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Research Article

Leaf Tissue Arrangement, Preliminary Phytochemical Investigation and Callus Induction from the Medicinal Hemi-parasite *Osyris alba* L.

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ABSTRACT

Osyris alba is a dioecious shrub used in traditional medicine for its bioactive secondary metabolites. It is used for its antiparasitic, antimicrobial and anti-bleeding properties. In the present study the leaf tissue arrangement, histochemsitry and callus formation of *Osyris alba* L. were investigated. Leaves (fresh and fixed) were observed by light, polarized oight and electron microscopy. Fresh stem was also observed by light microscopy. The sub-cellular localization of secondary metabolites was detected. Furthermore, in vitro production of its secondary metabolite, by using cell cultures, was also preformed. The compact leaves are amphistomatic, with a single layer of epidermal cells and contain idioblasts cells with crystals. They react positively to histochemical reagents for the major groups of secondary metabolites. In the preliminary histochemical screening, in vitro cultured cells also gave positive results. In conclusion, the medicinal nature of the plant is attributed to its phytochemical profile. Plant cell culture technologies can be used for the production of its bioactive molecules.

Keywords: leaf anatomy, histochemistry, Mediterranean, medicinal plant, callus.

INTRODUCTION

Osyris alba is a dioecious shrub of the Santalaceae family (order Santalales), widely spread in all territories of the Mediterranean basin, in Southeast Asia and in different localities of Turkey, Jordan and Palestine[1,2]. The woody members of the Santalaceae are divided into two groups according to their wood structure[3,4]. There are more than 400 species in the family, mainly of root (hemi) parasites, including *O. alba*, *Osyris lanceolata* and *Osyris quadripartita*[5]. However, within the sandalwood order Santalales, also exists free-living plants, such as those in the family Erythropalaceae[6]. The stem is woody, brown or dark green, reaching 30 to 150 cm in height. The plant has very small flowers (1 or 2 mm) which are either hermaphroditic or unisexual, with four yellow-green tepals and four stamens. The fruits are also small, red, fleshy drupes, 4 to 6 mm in diameter (Fig. 1.1). An interesting feature of this species, which is obviously a photosynthetic plant, is that it functions as a hemiparasite, taking advantage of haustoria formed on the roots. Haustoria tap into the roots of the host plant and extract their sap. The parasite has a wide host range of wild and cultivated species including forest trees, perennial woody herbs and fruit trees. It is said to produce long branches on *Cupressus sempervirens* and less developed branches under citrus trees (mainly grapefruit)[7]. Other common hosts are *Acacia cyanophylla*, *Casuarina equisetifolia*, *Ficus carica*, *Olea europaea*, *Prunus domestica*, *Pistacia palaestina*, *Thymus capitatus* and *Sarcpoterium spinosum*[5]. Previous phytochemical studies investigated the aqueous methanol and butanol extracts and revealed that they contain carbohydrates, amino acids, fatty acids, alkaloids (among them are osyrisine, quinolizidine and pyrrolizidine), flavonoids (e.g salvigenin, pachypodol, kumatakillin), triterpenoids (ursolic acid, oleanolic acid) and polyphenols (catechin, and catechin-3-O-a-L-rhamnopyranoside)[8,1]. In traditional medicine, *O. alba* is used for its antiparasitic properties against *Entamoeba histolytica* and *Giardia intestinalis*[1]. The antiamoebic and antigiardial properties are attributed to its phytochemical profile. Apart from *O. alba*, there are also other species in the genus *Osyris* which are known for their pharmaceutical uses. For instance, *Osyris wightiana*, whose bark infusion is administered to women after delivery to inhibit bleeding. It is used as an emetic, as well[9]. Roots of *O. lanceolata* are used in venereal diseases, menorrhagia, infertility and for their styptic effects on wounds[10]. These two species have also antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae* and *Candida albicans*[9,10]. Additionally, another species, *O. quadripartite* is reported to treat malaria[11]. In order to produce in vitro *O. alba* bioactive compounds, plant cell culture could be used as an expression system. Nowadays, the utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention[12-14]. By using different concentrations of hormones and changing culture conditions, the accumulation of secondary metabolites could be increased[15]. Although we there are various researches for

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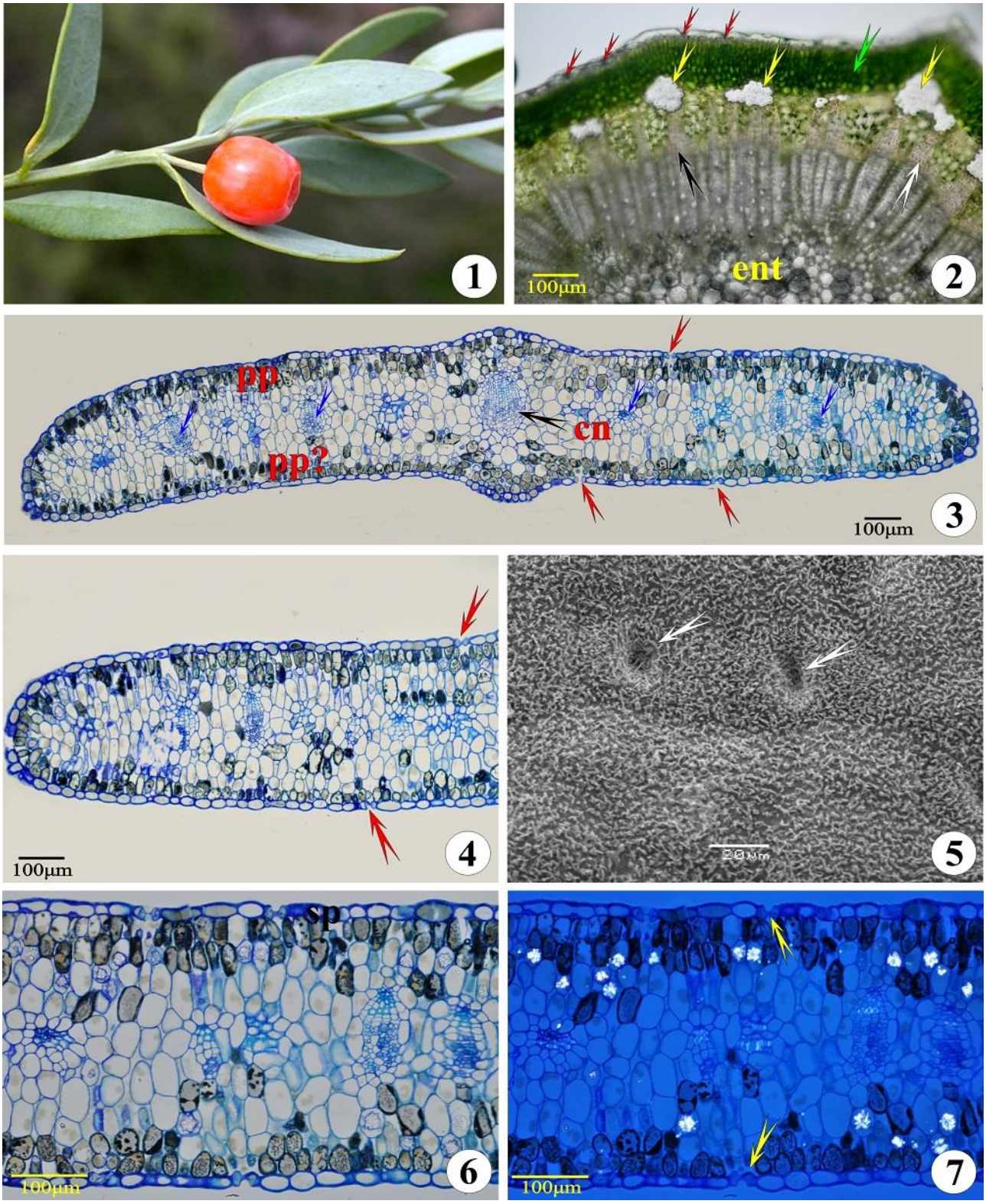

Figure 1: LM and SEM micrographs of the leaf and stem of *O. alba*. **(1)** The small leaves and the fruit **(2)** Cross section from the stem, green arrow indicate photosynthetic parenchyma, yellow arrows indicate sclerenchyma, red arrows indicate stomata, white arrow indicates phloem, black arrow indicates cambium. Pith is obvious (ent). **(3)** Cross-sections from plastic-embedded mature leaves. The adaxial layer of palisade parenchyma and epidermal cells accumulate osmiophic compounds. Conductive bundles (blue arrows), central nerve (cn), palisade (pp) and stomata (red arrows) are indicated. **(4)** Osmiophilic compounds within the epidermal cells and mesophyll can be observed. **(5)** SEM micrograph. Epicuticular wax coat is observed. **(6)** Crystals and a substomatal chamber can be seen. **(7)** Crystals under polarized light microscopy.

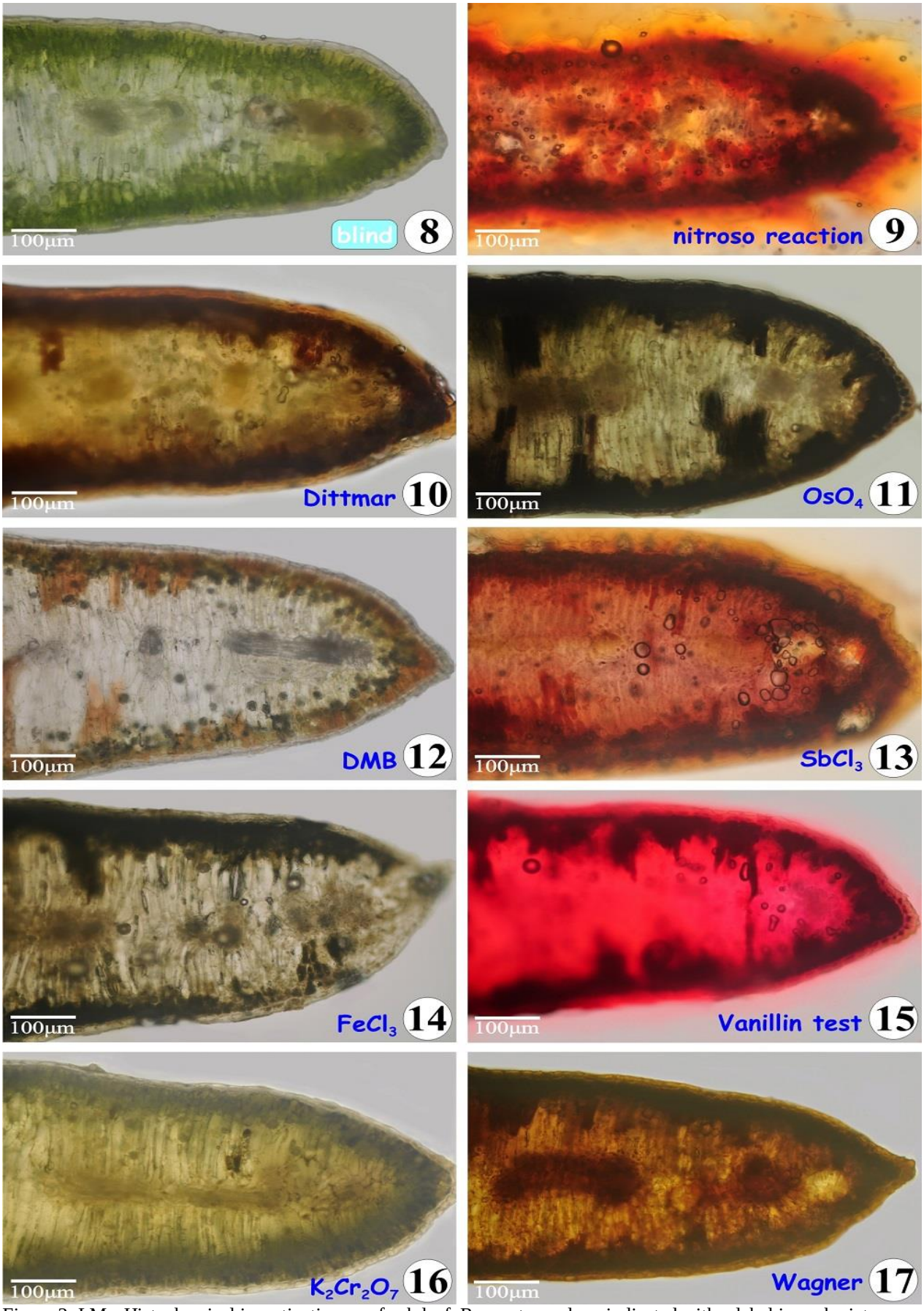

Figure 2: LM - Histochemical investigations on fresh leaf. Reagents used are indicated with a label in each picture.

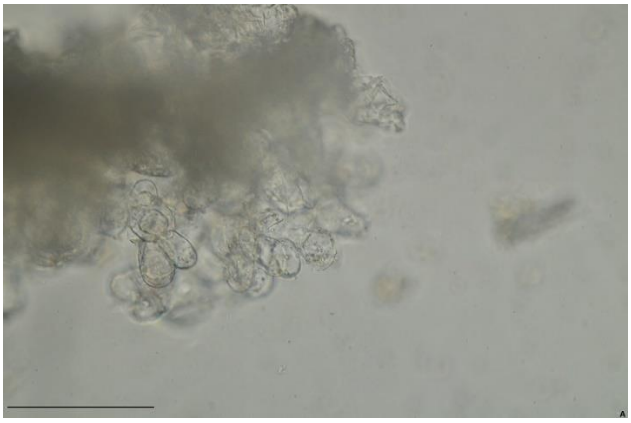

Figure 3: LM - Callus from *O. alba*. Callus cells, no stain added. Scale bars 100 µm.

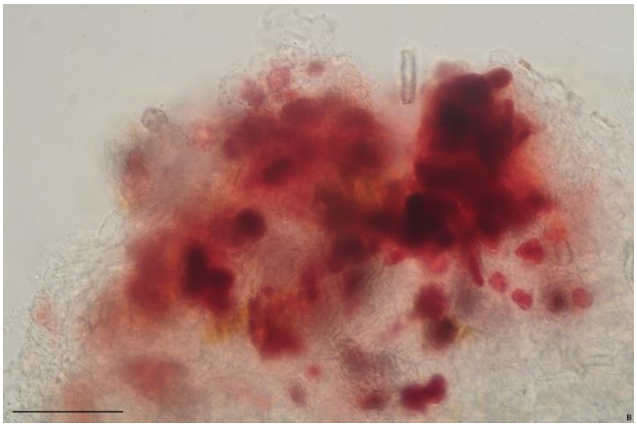

Figure 4: Callus cells treated with vanillin/HCl for flavonoids. Stained compounds escaped from the broken cells and the background appeared coloured. Scale bars 100 µm.

the traditional uses of the plant in remedies, there is no data on the anatomy of the leaves and sub-cellular localization of the secondary metabolite. In this paper the results of leaf tissue arrangement and a preliminary histochemical study (both on embedded and fresh leaf) of this peculiar species are presented. Furthermore, callus induction was performed in order to compare the nature of secondary metabolites to those biosynthesized from the plant.

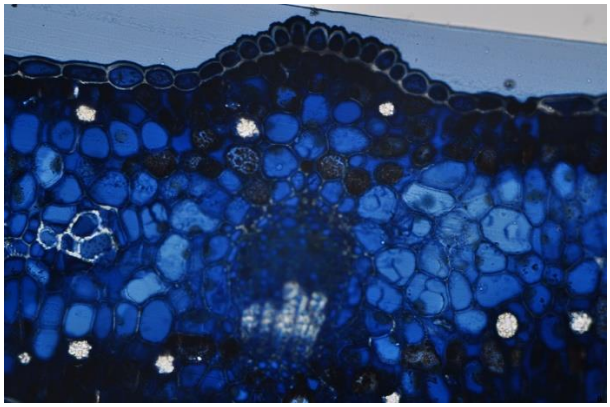

Figure 5: LM - Epoxy embedded leaves stained with Sudan black. Scale bars 100 µm.

MATERIALS AND METHODS

Plant Material

Young and mature leaves of *O. alba* plants growing wild in a natural formation east of Athens metropolitan area (37º57´76 N, 23o48´08 E, 365m elevation), were collected in late May 2013 and 2014. Identification of the plant was made by Prof. N.S. Christodoulakis, Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens and a voucher specimen was deposited at the University Herbarium. Some of the leaves were cut in to small pieces (1 x 1 mm) and fixed in phosphate buffered 3% glutaraldehyde (pH 6,8) at 0 ºC for 2 hours. A few larger pieces were dehydrated in graded acetone series, critical point dried, coated with gold and viewed with a JEOL JSM-6360 Scanning Electron Microscope. The rest of the tissue was post fixed in 1% osmium tetroxide in phosphate buffer, dehydrated in graded ethanol series and embedded in Durcupan ACM (Fluka, Steinheim, Switzerland). Semithin sections obtained from a LKB Ultrotome III, were placed on glass slides and stained with 0.5 toluidine blue O (in 1% borax solution), as a general stain, for light (LM) and polarized light microscopic observations. For scanning electron microscopy (SEM), the specimens, after fixation and dehydration, were critical point dried in a Balzers CPD 030 device and then coated with carbon in a vacuum evaporator[16,17]. Fixation was repeated, one year later, in early May 2014, and the embedded tissues were sectioned and observed, for cross-checking the results.

Histochemistry

Semithin sections of plastic embedded tissue (pet), were stained with: (a) saturated Sudan black B solution in 70% ethanol for the histochemical detection of lipids; (b) saturated alcian blue solution in 3% acetic acid for the detection of polysaccharides and (c) 1% aniline blue black in 70% acetic acid for the detection of proteins. Fresh leaves were sectioned by hand or with a cryotome (Leica CM1850, Germany) at -10 ºC and stained with traditional histochemical reagents: (a) osmium tetroxide for unsaturated lipids; (b) concentrated H_2SO_4 for sesquiterpenes; (c) vanillin/HCl for flavonoids; (d) antimony trichloride ($SbCl_3$) for terpene containing steroids; (e) Wagner's reagent for alkaloids; (f) Dittmar's reagent for alkaloids; (g) iodine – iodide solution for alkaloids; (h) potassium bichromate for tannins; (i) nitroso reaction for catechol tannins; (j) ferric chloride for polyphenols; (k) alcoholic vanillin / HCl (vanillin test) for phenolic compounds; (l) 4-nitrosophenol in concentrated H_2SO_4 for monoterpene phenols; (m) DMB (di methoxy benzaldehyde or veratral aldehyde) for phenolic tannin precursors. All stains were matched by controls[16,17]. All specimens were viewed with an OLYMPUS CX41 optical microscope. Original light micrographs were recorded digitally, using a Nikon D5000, 12.3 megapixel camera.

In vitro cell cultures

Pieces of leaf tissue were treated for in vitro cell culture. They were washed with tap water, counter washed with

Table 1: Histochemical preliminary screening on leaf (ND= Not Detected)

No		Secondary metabolite	Histochemical reagent	Result	
				Leaf	Callus
1	embedded	General stain	Toluidene Blue 'O'	+	Not executed
2		Polysaccharides	Alcian blue	ND	Not executed
3		Proteins	Aniline blue black	ND	Not executed
4		Lipids	Sudan black B	ND	Not executed
5	fresh	Lipids (unsaturated)	OsO_4	+	+
6		Terpenes	Concentrated H_2SO_4	+	+
7		Flavonoids	Vanillin / HCl	+	+
8		Steroids	Antimony trichloride	+	+
9		Alkaloids	Wagner's reagent	+	+
10			Dittmar's reagent	+	+
11		Phenols	Ferric chloride	+	+
12			Vanillin test	+	+
13			4 - nitrosophenol	+	+
14		Phenolic tannin precursors	Dimethoxy-benzaldehyde (DMB)	+	+

distilled, sterilized water for 5 min, rinsed with 70% ethanol for 30 s, immersed in 1% aqueous solution of hypochlorous acid (HOCl) and then rinsed three times with distilled, sterilised water. These pieces of leaf tissue were then transferred in 10cm Ø Petri dishes containing 1.1 g/500mL of Murashige and Skoog medium[18] ((0.8% agar, 2% sucrose, 0.2 mg/ml of culture medium synthetic auxin (2,4-dichlorophenoxyacetic acid) and 0.2 mg/ml of the cytokinin benzylaminopurine (BAP))[19,20]. They were incubated at 12 h daylight conditions, for 2 weeks, at 24 ◦C. All specimens were viewed with an OLYMPUS CX41 optical microscope. Original light micrographs were recorded digitally, using a Nikon D5000, 12.3 megapixel camera. SEM images are digital records from the instrument's built in camera.

RESULTS

Leaf morphology

Leaves are linear, lanceolate and rounded-at-the-tip. Their size is 15–35 mm long and 1–5 mm wide. They are alternate and smooth. The stipule is absent.

Leaf tissue arrangement

The leaves are rather compact. There is a single layer of epidermal cells which produces and accumulates numerous osmiophilic compounds. The external, periclinal cell wall of the adaxial epidermal cells seems to be lined with a rather thin layer of cutin (Fig. 1.3). The continuity of the epidermal tissue is often disrupted by stomatal complex. They can be found at both surfaces (adaxial and abaxial epidermis), making the leaf amphistomatic. Stomata seem a bit submerged in the epidermal tissue. They possess a compact palisade parenchyma adaxially and abaxially (amphipleurous leaf structure) while the spongy tissue seems to be to be confined in the middle of the leaf or missing. Below the epidermal tissue and just under the stomatal openings, distinct intercellular spaces appear, probably to facilitate aeration. The sub-epidermic photosynthetic parenchyma had high columnar cells with a dense cytoplasm which accumulated secondary metabolites. The rest layers of palisade are composed of rather shorter cells which rarely accumulate osmiophic compounds (Fig.1.4-5). Within the mesophyll cells, the osmiophillic content seems of phenolic nature while many cells accumulated large droplets of hydrophobic material. Additionally, numerous conductive bundles, composed of xylem and phloem, were observed. In the middle of the leaf, a large, central nerve was found, surrounded by a bundle sheath (Fig.1.3). Various different sizes of idioblast cells which host crystals were easily demonstrated by polarized light. These crystals are not artifacts since they were observed with polarized light both in thin sections of fresh leaves and semi-thin sections of plastic-embedded tissue (Fig.1.7). The topography of the leaf surface was studied further by using the SEM. The cuticle forms a waxy layer of relatively uniform thickness over the abaxial and adaxial surfaces and wax morphologies were observed. Stomata were a bit sunken (Fig. 1.5). Cross sections of fresh stem were also studied (Fig. 1.2). The surface of the young stem is glabrous, smooth and green. The mature bark is green to grey. The epidermis, the outermost covering of the stem, was easily observed. It consisted of a single layer of isodiametric, compactly arranged cells. Externally, it was covered by cuticle and epicuticular waxes. Below the epidermis, photosynthetically active parenchymatic cells were located. As seen in cross sections of the stem, groups of sclerenchyma fibres, with thick lignified secondary walls, gave structural support to the plant. Cambium and cambial rays were also observed. Periclinal divisions of the vascular cambium resulted in concentric layers of secondary phloem and xylem.

Callus and leaf Histochemical Investigation

Histochemical reactions were conducted on semi-thin sections from epoxy embedded and fresh leaves material using the proper reagents for each type of tissue. All sections of fresh leaves reacted positively with the histochemical reagents mentioned in Table 1 and Fig. 2. In tissue cultures (Fig. 3), calluses developed from leaf pieces after three weeks. Their cells are rather rod-shaped and virtually transparent prior to staining (Fig. 3). Cultured cell mass also gave positive reactions to the histochemical

reagents. The results are given in Table 1 and Fig. 4 (van/HCl). On the contrary, in epoxy embedded leaves, none of the specific histochemical reagents used gave positive reaction, thus no one of the studied macromolecules was detected (Fig 5).

DISCUSSION

The paper describes the leaf structure and sub-cellular localization of secondary metabolites in the medicinal plant *O. alba*. An additional histochemical screening was performed on plant tissue culture. The leaf is rather compact with many layers of palisade parenchyma. The abundance of this tissue is also reported in genus *Acanthosyris*, also member of the family Santalaceae[21]. Similar mesophyll arrangement has also been reported in previous studies for some other species of the family[22,23]. Stomata appear on both surfaces. Although rare among the Mediterranean plants, amphistomaty is considered as a major xeromorphic character for shortening the distance of CO_2 diffusion to mesophyll cells[24,25]. Additionally, the extra layers of palisade tissue are believed to increase the CO_2 absorbing surface of the mesophyll[26]. According to Metcalfe & Chalk[27], amphistomatic leaves are common in Solanaceae, although Cosa de Gastiazoro[23] described hypostomatic leaves in some species of the family. SEM microscopy revealed epicuticular wax which are common among most of the Mediterranean plant species[28]. Their main functions are to decrease surface wetting, moisture loss and reflect ultraviolet light. These structural characteristics give an additional advantage to *O. alba* to survive under the stressful Mediterranean conditions[24]. The presence of crystals in mesophyll cells has never before been reported for this species. Rajni was the first who mentioned the existence of crystals within the genus[29]. Crystals have also been observed in another common Mediterranean medicinal plants, such as *Ficus carica*[28] and *Urginea maritime*[30]. The presence of crystals is certainly not detrimental to the plant. Physical and chemical conditions, such as temperature, pressure, pH, and ion concentration, may affect crystal growth, habit, and properties but the precise controlling mechanism for crystal formation in plants is still unknown[31]. Generally, crystals in plants are formed by three minerals, calcium carbonate, calcium oxalate and silica[32]. Crystals might may have various roles in the plant. For instance, they can function as deterrents to herbivores, play structural roles, be important for pollen function while others are reported to be poisonous to humans, causing oral irritation and inflammation[33]. Sections of fresh and plastic embedded tissue were histochemically investigated for lipids, proteins, sugars and the accumulation of secondary metabolites by using specific histochemical reagents. According to the results of the preliminary phytochemical screening, leaves of *O. alba* produce a variety of secondary metabolites. Phenolic compounds and tannins were traced within the cells of the mesophyll tissue, along with terpenes. Plant tissue cultures also gave positive results to histochemical reagents. Our results are in agreement with similar researches which also compared the nature of secondary metabolite biosynthesized in plant to metabolites produces in *in vitro* cultures[28]. The biosynthesis and accumulation of secondary metabolites represent a chemical interface between plants and the environment and therefore, their synthesis is affected by environmental conditions[34]. Since *O. alba* is growing under the stressful of Mediterranean climate, it is expected to accumulate various secondary metabolites.

CONCLUSIONS

Specific anatomical adaptions and biosynthesis of secondary metabolites enable *O. alba* to withstand the unfavorable environmental conditions. The production of those bioactive molecules, apart from having a phytoprotective role in the plant, can be used in biotechnology, in plant cell culture, as an alternative source for the production of high-value secondary metabolites. This is the first report which gives a detailed description of leaf tissue arrangement, histochemical profile and callus induction of *O. alba*.

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