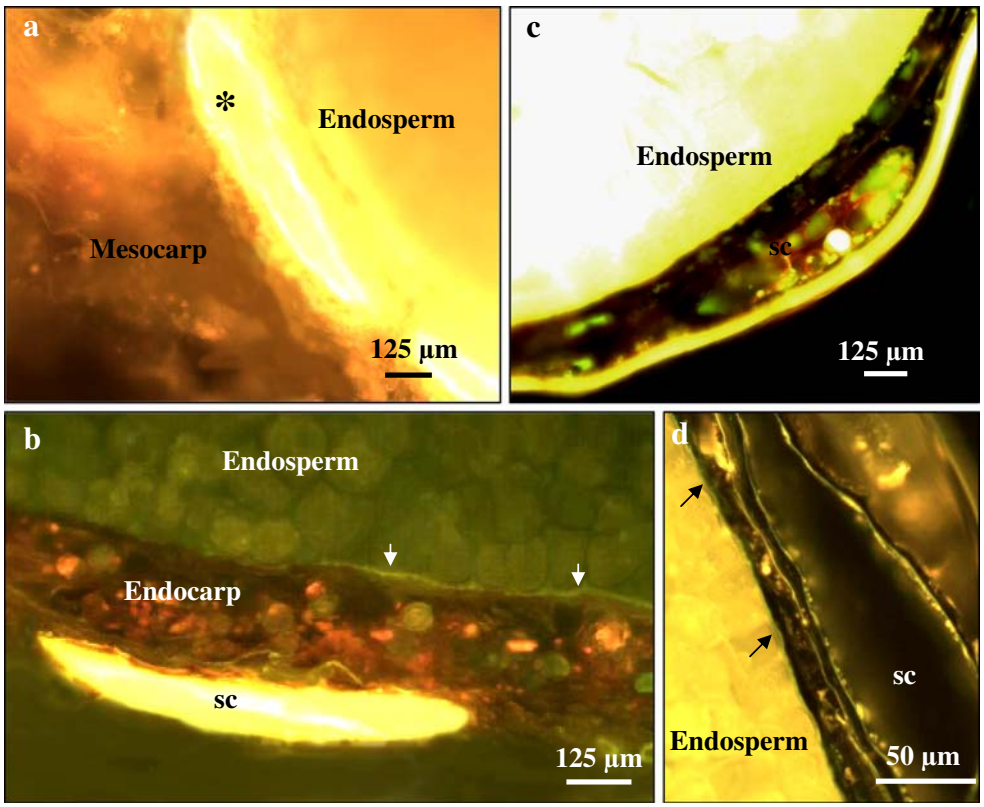

Fig. 4 Fluorescence micrograph of secretory canals (sc) and the endocarp layer. **a** Autofluorescence of immature fruit showing the high yellow fluorescence of secretory canals (*asterisk*). **b** Autofluorescence of secretory canals of mature seeds showing the accumulation of essential oils and showing the autofluorescence in the interface between the endosperm and the endocarp (*arrows*). **c** Micrograph of neutral red-stained section showing the accumulation of essentials oils in the secretory canals. **d** TBO-stained section showing a positive reaction in the intersection of endosperm and endocarp layers indicating the presence of polyphenols (*arrows*)

dark brown or dark blue coloration (Table 1, Fig. 3b, c). A blue coloration was also observed in the interface between the endocarp layer and the endosperm tissue (Table 1 and Fig. 3b) as well as in the inner surface of secretory canals (Table 1 and Fig. 3b). Our results tend to prove that seed envelopes of *C. maritimum* L. are rich in *O*-dihydroxyphenols. They also confirm previous reports on the accumulation of coumarins and tannins in *C. maritimum* L. (Glowniak et al. 2006).

The fluorescence microscope observation of secretory canals of either pre-mature or mature seed showed high yellow autofluorescence, characteristic of essential oils (Table 2 and Fig. 4a, b). Despite the fact that the occurrence of essential oils has been already documented in *C. maritimum* L. fruit (Glowniak et al. 2006; Oscan et al. 2006), this is the first report describing the localization of essential oils in the seed envelope of this species. The fluorescent light is mainly emitted by terpenoid compounds such as *p*-cymene and to a lesser extent by other sources such as flavones (Gersbach 2002). Oscan et al. (2006) found that the oil extracted from *C. maritimum* L. was rich in *p*-cymene. The neutral red, a lipid-detecting fluorochrome, was used to assess the presence of essential oils in the secretory canals, as revealed by high lemon yellow and green fluorescence (Fig. 4c). The two colorations are lipid-specific and are indicative of differences in the lipid composition (Kirk 1970; Bandyopadhyay and Hamill 2000). Although lipids are common reserves in the

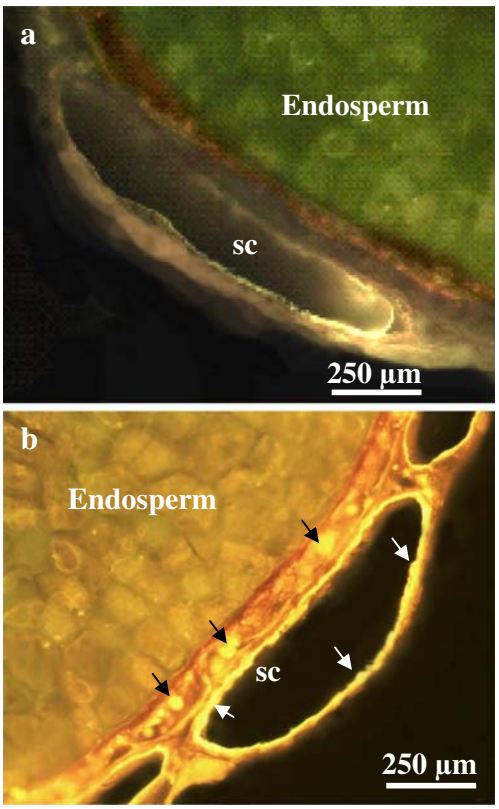

Fig. 5 Fluorescence micrograph of **a** nonstained section (control) and **b** $AlCl_3$-stained section showing a bright yellow fluorescence of deposit compounds in the inner surface of the secretory canals (sc) (*arrows*)

Apiaceae seeds, essential oils and triacylglycerols were accumulated in distinct compartments, such as the secretory canals and the seed endosperm (Glisic et al. 2007), which is consistent with our findings in *C. maritimum* L.

In the endocarp layer, a red fluorescence indicative of chlorophyll was observed (Fig. 4b). The autofluorescence showed a yellow green fluorescence at the limit of endocarp layers and the endosperm (Fig. 4b). Zobel and March (1993) showed the same fluorescence in the fruit of the Apiaceae *Daucus carota* and concluded that this fluorescence is due to furanocoumarins. Furthermore, when stained with TBO, this layer appeared to be black under fluorescence microscopy (Fig. 4d). This confirms that this layer was composed of phenolic compounds.

The sections stained with $AlCl_3$, a fluorochrome used for flavonoid detection, showed a bright yellow fluorescence as compared with nonstained sections (in the inner surface of secretory canals; Table 2, Fig. 5a, b). Furthermore, yellow fluorescent bodies were observed in the endocarp layer, indicating the presence of flavonoids. These findings agree with a previous report on the high accumulation of flavonoids in the different organs of *C. maritimum* L. (Glowniak et al. 2006).

In conclusion, the present study brought new insights regarding the structure and the localization of bioactive substances of *C. maritimum* L. in the seed envelope. Essential oils and polyphenols, mainly flavonoids, were detected in the secretory layer, while *O*-dihydroxyphenols, flavonoids, and polysaccharides were accumulated in the endocarp. Finally, this halophyte could be valorized as a potential source of natural antioxidant compounds, useful for medicinal applications.

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