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# Chapter 12

# Ultrastructural response of a Mediterranean shrub species to $O_3$

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**Abstract**

Mediterranean vegetation (especially evergreen shrubs) is regarded as resistant to ozone ($O_3$) because of its avoidance mechanisms (summer stomatal closure, low stomatal conductance, low gaseous exchange rates, slow growth). Nevertheless, visible symptoms of ozone damage were observed on some species (*Arbutus unedo, Pistacia lentiscus*) both in natural and controlled conditions. This paper reports the results of a survey on the ultrastructural characterization of the visible symptoms (red stippling) occurring on strawberry trees (*Arbutus unedo*) in controlled conditions (fumigation chambers), i.e., treated with a chronic exposure to two realistic $O_3$ doses (50 and 100 ppb for 21 days, 5 h $day^{-1}$) on recently mature leaves. The findings, obtained with light and transmission electron microscopy, show that the most important responses are in plants treated with 100 ppb $O_3$, and are localized in the epidermis-cuticle complex. The thickness of the cuticle increases and changes also occur in the reticular structure of its lower layer. In the epidermal cells of 100 ppb $O_3$-treated leaves, tannins embed the outer primary wall. In the mesophyll, we observe the alteration of tannins contained in the vacuoles. Results suggest that *Arbutus unedo* increases its defenses as an active response to ozone stress.

## 1. Introduction

Ozone ($O_3$) is one of the most widely spread pollutants in Europe and North America, and its concentration in the troposphere is increasing (Allegrini and Brocco, 1995; Fowler et al., 1999). Ambient levels of $O_3$ are already known to be high enough to cause extensive visible injury in forest vegetation, both on conifers (Miller and McBride, 1999) and broadleaved trees (Skelly et al., 1987). In Europe, ambient concentrations of this pollutant are known to be

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particularly high in the Mediterranean region because of the high summer temperatures and radiation levels (Butkovic et al., 1990; Gimeno et al., 1994; Millán et al., 1996, 1997). In many sites around the Mediterranean basin, the 24-h mean of the $O_3$ concentration from May to September was, in recent years, above 40 ppb with peaks (hourly means) that occasionally exceeded 100–150 ppb (Velissariou and Skretis, 1999; Chaloulakou et al., 1999; Soda et al., 2000). During the hottest and driest periods of the year, Mediterranean forest species have low gas exchange rates, thus avoiding the foliar uptake of $O_3$ (Rhizopoulou and Mitrakos, 1990; Tretiach, 1993; Gucci et al., 1999), but visible symptoms on leaves were recorded in several species. Ozone-induced symptoms occur primarily on *Pinus halepensis* Mill. (chlorotic mottle, see Gimeno et al., 1992; Velissariou et al., 1992; Barnes et al., 2000), but some evergreen sclerophyllous shrubs are also known to exhibit stippling and/or change in color. Among these, *Arbutus unedo* L. (strawberry tree) is probably the most sensitive species (Skelly et al., 1999; Sanz and Millán, 2000).

The present study reports structural effects in epidermal cells on the upper leaf surface of recently mature leaves of *A. unedo* caused by chronic exposure to ozone (50 and 100 ppb for 21 days, 5 h $day^{-1}$).

## 2. Materials and methods

Two-year-old seedlings of strawberry tree derived from a single mother plant were grown in pots containing a fertilized compost of peat, perlite, and natural soil in natural conditions under a shade awning until the time of the experimental fumigation. All containers were regularly provided with optimal water supply by means of an automatic drip irrigation system. Plants, selected by phenotypical homogeneity, were pre-adapted to greenhouse conditions a week before the treatment.

Fumigations were performed in a set of Perspex chambers, each measuring $0.90 \times 0.90 \times 0.65$ m, continuously ventilated with charcoal-filtered air (two complete air changes/min). Ozone was produced by electric discharge with an air-cooled generator (Fischer 500, Zurich, CH) supplied with pure oxygen, and was mixed with the inlet air entering the fumigation chambers. The concentration of $O_3$ at plant height was continuously monitored with a photometric ML8810 analyzer (Monitor Labs, San Diego, USA). More details are reported elsewhere (Lorenzini et al., 1994). The target doses were 50 and 100 ppb for 21 days (5 h day $^{-1}$, from 09.00 to 14.00, solar time). Control plants were exposed only to charcoal-filtered air ($< 3$ ppb). Five individuals were used, each randomly allocated to a fumigation chamber and the exposure was performed in the summer of 1996.

For light microscopy observations, five leaves were examined from each plant. From the median intervenial zone of each leaf, four samples $3 \times 5$ mm wide were collected and fixed in 4% formalin. Of these, two samples per leaf were cut using a freezing microtome (semithin cross-sections 30 µm thick) and stained with Floral Yellow 088 ($C_{22}H_{16}O$, Sigma, Italy) to highlight lipids (Brundrett et al., 1991). The other two samples per leaf were dehydrated in an ethanol series, embedded in LR White Resin (London Resin Company Ltd.) and cut using a Reichert OM-U3 Ultramicrotome glass blade to a thickness of 1 µm. These sections were stained with Schiff reagent plus Calcofluor (Mori and Bellani, 1996), to display the cellulosic matrix of the wall and subsequent modifications (caused, for example, by apposition of phenolic substances and lignification process—see Bussotti et al., 1997).

For transmission electron microscopy, a further two samples from each leaf were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer, pH 7.2. After 20 h at 5 °C, the samples were rinsed twice, each time for 10 min in the same buffer, postfixed in 2% $OsO_4$ in the same buffer for 2 h and, later, dehydrated in an acetone series, spending 10 min at each stage of the dehydration series. Finally, after two 5-min rinses in propylene oxide, the samples were embedded in resin, according to Spurr's procedure (Spurr, 1969). A Reichert Ultracut S microtome with a diamond knife was used to cut ultrafine sections (0.09 µm). These sections were stained with uranyl acetate and lead citrate and observed under a Carl Zeiss EM9-S2 microscope. Electron-microscopy observations show phenolic compounds as electron-dense structures, due to the osmiophilic properties of these compounds (Parham and Kaustinen, 1976; Bussotti et al., 1998).

## 3. Results

After 15 days of $O_3$ exposure, reddish intervenial spots (Fig. 1(A), (B)) were observed on the adaxial surface of leaves treated with 100 ppb $O_3$. In plants fumigated with 50 ppb $O_3$, visible injury was scarce and appeared only at the end of experimental period (21 days).

Observations with LM and TEM were performed in sections of $O_3$-treated leaves from both symptomatic and asymptomatic patches. The results described in this paper refer to observations in symptomatic patches, because the green areas did not differ from controls. *Arbutus* leaves characteristically have a 2-layer palisade parenchyma. The layer next to the upper leaf surface shows vacuoles filled with phenolic content (identified as tannins by Gravano et al., 2000). Vacuoles of epidermal cells do not stain as phenolic substances. Outer walls and cuticles of the abaxial and adaxial epidermal cells are thick (see Gravano et al., 2000).

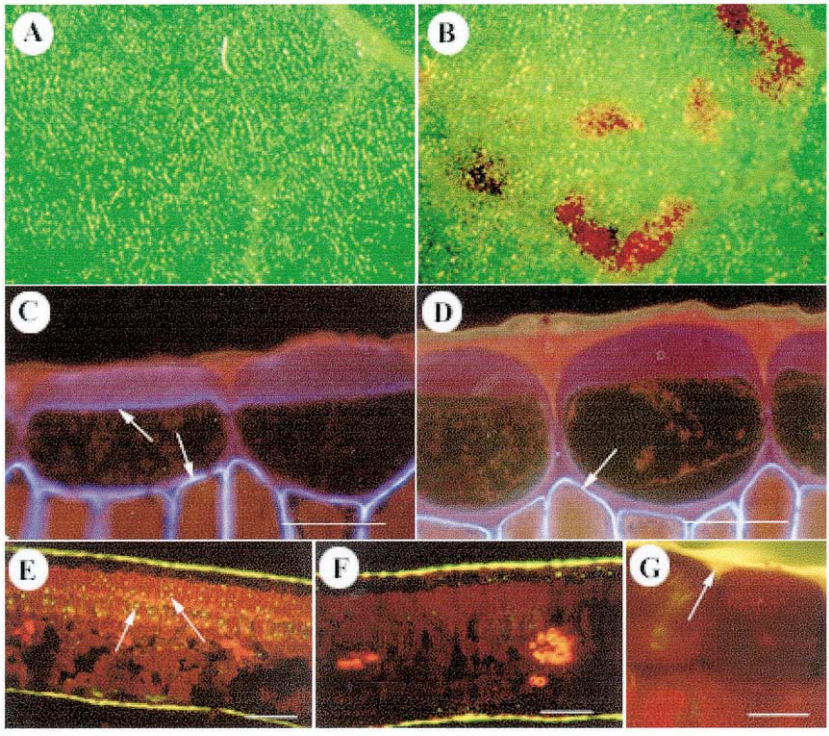


*Figure 1.* (A)–(G): Macroscopic and LM observations. (A), (B): Upper surface of control (A) and 100 ppb $O_3$-treated leaves (B). Typical ozone symptoms (reddish spots) are visible in the latter. (C), (D): Samples stained with Calcofluor + Schiff reagent; arrows indicate the cellulosic matrix of the wall (A = Control; B = 100 ppb $O_3$-treated sample), bars = 20 µm. (E)–(G): Samples stained with Floral Yellow. (E), (F): arrows indicate the pattern of lipidic bodies (E = Control; F = 100 ppb $O_3$-treated sample, bars = 0.1 mm); (G) (100 ppb $O_3$-treated sample): arrows indicate the cuticular nails, bar = 20 µm.

Reactions to the 100 ppb $O_3$ treatment were observed with a light microscope (Fig. 1(A)–(D)), whereas no changes were detected in the 50 ppb $O_3$ treatment compared with the filtered air control. Fig. 1(C), (D) (Calcofluor + Schiff reagent) illustrates the effects of the 100 ppb $O_3$ treatment in the walls of the adaxial epidermal cells. The cellulose-pectic component (that stains light blue) was clearly visible in the control leaves (Fig. 1(C), arrow), but in the 100 ppb $O_3$ treatment (Fig. 1(D)), in the outer wall, the fluorescent response of cellulose disappeared and walls stained red. This indicated the presence of modification processes, probably apposition of phenolic substances (Bussotti et al., 1997). In 100 ppb $O_3$-treated leaves the typical cellulose response was evident only in the walls of the mesophyll cells (Fig. 1(D), arrow).

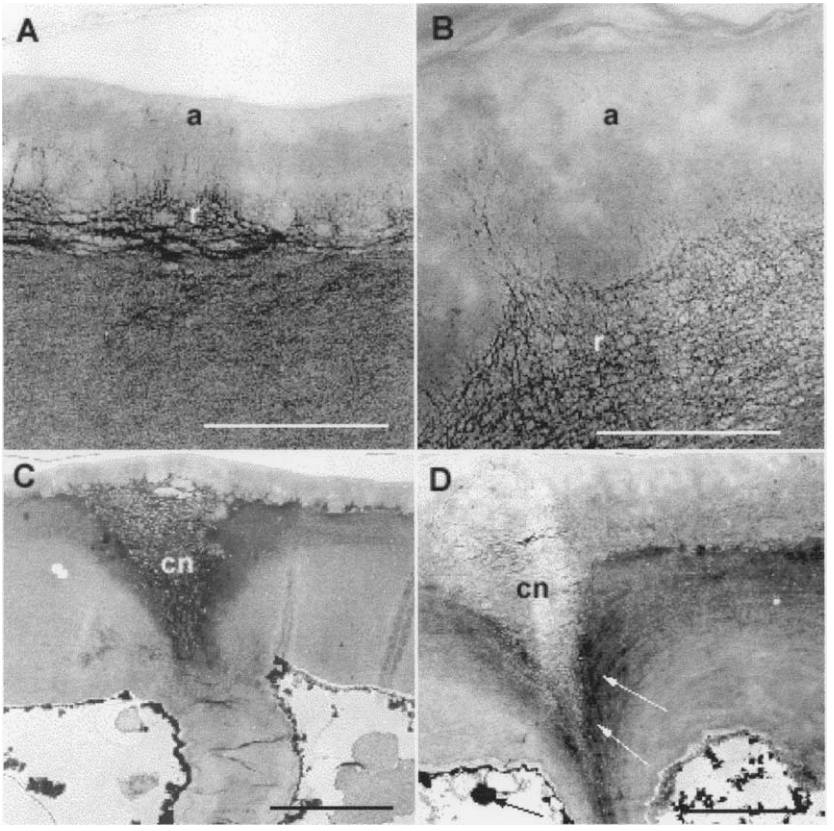


*Figure 2.* (A)–(F): Electron microscope observations. (A), (B): Bi-layered cuticle, (a) amorphous and (r) reticulate layer (A = Control; B = 100 ppb $O_3$-treated sample), bars = 0.5 µm. (C), (D): Cuticular nail (cn) (C = Control; D = 100 ppb $O_3$-treated sample). Arrows indicate the phenolic substances embedding the wall, bars = 2 µm.

Fig. 1(E)–(G) shows the pattern of lipids: lipidic bodies were notably present in the palisade cells of filtered-air control leaves (Fig. 1(E)), and they appeared localized in the epidermal cells in those subjected to fumigation (Fig. 1(F)). In Fig. 1(G), lipidic substances filled the cuticular pegs in fumigated leaves.

The results of TEM observations (means) are shown in Figs. 2 and 3. Only the controls and 100 ppb-treated leaves are shown, because the 50 ppb-treated leaves did not differ from controls. Fig. 2(A), (B) shows the condition of the cuticle of the adaxial surface in controls (Fig. 2(A)) and in samples from the 100 ppb $O_3$ treatment (Fig. 2(B)). The cuticle of *A. unedo* is composed of a reticulate and an amorphous layer (*Pyrus* type: see Gouret et al., 1993), and

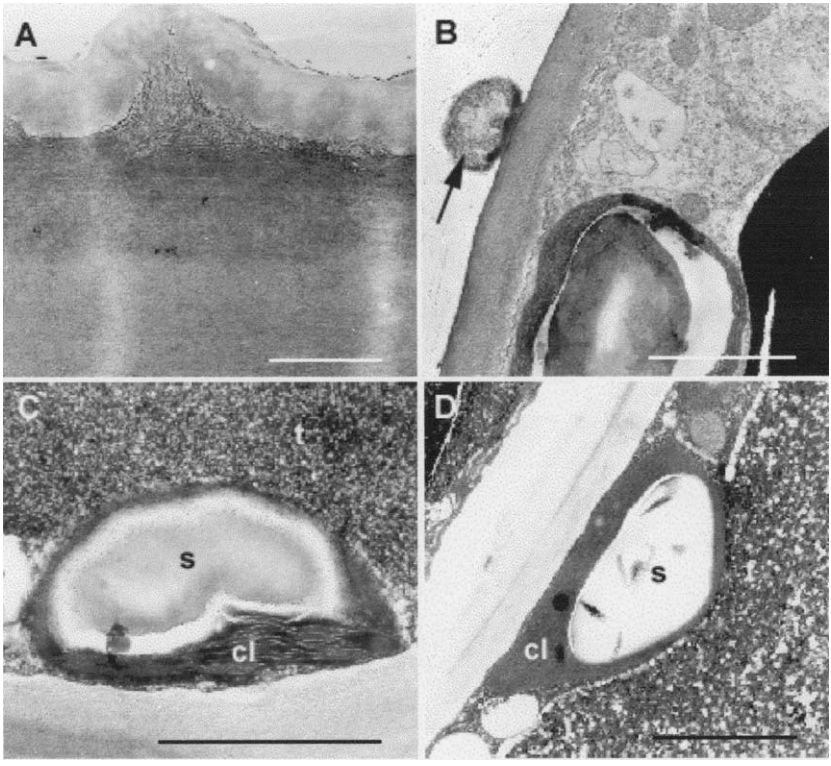


*Figure 3.* (A)–(D): Electron microscope observations. (A), (B): 100 ppb $O_3$-treated samples. A: Cuticular ridge; B: Cell wall projection in the mesophyll cell (arrow), bar = 1 µm. (C), (D): Condition of the mesophyll cells (C = Control; D = 100 ppb $O_3$-treated sample): (t) tannins; (cl) chloroplast; (s) starch grain, bars = 1 µm.

both of them had developed considerably by the end of this treatment. Cuticular nails (Fig. 2(C), (D)) had a finely reticulated structure in the controls (Fig. 2(C)), whereas in the treated leaves (Fig. 2(D)) that structure appeared amorphous. In the 100 ppb $O_3$ treatment, amorphous electron-dense material (probably tannins: Fig. 2(D) arrow) was found embedding the outer part of the primary cell wall. Cuticular ridges (Fig. 3(A)) and probably cell-wall projections (or exudates) at the mesophyll cell surface facing the intercellular space (Fig. 3(B)) were observed in the samples from the 100 ppb $O_3$ treatment. Fig. 3(C), (D) shows electron-dense vacuolar material particularly in the cells from the adaxial palisade layer, which is more homogeneous in the control (Fig. 3(A)) compared with the granulated appearance in the $O_3$-treated sam-

ples. Chloroplasts (Fig. 3(D)) show a normal lenticular shape both in controls and in the 100 ppb $O_3$ treatment.

## 4. Discussion and conclusions

The behavior we observed suggests the presence of strong active responses located primarily in the epidermal layer. The epidermis represents the interface between plant and environment, so it is the seat of important biochemical protection processes (Bell, 1981) that include an additional synthesis of cutine with the thickening of the cuticular layer, phenolic compounds embedded in the cell wall, and an increased synthesis of polysaccharides that help to build up the cell wall. Ozone is known to be involved in all these processes. Percy et al. (1992) observed the increase of the cuticular membrane thickness in red spruce (*Picea rubens* Sarg.) exposed to $O_3$ and acid fog. Phenols are involved in several protection processes against biotic and abiotic stress factors (Bennett and Wallsgrove, 1994; Rosemann et al., 1991; and Kangasjärvi et al., 1994) have reported on the role of $O_3$ in the formation of enzymes as precursors of phenolic compounds. Tannins filling the adaxial epidermis wall were found in *Fagus sylvatica* L. (Bussotti et al., 1998) and *A. unedo* (Gravano et al., 2000) in response to water stress, and also in *Fraxinus excelsior* L. as response to the influence of ozone (Günthardt-Goerg et al., 2000).

In the mesophyll, the main alterations related to the effects of $O_3$ were found in the vacuolar content of tannins and the cell walls, and in the lipids pattern. The observed granulation of tannins in response to the treatment is consistent with the process described by Kärenlampi (1986) and Pääkkönen and Holopainen (1995) in pines and birch as a response to $O_3$ fumigation. Wall excrescences and protuberances were observed by Günthardt-Goerg (1996) in ozone-treated broadleaves. As far as lipids are concerned, previous studies by our group (Soda et al., 2000) report an accumulation of these substances in ozone-exposed leaves, this behaviour was associated with the degeneration of organelle membranes. In the present study, lipidic bodies did not increase in the mesophyll of the treated leaves but, on the contrary, they disappeared. Their disappearance may be related to a mobilization of reserves necessary to build up the epidermal structures.

This experiment focused on the early active responses at the epidermis level and did not investigate the mesophyll alterations, namely the condition of chloroplasts. In much of the literature (Sutinen et al., 1990; Selldén et al., 1996), these organelles are considered the structures most sensitive to ozone and the fact that we observed no change in these organelles cannot allow us to rule out damage entirely. It simply suggests that, in *A. unedo*, the active

responses occur earlier than the organic damage, and symptoms are not necessarily associated with mesophyll injuries (as was reported by Moss et al., 1998; Evans and Miller, 1972). On the other hand, active responses imply a different metabolism of the reserves, so that they are employed to increase defense mechanisms rather than growth (Heath, 1999).

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