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Effects of ozone on the foliar histology of the mastic plant (*Pistacia lentiscus* L.)

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"Capsule": *Ozone causes alterations in the mesophyll, the conductive tissue and the secretory channels of the mastic plant (Pistacia).*

Abstract

An open-top chamber study was conducted to investigate the tissue and cellular-level foliar effects of ozone (O_3) on a Mediterranean evergreen species, the mastic plant (*Pistacia lentiscus* L.). Plants were exposed at three different O_3 levels, and leaf samples were collected periodically from the beginning of the exposure. Although no visible foliar injury was evident, alterations of the plastids and vacuoles in the mesophyll were observed. Senescence processes were accelerated with an anomalous stacking of tannin vacuoles, and a reduction in the size and number of the chloroplasts. Overall, most of the modifications induced by O_3 were consistent with previously reported observations on deciduous broadleaf species, with the exception of alterations in the cells covering the secretory channels, reported here as a new finding. Comments on the feasibility of using microscopy to validate O_3 related field observations and subtle foliar injury are also given.

Keywords: Ozone; Foliar injury; Leaf anatomy; Mediterranean species; Mastic plant; *Pistacia*; Spain

1. Introduction

At the present time, ozone (O_3) is considered to be the most important air pollutant worldwide (Krupa et al., 2001). During the past decades, a global increase in the lower troposphere O_3 levels has been observed over the Northern Hemisphere. This has been attributed primarily to the increases in anthropogenic O_3 precursors (Volz and Kley, 1988). The Mediterranean Basin is one of the critical areas for photo-oxidant formation (Millán et al., 1992). It is regarded as a large photochemical reactor where intense solar radiation, high temperatures and the particular re-circulation dynamics of the polluted air masses favour the formation and accumulation of O_3 (Millán et al., 1996, 1997, 2002; Sanz and Millán, 1998). As a consequence, O_3 concentrations reach phytotoxic levels (Bussotti and Ferretti, 1998; Fumagalli et al., 2001; Reinert et al., 1992; Sanz and Millán, 1998, 2000; Velissariou et al., 1992).

Visible foliar injury is one of the most striking effects of O_3 on many native plant species in central and southern Europe (Cozzi et al., 2000; Gimeno et al., 1992; Innes et al., 2001; Novak et al., 2003; Sanz et al.,

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2001a,b; Skelly et al., 1999; VanderHeyden et al., 2001). In that context, there are many studies on the effects of O_3 on conifer and deciduous broadleaf species, but few on Mediterranean evergreens. A fumigation experiment with the dominant Mediterranean sclerophyllous helm oak (*Quercus ilex*) showed that it is tolerant at realistic O_3 concentrations, probably due to its morphology, anatomical structure and ecological adaptation (Manes et al., 1998). In contrast, others found visible foliar injury symptoms (dark pigmented stipples), and a reduction in stem diameter in *Q. ilex* subsp. *ballota* (Inclán et al., 1999). Ozone effects have been also reported on other Mediterranean species such as *Arbutus unedo*, *Ceratonia siliqua*, *Laurus nobilis*, *Olea europaea* subsp. *sylvestris*, *Phyllirea latifolia*, and *Quercus coccifera*, including impairment of net photosynthesis, increased anti-oxidant levels, and reductions in stem growth (Elvira et al., 2003, Inclán et al., 1999; Paoletti et al., 2003). Ozone-like visible injury has been observed in a few evergreen species in the field (Cozzi et al., 2000; Sanz et al., 2003), and has also been induced in seedlings under controlled conditions in Open-Top Chambers (OTCs) and Continuous Stirred Tank Reactors (CSTRs) (Skelly et al., 1999; Sanz and Millán, 2000; Sanz et al., 2001a,b, Orendovici et al., 2003). Some of the symptomatic species found in those fumigation experiments were *C. siliqua*, *Fraxinus ornus*, *Fraxinus angustifolia*, *Pistacia lentiscus*, and *Pistacia terebinthus*, although highly variable in their sensitivity.

Compared to the cellular-level effects of O_3 on conifers (Barnes et al., 1999; Dalstein et al., 2002; Soda et al., 2000), and many deciduous broadleaf species such as *Betula pendula*, *Fagus sylvatica*, *Fraxinus excelsior*, *Prunus serotina* and *Sorbus aucuparia* (Günthardt-Goerg et al., 2000; Oksanen et al., 2001; Pääkkönen et al., 1998), very little information is available on evergreen Mediterranean species. Bussotti et al. (2003) reported some ultra-structural changes in *A. unedo* following O_3 fumigation, including an increase in thickness of the cuticle, degeneration of the cytoplasm in the epidermal cells and tannin deposition on the outer primary walls of those cells.

The present study has been carried out in connection with the surveys of visible injury in native plants, launched by the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP-Forests) (Sanz et al., 2003; http://gva.es/ceam/ICP-forests). It is the first in a series on histological and cytological effects of O_3 on plants in the Mediterranean Basin. *P. lentiscus* (mastic plant, lentisc) is distributed throughout the entire Mediterranean basin, being characteristic of the native shrub land (with *Q. cocciferae–Pistacietum lentisci* Br.-Bl.). It is an evergreen bush of 1–2 m height, although it can grow up to 6–7 m under favourable conditions. It is rich in resin (mastic) and in tannins (Costa, 1999). Mastic is used in chewing gum. The berries are used for making perfume and sometimes sweets or liqueurs.

2. Materials and methods

2.1. Experimental site

The study was conducted at "La Peira" (Benifaió, 39°16′14.8″N, 00°26′59.6″W, 30 m MSL), 20 km South of Valencia (eastern Spain), in a rural area with no important air pollutant sources in the vicinity (Sanz, 1996).

2.2. Plant material

P. lentiscus saplings were obtained from a regional nursery (Viveros Todolí, Alicante) and were kept in filtered air for 14 months before the exposures. The saplings were grown in 7 l containers filled with 60% peat moss, 20% coco-peat, and 20% sand (pH ~7.0). A slow release fertilizer was incorporated (Osmocote plus, N:P:K 15:12:13 + additional micronutrients). Plants were irrigated regularly to avoid any drought stress.

2.3. Ozone exposures

The experiment was conducted with three NCLAN (National Crop Loss Assessment Network, US.EPA) type Open-Top Chambers (OTCs). Air quality inside and outside the chambers was continuously measured (sharing the monitoring instruments between treatments, in set time cycles) with ozone (Dasibi 1008-AH, Environmental Corp.), and nitrogen oxides' (Dasibi 2108, Environmental Corp.) monitors, calibrated periodically. Additional metereological data (e.g. temperature, precipitation, wind direction and speed) were also recorded. Saplings were exposed to three different treatments: charcoal filtered air (CF), non-filtered air + 40 ppb (NF + 40), and non-filtered air + 80 ppb (NF + 80) O_3. Since during the previous years several lentisc saplings were also grown in the ambient air and its O_3 levels did not result in visible foliar injury symptoms, a chamber-less, ambient air treatment was not established. Plants were fumigated from 29 May to 7 November 2002, 8 h a day, from 10:00 to 18:00 h, 7 days a week. Ozone was generated from oxygen using a high-voltage electrical discharge generator (SIR S.A.). Leaf samples for microscopy were collected before exposure (20 May 2002), and then weekly after the exposures started, except at the end of the exposures, when sampling was done once every 2 or 3 weeks.

In addition to daily (24 h) and 12 h (8:00–20:00 h CET) mean O_3 concentrations, the accumulated O_3

exposure over a threshold of 40 ppb (AOT40, Fuhrer et al., 1997) was also calculated from hourly averages, during daylights hours with solar radiation >50 W m^{-2} (Table 1).

2.4. Microscopy methods

Leaf samples were fixed in situ with FAA (formyl acetic alcohol). After washing with a 0.1 M phosphate buffer (pH 7.4), they were dehydrated by means of an ethanol series. Some of the samples were then embedded in paraffin wax (Histosec, Merck), with a melting point of 56–58 °C; isoamyl acetate was used as an intermediate solvent between ethanol and the paraffin. The infiltration time was 30 min and the temperature was 60 °C. The resulting blocks were then cut in 8 μm sections with an Anglia Scientific microtome and stained with safranin and fast green (Johansen, 1940).

Other samples were embedded in LR-White medium grade acrylic resin (London Resin Co.). The sectioning of those blocks was done with a Sorvall MT 5000 ultramicrotome (Knifemaker, Reichert-Jung) provided with special glasscutters (45°) (Leica 6.4 mm Glass Strips). This microtome allowed for semi-thin sections (1.5 μm). Those samples were stained with toluidine blue (1%).

Sections were observed and photographed with an Olympus Provis AX 70 brightfield microscope fitted with an Olympus Camedia C-2000 Z camera. Additional samples to be observed through scanning electron microscopy were dehydrated in an ethanol series and dried to critical point in a Denton DPC-1 apparatus, coated with a gold–palladium mixture using an Edwards S-150 marker and observed under a Hitachi S-500 scanning electron microscope (University of Valencia Electron Microscope Service).

3. Results

3.1. Modifications in the anatomy

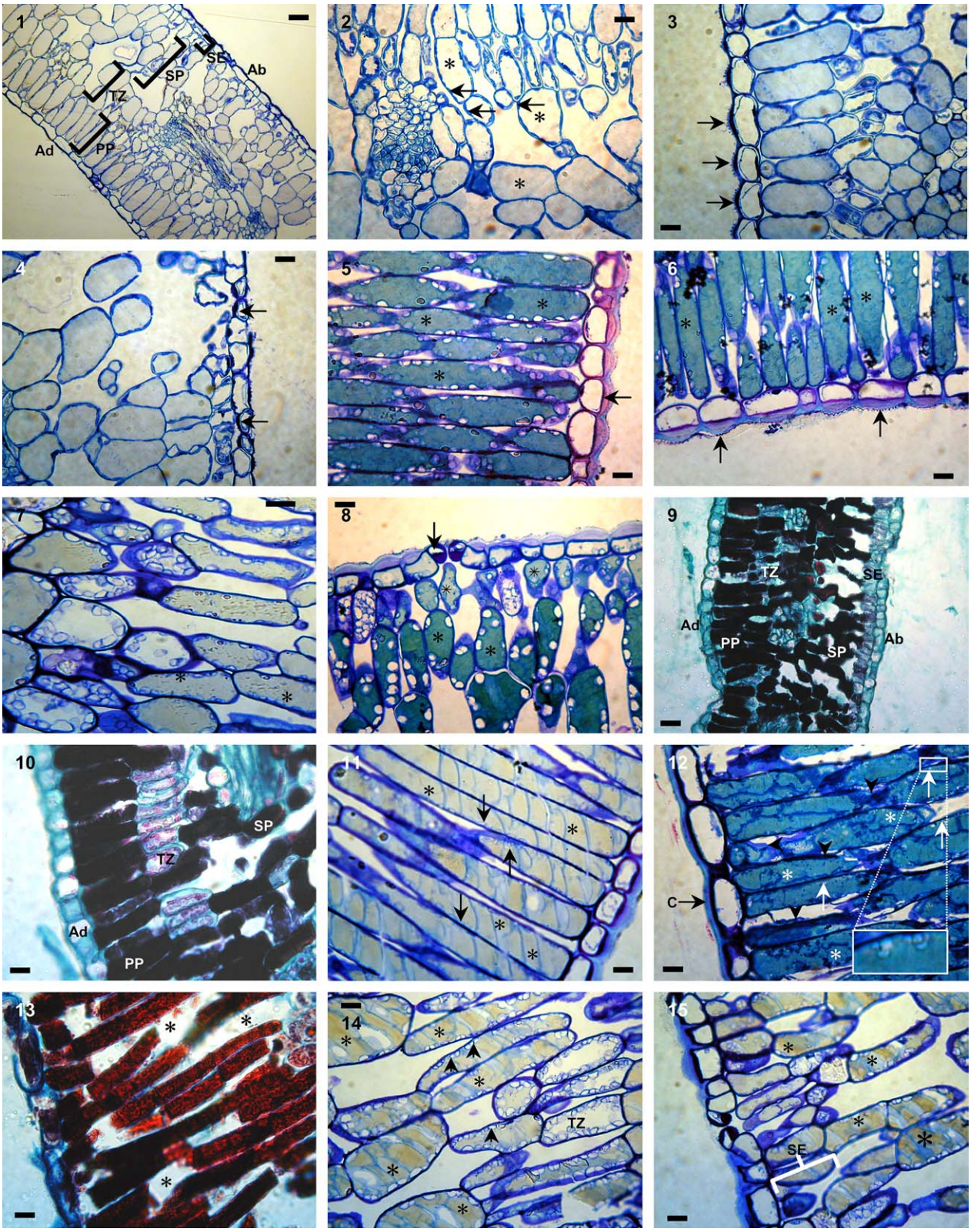
3.1.1. Effects of leaf age

Over time, natural senescence processes affected the anatomy of the leaves exposed to sub-ambient levels of O_3 (CF). Clear differences could be seen between young and mature leaves. In young leaves (Figs. 1–4, semi-thin cross sections 1.5 μm thick), a single layer of palisade parenchyma (PP), a transition zone and a spongy parenchyma with few intercellular spaces were present. The plastids in all the parenchyma cells were small and almost free of starch (Fig. 2). The cells showed a very thin primary wall and a transparent vacuole content. The epidermis (Ad and Ab, of the adaxial and abaxial sides of the leaves, respectively) is covered by a very thin although well ornamented cuticle (Fig. 3). There was a slight difference between the sub-epidermal layer (SE) and the spongy parenchyma layer (SP), the cells of the former being smaller in size. The transition zone (TZ) was hardly distinguishable. The stomata scarcely developed upper walls, and the cytoplasm of the occlusive cells occupied more than 50% of the cellular volume (Fig. 4).

In mature leaves (Figs. 5–8, semi-thin cross sections 1.5 μm thick; and Figs. 9, 10, paraffin-embedded 8 μm thick cross sections) the palisade parenchyma was structured in two layers, both made up of cells with vacuoles. The vacuoles showed a very dense content, most probably tannins, with an affinity for safranin (Figs. 9, 10). The plastids were large, spherical and full of starch (Fig. 5). The cell wall was thin. The cells in the transition zone were intermediate in size, without dense vacuolar content and with abundant, large plastids (Fig. 7). The epidermis developed a much thicker cuticle, with less ornamentation than in young leaves (Figs. 5, 6). The spongy parenchyma followed the same pattern

Table 1
AOT40 (ppb h, for daylight hours with solar radiation >50 W m^{-2}), 12-h (from 8:00 to 20:00 CET) and daily (24-h) means for the successive plant sampling days since the beginning of the fumigations (the first plant sampling (20 May 2002) was carried out before the beginning of the fumigations on 29 May 2002; CF = charcoal filtered and NF = non-filtered air)

Plant sampling date	AOT40 (ppb h)			12-h mean (ppb)			24-h mean (ppb)		
	CF	NF + 40	NF + 80	CF	NF + 40	NF + 80	CF	NF + 40	NF + 80
20/05/02	–	–	–	–	–	–	–	–	–
05/06/02	0	2639	4381	11.8	68.8	89.7	11.8	41.9	51.4
12/06/02	0	4688	8929	12.9	65.9	90.9	13.6	41.2	53.1
19/06/02	1	7177	13315	13.8	67.8	93.2	13.2	41.3	53.4
26/06/02	1	9788	18120	14.2	66.8	91.6	13.2	40.7	52.4
03/07/02	1	11836	21936	14.8	66.4	90.8	14.9	42.0	53.7
10/07/02	1	14169	25860	14.6	66.2	89.7	14.8	41.9	53.1
17/07/02	3	16356	30051	14.7	65.7	89.3	14.8	41.5	52.8
26/07/02	3	19458	35193	14.8	65.8	88.7	14.6	41.3	52.3
16/08/02	3	24631	46007	14.1	63.4	86.2	14.0	39.8	50.7
05/09/02	3	28827	53888	13.6	61.2	83.2	13.5	38.2	48.6
07/10/02	3	39672	64733	12.4	59.1	78.8	12.6	36.7	46.0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as the palisade parenchyma with reduced intercellular spaces. Cells of the sub-epidermal layer also showed vacuolar content, although less dense than in the spongy parenchyma, with large and abundant plastids (Fig. 8). The stomata increased the thickness of the wall at the expense of the cytoplasmic content, which was less than 50% (Fig. 8).

3.1.2. *Effects of O_3*

In comparison with plants grown at sub-ambient O_3 levels (CF), O_3 enriched treatments (NF + 40 and NF + 80) strongly accelerated leaf senescence. Furthermore, O_3 also induced other more characteristic histological alterations (described in the following section). At the beginning, plants exposed to both NF + 40 and NF + 80 regimes exhibited similar modifications, but clearly longer exposure periods resulted in more intense effects on the tissues and cells in plants from the NF + 80 treatment.

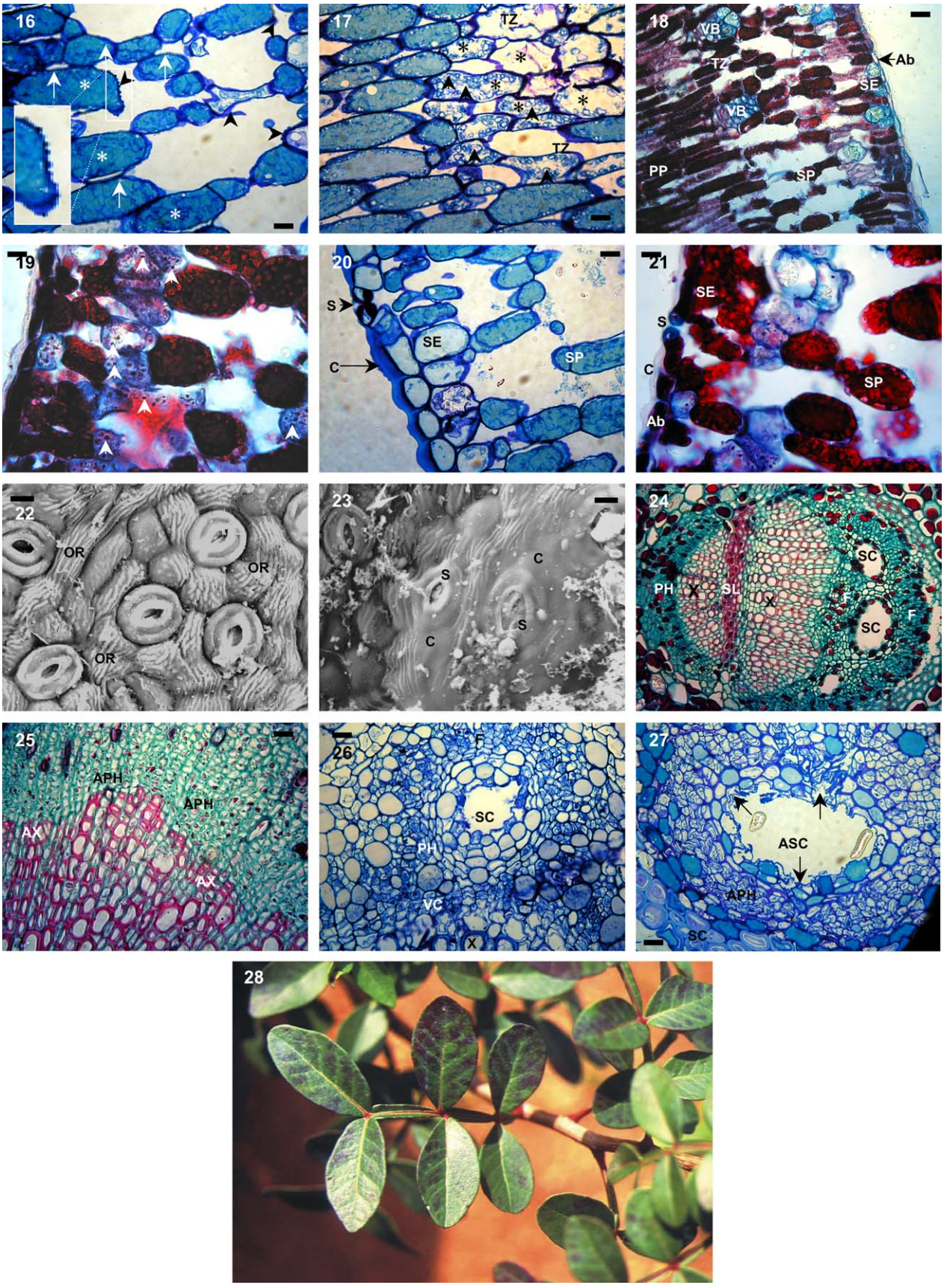
3.1.2.1. The palisade parenchyma. Figs. 11 and 12 (semithin sections) show initial effects of O_3 on the palisade parenchyma cells. Tannin vacuoles in the affected areas changed, with one super-imposed on the other, giving the cell an overall striated look (Fig. 11). Staining with toluidine blue produced a variable coloration of the contents of the vacuoles; some were orange while others were more or less intense blue. The density of the vacuolar content was notably lower. With higher O_3 doses and longer exposure times, the vacuoles degenerated completely; their tonoplast disintegrated and their content was mixed with the cytoplasm (Fig. 12). Chloroplasts in the affected zones tended to decrease in both size and number. After approximately three weeks at the highest O_3 treatment (NF + 80 ppb), several darker granulations resembling plastoglobuli appeared in the centre of the chloroplasts (Fig. 12). During the course of approximately one month, there was a progressive degeneration by zones, in the cell walls of those parenchyma cells. They become deteriorated and acquired a rough appearance. As a result, there was an increase in the size of the intercellular spaces (Fig. 13).

3.1.2.2. The spongy parenchyma and transition zone. In the spongy parenchyma, plants from the NF + 40 ppb treatment, showed a series of alterations with respect to the corresponding pattern described for unaffected leaves after a 7-day exposure (Figs. 14, 15). As was the case with the palisade parenchyma, a stacking of the tannin vacuoles with a variable aspect was observed. There was also a reduction in the size and number of chloroplasts, although it was less marked than in the palisade parenchyma. Similar alterations also occurred in the sub-epidermal layer (Fig. 15), but the transition zone was scarcely affected (Fig. 14).

At a higher O_3 dose (NF + 80 ppb) and longer exposure times (1 month), the degenerative processes become more acute. Vacuoles disappeared, with their content mixed with the cytoplasm (Figs. 16, 17, semithin sections, and Fig. 18, in paraffin). The chloroplasts degenerated and plastoglobuli were formed. The walls became more irregular and deteriorated. In some areas, wart-like protrusions of the cell walls appeared (Fig. 16). The intercellular spaces became larger (Figs. 16, 18). The plastids in the transition zone were reorganized, occupying the middle of the cell, probably due to the disappearance of the central vacuole (Fig. 17). These plastids also showed a more irregular and less refringent appearance.

Safranin and fast green staining revealed the presence of granulations in the chloroplasts with a high affinity for safranin (Fig. 19). They were especially visible in

Figs. 1–15. **Fig. 1.** Cross section of a juvenile leaf. Ad: adaxial epidermis; Ab: abaxial epidermis; PP: palisade parenchyma; TZ: transition zone; SP: spongy parenchyma; SE: sub-epidermal layer. (×200). **Fig. 2.** Detail of the transition zone showing the aspect of the plastids (arrows) and the vacuoles (⁎) in the parenchyma cells. (×1000). **Fig. 3.** Details of the adaxial epidermis, palisade parenchyma and transition zone. Note the ornamentation on the thin cuticle (arrows) (×1000). **Fig. 4.** Details of the abaxial epidermis, spongy parenchyma and sub-epidermal layer. Two stomata are marked with arrows (×1000). **Fig. 5.** Details of the adaxial epidermis and the palisade parenchyma in a mature leaf. Note the thicker cuticle and ornamentation (arrows). Parenchyma cells (⁎) showing a dense vacuolar content and plastids (×1000). **Fig. 6.** Details of the adaxial epidermis and palisade parenchyma in a mature leaf. Note the thick cuticle and the ornamentation (arrows). Parenchyma cells (⁎) with a dense vacuolar content and plastids. (×1000). **Fig. 7.** Details of the transition zone (⁎) and the spongy parenchyma, with numerous large plastids; it has a less dense vacuolar content than the palisade parenchyma. (x1000). **Fig. 8.** Details of the spongy parenchyma (⁎), the sub-epidermal layer (⁎) and the abaxial epidermis. In mature leaves, stomata (arrow) show a more swollen cell wall and a lower cytoplasmic content than the stomata of young leaves (×1000). **Fig. 9.** An 8 μm-thick cross section of a paraffin-embedded mature leaf. Ad: adaxial epidermis; PP: palisade parenchyma; TZ: transition zone; SP: spongy parenchyma; SE: sub-epidermal layer; Ab: abaxial epidermis. (×200). **Fig. 10.** Details of an 8 μm-thick cross section of a paraffin-embedded mature leaf. Ad: adaxial epidermis; PP: palisade parenchyma; TZ: transition zone; SP: spongy parenchyma (×400). **Fig. 11.** Details of a cross section of the palisade parenchyma of a leaf exposed to NF + 40 ppb O_3 for a week. There is a stacking of the vacuoles (⁎) and a decrease in the number and size of the chloroplasts (arrows) (×1000). **Fig. 12.** Details of a cross section of the palisade parenchyma of a leaf exposed to NF + 80 ppb O_3 for a month. A degeneration of the vacuoles (⁎), chloroplasts (white arrows, enlarged detail) and cell wall (arrow heads) is observed. C: cuticle (×1000). **Fig. 13.** Details of a cross section of the palisade parenchyma of a leaf exposed to NF + 80 ppb O_3 for a month and then embedded in paraffin. There is a degradation of the cell walls and an increase in the intercellular spaces (⁎) (×1000). **Fig. 14.** Details of a cross section of the spongy parenchyma of a leaf treated with NF + 40 ppb O_3 for one week. There is a stacking of the vacuoles (⁎) and a decrease in the size and number of the chloroplasts (arrows), although to a less degree than in the palisade parenchyma. TZ: transition zone (×1000). **Fig. 15.** Details of a cross section of the spongy parenchyma and the abaxial epidermis of a leaf treated with NF + 40 ppb O_3 for one week. Cells of the sub-epidermal layer (SE) experienced similar changes as those of the spongy parenchyma. Stacked vacuoles (⁎) (×1000).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those cells lacking tannins, since those compounds also react with safranin resulting in the masking of the granulations. At the end of the treatment, however, granulations became denser with a higher affinity for the safranin, thus becoming recognizable even in cells rich in tannins.

3.1.2.3. The epidermis and the stomata. The cuticular ornamentations that were very sharp in the control plants (CF) (Figs. 3–6, 22) became smooth, flat and waxy in appearance in the O_3-treated plants (Figs. 20, 21, 23). A thickening of the adaxial cuticle was also observed (Fig. 11), due mainly to the increase in the size of the cuticular layer. Microscope sections of leaves exposed to the O_3 enrichment showed a considerable increase in the thickness of the external tangential walls of the epidermal cells. The thickening was produced by the accumulation of a dense material, with affinity for toluidine blue (staining dark blue, in contrast with the light blue colour of the lignified secondary walls) (Fig. 12). In the abaxial epidermis, the O_3-treated plants (Figs. 20, 21) exhibited a considerable reduction in cytoplasmic volume, which was about 30% after only a month, due to a substantial increase in the thickness of the walls, especially the upper. Time, as it progressed accelerated the accumulation of wall material in the O_3-treated plants.

Anomocytic stomata also changed as a result of the O_3 action. In the cross sections of the stomata from the control-plants, the lower wall of occlusive cells was much thicker than the upper. Between both walls, the cytoplasm occupied about 50% of the total cell volume (Fig. 4) in juvenile leaves, and less in mature leaves (Fig. 8). In O_3-fumigated plants, the wall thickness of the occlusive cells increased, finally becoming inoperative (Figs. 20, 23 compared with Fig. 22).

3.1.2.4. The conductive bundles and the secretory channels. In contrast to the vascular bundles in the unaffected leaves (Fig. 24), in the xylem of the O_3-treated leaves, an increase in the thickness of the lignified secondary wall reinforcements was observed, resulting in a reduction of the lumen diameter of vascular elements (Fig. 25) in the metaxylem area. The phloem bundles in the O_3-treated plants also changed in appearance: the walls turned sinuous and slightly thicker, thus reducing the lumen of the cells, especially in the older leaves. In addition, the contents of some of the phloem cells became denser (Fig. 25), more visibly so in those that surrounded the secretory channels (Fig. 27).

The secretory channels in the O_3-treated leaves also seemed to undergo modifications; the cells that covered the secretory channels seemed to be in a process of degradation (Fig. 27) or in some way altered when compared with the cells that covered the normal channels (Fig. 26).

3.2. Visible injury

During 2002, plants showed no visible injury on the leaves or only a very slight intervenial reddening (AOT40 for the entire study period in NF + 80 ppb treatment was 64,733 ppb h, Table 1). In contrast, in a previous experiment conducted in 1998, plants developed clear O_3-induced foliar injury symptoms after just 14 days of exposure (AOT40 = 8808 ppb h):

Figs. 16–28. **Fig. 16.** Details of a cross section of the spongy parenchyma of a leaf treated with NF + 80 ppb O_3 for one month. Vacuoles (*), degenerated chloroplasts (white arrows), and wart-like protrusions of the cell walls (arrow heads; enlarged detail) (×1000). **Fig. 17.** Details of a cross section of the transition zone (TZ) of a leaf treated with NF + 80 ppb O_3 for one month. There is a degeneration of the vacuoles (*) and a reorganization of the TZ chloroplasts (arrows) (×1000). **Fig. 18.** Details of the cross section of the mesophyll of a leaf treated with NF + 40 ppb O_3 for 1 week and then embedded in paraffin. With respect to control leaves, there is an enlargement of the intercellular spaces and an accumulation of tannins in the vacuoles of the SE layer cells. SP: spongy parenchyma; PP: palisade parenchyma; TZ: transition zone; SE: sub-epidermal layer; VB: vascular bundle; Ab: abaxial epidermis (×400). **Fig. 19.** Details of the cross section of the spongy parenchyma and sub-epidermal layer of a leaf treated with NF + 80 ppb O_3 for 1 month and then embedded in paraffin. Granulations with an affinity for safranin were observed in chloroplasts of those cells not accumulating tannins (arrows) (×1000). **Fig. 20.** Semi-thin cross section of a leaf treated with NF + 40 ppb O_3 for 1 month. Cuticle (C) increased in thickness and the waxy ornamentation disappeared. The external tangential walls of the occlusive cells of the stomata (S) became thickened. SP: spongy parenchyma; SE: sub-epidermal layer. (×1000). **Fig. 21.** Details of spongy parenchyma (SP) and abaxial epidermis (Ab) of a leaf treated with NF + 40 ppb O_3 for 1 month and then embedded in paraffin. The increase in cuticle (C) thickness and the disappearance of the waxy ornamentation can be observed. Also apparent is the increase in the thickness of the external tangential walls of the occlusive cells in the affected stomata (S). SP: spongy parenchyma; SE: sub-epidermal layer. (×1000). **Fig. 22.** Scanning electron microscope view of the abaxial epidermis of a healthy mature leaf. One can see totally functional stomata and waxy ornamentations (OR) in the cuticle (×2000). **Fig. 23.** Scanning electron microscope view of the abaxial epidermis of a leaf treated with NF + 40 ppb O_3 for 1 month. The leaf shows changes in the cuticle (C) and in the stomata (S) (×2000). **Fig. 24.** Details of the central vascular bundle in an unaffected leaf. X: xylem; SL: sclerenchyma; PH: phloem; SC: secretory channel (×400). **Fig. 25.** Details of the xylem and the phloem in leaf treated with NF + 80 ppb O_3 for 2 weeks. In the xylem, the lowered capacity of the conductive bundles and the increased thickness of the walls can be observed. Also apparent are the alterations produced in many of the phloem cells, with a much more irregular morphology, and accumulation of a substance with affinity for safranin. AX: altered xylem; APH: altered phloem. (×1000). **Fig. 26.** Details of a secretory channel in a control leaf. The cells covering the channel are seen to be normal, as is the phloem. CS: secretory channel; PH: phloem; X: xylem; VC: vascular cambium. (×1000). **Fig. 27.** Details of a secretory channel in a leaf treated with NF + 80 ppb O_3 for 3 weeks. The cells covering the channel present a clearly altered appearance (arrows), as does the phloem. ASC: altered secretory channel; APH: altered phloem; SC: sclerenchyma. (×1000). **Fig. 28.** Ozone-induced visible foliar injury on *P. lentiscus*.

intervenial areas of the older leaves became reddish, while veins remained green (Fig. 28). Symptoms were more intense in the oldest leaflets of the composite leaves, but did not affect the abaxial side. Similar symptoms were also observed during exposures in 2001, but plants developed readily visible symptoms only after 141 days (AOT40 74,036 ppb h).

4. Discussion

The present study showed that under the experimental conditions, O_3 had a deleterious effect on the leaves of *P. lentiscus*. As a summary, the following anatomical effects have been observed. (1) *Modifications in the palisade parenchyma*: (a) chloroplasts in the affected zones tended to decrease both in size and number and finally degenerate; plastoglobuli appeared in their stroma; (b) vacuoles were altered and later degenerated and their tonoplast disintegrated; (c) tannins were at first anomalously stacked, and later homogeneously distributed within the vacuoles; (d) cell walls became thickened and developed wart-like protrusions; (e) mesophyll cells collapsed, and there was an increase in the size of intercellular spaces. (2) *Modifications in the spongy parenchyma*: similar to those of the palisade parenchyma, with a granulation of the stroma. (3) *Modifications in the epidermis and stomata*: external tangential walls of the epidermal cells became thickened, consequently reducing cytoplasmic volume of the cells, and stomata finally become inoperative. (4) *Modifications in conductive bundles*: an increase in the lignified secondary wall reinforcements in the metaxylem area, and alterations of the wall and cellular content of the phloem cells were observed, resulting in a reduction of cell lumen for both conductive tissues. (5) *Modifications in the secretory channels*: the cells covering the secretory channels partly degenerated.

These results were consistent with previous observations of the O_3 effects reported for several deciduous trees and conifers. Mesophyll cells are preferential targets for O_3 (Guderian et al., 1985). Several authors have described modifications similar to those observed here in the chloroplasts after O_3 exposures: a reduction in size (Sutinen and Koivisto, 1995), production of plastoglobuli (Miyake et al., 1989; Mikkelsen and Heide-Jørgensen, 1996), and granulation of the stroma (Sutinen and Koivisto, 1995; Holopainen et al., 1996). Thickened mesophyll walls and wart-like protrusions of the cell walls described in the previous section are also known to be induced by O_3, and are indicative that the pollutant had induced a slow intercellular oxidative response (e.g. Günthardt-Goerg et al., 1997). Those protrusions can also be induced by other agents such as heavy metals (e.g., zinc), and have also been interpreted as indicative of apoplastic oxidative burst (Günthardt-Goerg and Vollenweider, 2002). In several species, accumulation of secondary compounds, mainly phenols, is enhanced by O_3 (Vollenweider et al., 2003). Accumulation of phenolic compounds (including tannins) is considered as an active response of the plant to differing environmental stresses: O_3, but also other pollutants (Pasqualini et al., 2003), low nutrient supply, or environmental factors such as drought (Bussotti et al., 1995). That is a very important issue requiring further, detailed investigation under ambient conditions.

An increase in the cuticle thickness was observed in *A. unedo* after O_3 fumigation (Bussotti et al., 2003). In birch leaves, O_3 induced an increase in cell wall thickness in the veins, and vacuoles were filled with a coarse tannin precipitate (Günthardt-Goerg and McQuattie, 1998). In the petioles of ozonated plants of that species, the phloem tissue was deformed (Matyssek et al., 1992). In stems, a reduction in the xylem ray and tracheid cells has also been reported, due to an inhibition of the cambial activity of xylem growth, and an increased number of lignified tracheids (Matyssek et al., 2002). All those alterations, especially in the phloem, were probably relevant for transport and carbon allocation within the plant. However, as far as we know, the modifications in the secretory channels as a response to O_3 are reported here for the first time. Those channels are responsible for mastic secretion, a resin rich in tannins. Secretory cells of the channels experience similar changes in their tannin content as those reported for the mesophyll cells, and the cells finally become deformed and partly broken. Those alterations have also been observed in a parallel experiment with the congeneric plant *P. terebinthus* (unpublished).

Ozone affects the evergreen mastic at the cellular level along similar patterns as in deciduous plants. However, the presence of a thick epidermis and a relatively high specific leaf area (SLA, area of leaf divided by its dry mass) makes symptom expression of the injury in this species less apparent than in plants with thinner leaves and epidermis. Low SLA and large intercellular spaces in the leaves are features considered to favour symptom expression in plants (Gravano et al., 2003). Therefore, hardening of plant leaves may be an important factor affecting symptom onset and development in evergreen plants, and may partly explain the variation among individuals and years. Since *P. lentiscus* is a perennial with extended foliar longevity, it would be of interest to elucidate whether the observed adverse effects of O_3 are persistent for more than a single growing season or whether carry-over effects occur or whether repair mechanisms are able to compensate the stress during periods of low O_3 (autumn and winter). A "memory" effect has been suggested for conifers, where symptom development may be delayed by many months after the O_3 exposure (Sandermann, 1996) and also in grape, where

the response of plants to O_3 at a given time depends on the previous year exposures (Soja et al., 2003).

Caution should be used in the extrapolation of the results from the study to field conditions. In addition to the artificial experimental conditions, there are uncertainties in scaling the O_3 response of seedlings and saplings to mature plants, seedlings often having for example, higher stomatal conductance than adult plants (Chappelka and Samuelson, 1998). Higher stomatal conductance enhances O_3 uptake, and may favour deleterious effects at the cellular level. Water availability, nutrient status of the plants, air temperature and humidity, wind speed, and incident light levels are known to affect gas uptake rates (US-EPA, 1996). Particularly relevant for Mediterranean conditions are soil moisture and Vapour Pressure Deficit (VPD) governing stomatal conductance and hence, O_3 uptake. Soil moisture affects the onset, development and severity of foliar O_3 injury in some species, with more symptoms under well-watered conditions (Schaub et al., 2003). *P. lentiscus* is a drought-avoiding species, with a prompt response to drought stress by stomatal closure, to prevent severe tissue dehydration (Vilagrosa et al., 2003); and consequently decreased O_3 flux into leaves. However, in some parts of the Mediterranean, high O_3 episodes also occur during spring (Sanz et al., 2001a,b), when stomatal conductance is not impaired by the mid-summer drought. Possible effects of those O_3 episodes on vegetation remain to be established under the Mediterranean conditions. Ozone-like foliar injury symptoms (reddening affecting the intervenial areas of the upper surface) on *P. lentiscus*, have been observed sporadically under field conditions (Sanz and Millán, 2000). Homogeneous reddening, affecting also the veins, has been observed in the field and might be attributed to O_3. It has been suggested that microscopy (the presence of characteristic cellular alterations as those described in this study) may be a complementary tool to field observations in discriminating possible O_3 effects (Sutinen and Koivisto, 1995; Vollenweider et al., 2003) and early detection, before the onset of visible injury. From a practical viewpoint, however, in extensive monitoring studies of the effects of O_3 on natural vegetation, microscopy may be quite time consuming and may not always be sufficiently specific to be used alone.

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