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The effect of simulated acid rain on the leaf structure of *Laurus nobilis* L., an injury resistant species

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Leaves of *Laurus nobilis* were subjected to artificial acid precipitation of various concentrations for a period of two months. Leaf sections were examined during the period of the acid rain application and after it using light microscopy as well as transmission electron microscopy. Leaves of *Laurus nobilis* resist to structural deformation and cellular damages, usually caused by acid rain, remaining productive for the time span our investigation lasted. The use of this species for environmental restorations or reforestations is suggested and has to be encouraged.

Key words: Air pollution, evergreen sclerophyllous species, mediterranean plants, simulated precipitation, structural deformations.

INTRODUCTION

Acid rain affects the structure and function of plants and plant communities and has long ago been a matter of consideration. Limitation of photosynthesis and bleaching, followed by necrotic spots have been reported for the highly susceptible lichens (Sigal & Johnston, 1986; Diamantopoulos *et al*., 1992; Tarhanen, 1998), while forests (Zobel & Nighswander, 1991; Fan, 2000) and agricultural crops (Irving & Miller, 1981; Caporn & Hutchinson, 1986; Percy & Baker, 1987; Knittel & Pell, 1991; Temple *et al*., 1992) are also dramatically affected. Limitations on tree growth (Jensen & Patton, 1990) and reproduction (Funk & Bonde, 1986) have been further observed. Besides visible foliar injury in affected plants (Adams *et al*., 1984; Shan, 1998), modifications of epicuticular wax structure and cuticle thickness (Percy & Baker, 1987; 1990), accumulation of phenolics in the mesophyll cells (Zobel & Nighswander, 1991) and necrotic tissues (Leith *et al*., 1989; Psaras & Christodoulakis, 1987a; Zobel & Nighswander, 1991; Knittel & Pell, 1991) have been reported for many species. In addition, simulated acid rain has been considered to affect the metabolic activity (Roy-Arcand *et al.*, 1989), photosynthesis (Velikova *et al.*, 1999) and ultrastructure of chloroplasts (Stoyanova & Velikova, 1997; Gabara *et al.*, 2003) and mitochondria (Gabara *et al.*, 2003).

At the very opposite, some drought resistant mediterranean evergreen sclerophylls revealed an incredible resistance to air pollution-induced structural and ultrastructural deformations (Christodoulakis & Fasseas, 1990; Christodoulakis & Koutsogeorgopoulou, 1991; Christodoulakis, 1993) compared to other species (i.e. *Citrus aurantium* or *Phlomis fruticosa*) growing in the same areas (Psaras & Christodoulakis, 1987a,b).

Taking into account that: a) the acid rain "syndrome", besides the direct effect on plant life, also has a serious effect – partly because of the deforestation it causes – on the leaching of nutrients from the soil and the consequent disturbance of the aquatic life, b) the fact that the case of acid rain in Greece has been disregarded in general, mainly because of the relatively low annual rainfall, the blowing winds and the buffering effect of the calcareous soils, c) the surprising fact that no damage has been reported so far for the evergreen mediterranean formations growing wild on the mountain slopes surrounding Athens metropolitan area, and d) the challenge to check whether the resistance of the mediterranean evergreen sclerophyllous species to air pollution-induced damages extends to acid-rain-induced deformations and injuries, we decided to undertake this

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investigation using the laurel tree (*Laurus nobilis* L.), a species with well documented resistance to air pollution-induced leaf injuries (Christodoulakis & Fasseas, 1990; Christodoulakis, 1993).

MATERIALS AND METHODS

a) *Site and plant description*: This research was performed on the western slope of mount Hymettus, near the St. John abbey, where a typical mediterranean plant formation grows wild. This site, often exploited for ecological investigations, is considered to be practically non-polluted. Individuals of *L. nobilis* growing wild on this area were selected and a young, (one-year old) branch was marked on each plant. Plants to be treated were grouped as follows: *group 1* – untreated plants (10 individuals), *group 2* – plants treated with simulated rain (deionized water) (10 individuals), *group 3* – plants treated with simulated acid rain (pH 4.0) (15 individuals) and *group 4* – plants treated with simulated acid rain (pH 2.5) (15 individuals).

b) *Simulated rain*: Artificial acid rain was prepared just prior to each application by acidifying distilled water with a 1:2 mixture (by volume) of HNO_3 : H_2SO_4 (pH values of 4.0 and 2.5 respectively). The precipitation simulant was applied to each individual by atomization, early in the morning, at five-day intervals, for a period of more than two months (7 October to 20 December 2002). The soil bellow each plant was covered with absorbing paper to avoid its acidification.

Plants of *group 1* received no treatment. Plants of *group 2* were treated only with deionized water (pH 5.9). Each plant of *group 3* and *group 4* was exposed to a different pH level, while the amount of the precipitation simulant that each plant received, each time, was equal to 1/10th of the mean monthly precipitation recorded for the area. This amount was enough to be applied to run-off on the previously selected branch of each individual, but not sufficient to penetrate the soil and alter its properties. Much thought was spent for the composition of the acidifying mixture and the pH value of the simulated rain. Facts such as i) the main air pollutants in the metropolitan area of Athens are sulphur and nitrogen oxides (from motor vehicles and coal or oil furnaces) and, ii) the pH value for the total volume of the precipitation during an eight-month period each year was found to be less than 5.6, for 69% of the samples collected for a period of 7 consecutive years (Ministry of Environment, Air Quality Dir., 2000), were strongly considered. Thus, the mixture of HNO_3 and H_2SO_4 at the pH values of 4.0 and 2.5, was adopted as more realistic.

c) *Microscopy*: Besides macroscopical observations for visible damage on the leaf surface, one mature sun leaf was detached from each branch, two days after each application. All leaves were grouped according to the treatment, cut into small pieces and fixed immediately in phosphate buffered 3% glutaraldehyde at 0°C.

Leaf tissue was post fixed with 1% OsO_4, dehydrated in a graded ethanol series and embedded in Durcupan ACM resin (Fluka). Glass-mounted, semithin sections (3-5 μm in thickness, obtained with an LKB - Ultrotome) were stained with toluidene blue O and viewed with a Zeiss Axioplan light microscope. Conventional light micrographs were taken on a Kodak, EliteChrome, Extra Colour 100 transparency film. All pictures were also obtained with a Nikon D-100, 6.1 effective megapixels digital camera.

Ultrathin sections (30-50 nm in thickness, cut with an LKB - Ultrotome) were double stained with uranyl acetate and lead citrate and observed with a Phillips 300 transmission electron microscope.

A part of each leaf was kept for peeling (by boiling leaf pieces in 65% HNO_3) the epidermal tissue and observing the stomatal arrangement, frequency and index.

RESULTS

After the two-month treatment, the leaves from the plants of all four groups presented no macroscopical difference. To avoid detachment of a large number of leaves from the selected plants, the leaf area was measured *in situ* by scanning about fifty leaves with a portable scanner, printing their outlines on a Xerox paper of known weight per surface unit (80 gm^{-2}), cutting and weighing. The leaf areas (leaf projection on the paper) were: *group 1* (untreated) 325 ± 15 mm^2, *group 2* (deionized water) 392 ± 17 mm^2, *group 3* (pH 4.0) 318 ± 21 mm^2 and *group 4* (pH 2.5) 316 ± 19 mm^2.

Light microscope observations revealed that leaves from plants subjected to simulated acid rain (Figs 5, 7) appear more xeromorphic compared to leaves originating from untreated plants. They are

generally thinner than normal (untreated) leaves (Fig. 1). The latter have the two layers of palisade tissue more or less equally developed. Only the cells of the upper layer accumulate phenolics (Figs 1, 2). The vacuoles in the cells of the second layer, remain free of these secondary metabolites thus numerous osmiophillic oil droplets are observed. Oil droplets are also present in the chloroplasts of the palisade cells of both layers. Phenolics appear to be present in some of the cells of the spongy parenchyma. They also occur in all epidermal cells which appear to be lined with a thick cuticle (see Fig. 2, adaxial leaf surface).

Leaves subjected to distilled water misting (*group 2*, Fig. 3) respond differently. They appear thicker, less compact, phenolics are occasionally present, raphide crystals seldom appear in the vacuoles and spongy cells are separated by larger intercellular spaces. Numerous, small oil droplets can be observed in all cells of this type of leaves (Fig. 4), while epidermal cells seem to insist on the "phenolic habit".

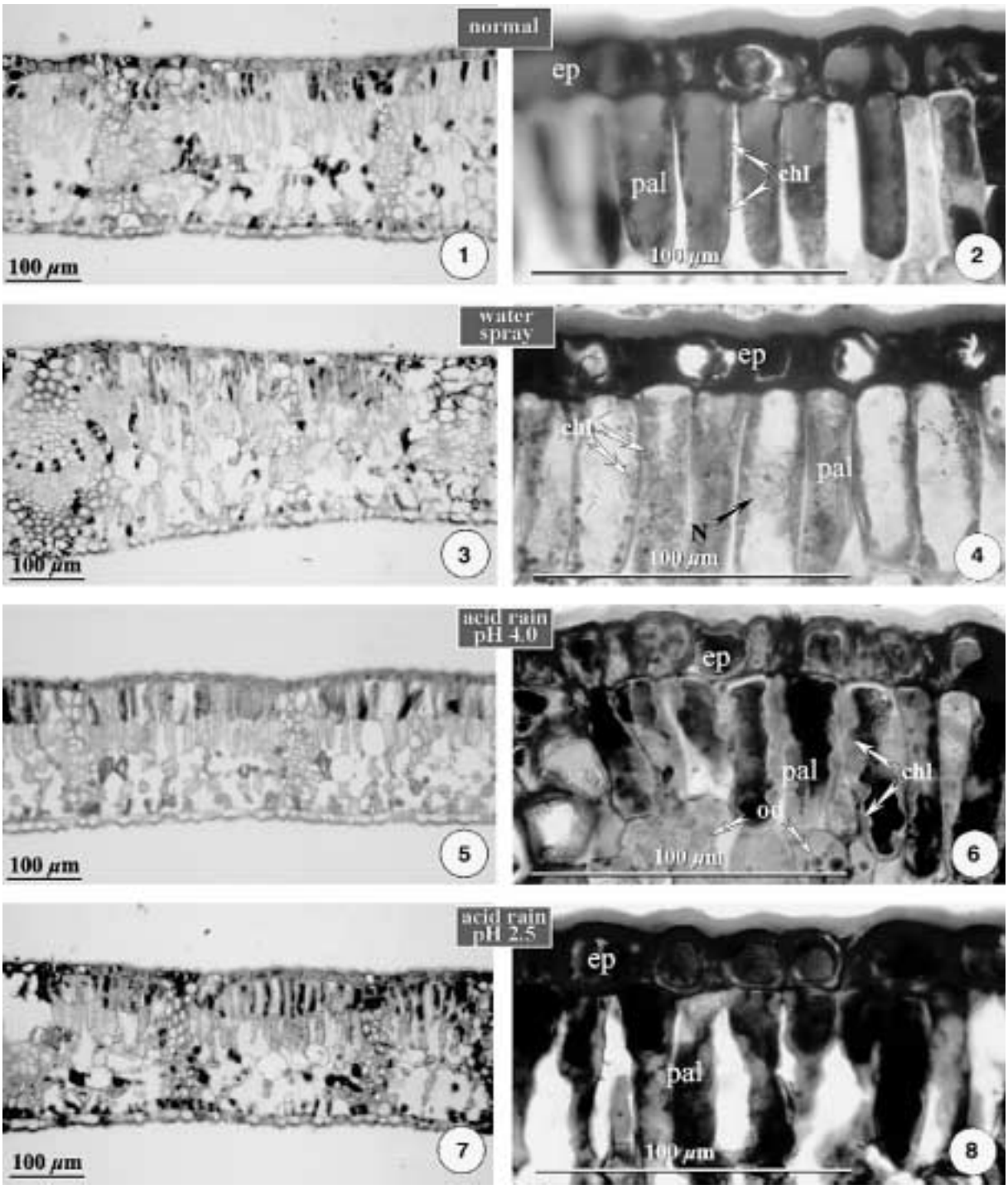
Leaves originating from plants treated with acid rain at pH 4.0 (*group 3*, Figs 5, 6) appear to have a more developed and compact palisade accumulating phenolics, mainly in the vacuole of the cells of the first layer. The second layer appears more stressed than in normal leaves, as the cells are more densely stained due to their secondary metabolites, mainly of lipid nature (even in texture, less osmiophilic than phenolics) (Fig. 6). The same is true for the spongy parenchyma (Figs 5, 6). Epidermal cells accumulate phenolics and possess thick cuticle (Fig. 6).

When the pH of the simulated acid rain was as low as 2.5 (*group 4*, Fig. 4), leaves appeared seriously stressed. Epidermal cells are completely impregnated with condensed phenolics (Figs 7, 8), as usually happens under stressful conditions. Both layers of palisade tissue accumulate these secondary metabolites which now appear to be strongly osmiophillic. The mechanical elements accompanying the conductive tissue are more lignified, as their intense staining indicates (Fig. 7). A thick cuticle, densely stained with toluidene blue O, is present as well (Fig. 8). All these structural characteristics attributed to leaf structure have been commonly observed in severely water stressed mediterranean evergreen sclerophyllous individuals.

Observations of peeled lower epidermal tissue (hypostomatic leaves) revealed that stomatal frequencies do not present a statistically significant variation among the leaves of the four groups. Stomatal frequency was 307 ± 41 for normal leaves, 296 ± 39 for deionized-water treated leaves, 315 ± 37 for acid rain treated leaves (pH 4.0) and 317 ± 32 for acid rain treated leaves (pH 2.5) ($x \pm$ S.E.M.).

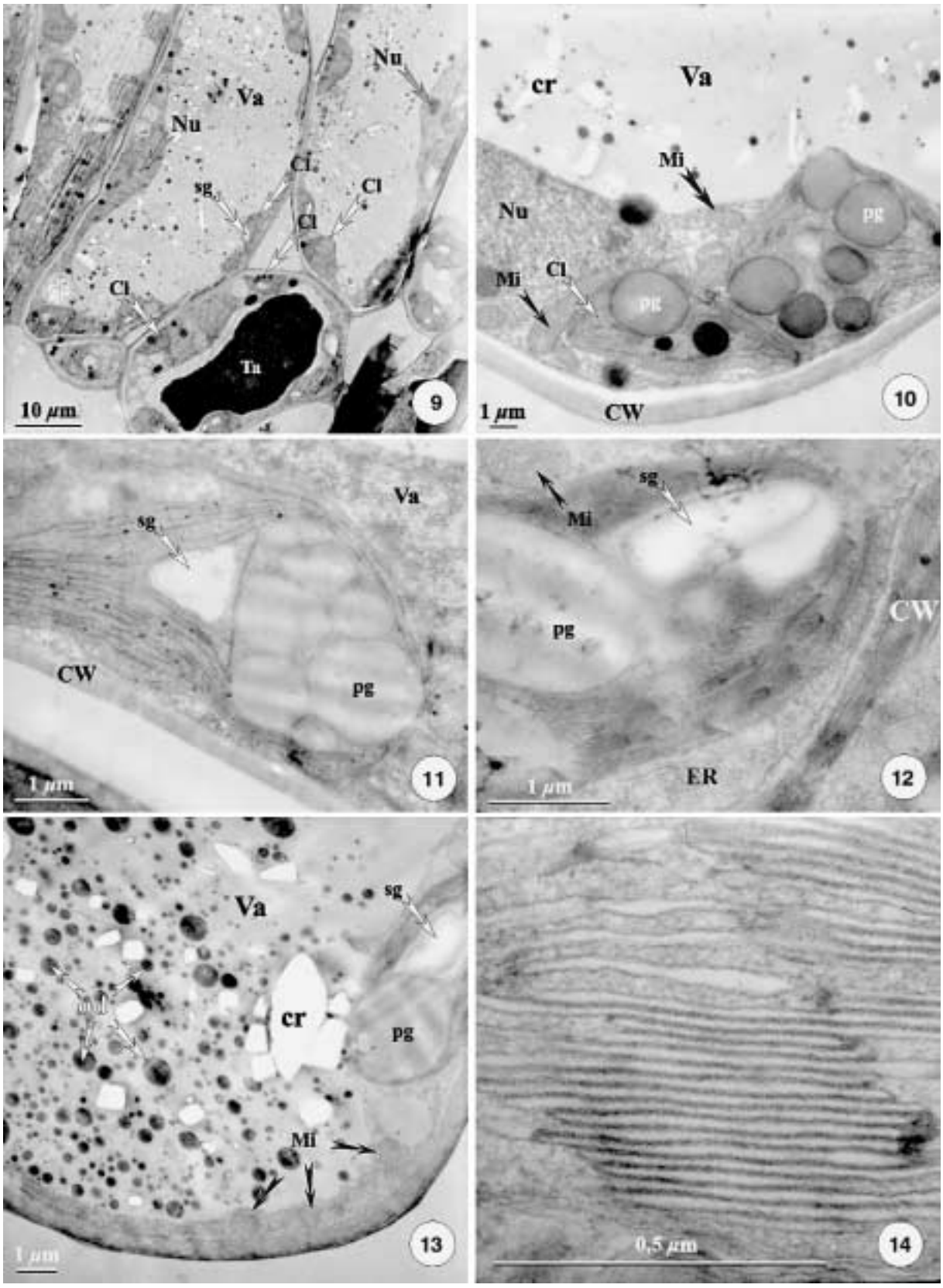
It has been well documented, some decades ago, that unfavourable mediterranean climatic conditions (hot and arid summers followed by short but cold winters) forced plants to develop a stress evading strategy known as sclerophylly (hard-leaf strategy). Some characteristics observed in normal leaves of *L. nobilis* at the ultrastructural level, such as the accumulation of tannins, oil droplets and crystals in the vacuoles and the presence of many plastoglobuli in the chloroplasts (Figs 9, 10), reveal the dedication of the leaves to this strategy. In our investigation, electron microscope observations were focused on the structure of chloroplasts and mitochondria, organelles known to be susceptible to injuries from air pollution and acid rain. Series of ultrathin sections from leaf material fixed at the end of the treatment period, two days after the last rain simulant was applied, were observed.

Leaves treated with pH 4.0 acid-rain simulant, apart from developing a more xeromorphic structure, they present no sharp difference from the untreated leaves, at the ultrastructural level. Surprisingly, the same was observed in the cells of leaves having undergone the two-month treatment with acid rain buffered at pH 2.5. Mitochondria and their internal membranes remain intact (Figs 12, 13). Chloroplasts retain a well-developed system of thylakoids while starch grains are indicative of their activity (Figs 11, 12). Chloroplasts of the second layer of the palisade cells appear with increased size although their number seems reduced compared to that of normal leaves. We may assume that this is the only impact of this very low pH simulated acid rain. Numerous large plastoglobuli of low electron density appear in the chloroplast stroma (Figs 11, 12 and 13). A close look at the chloroplast thylakoid system rarely revealed some slightly swollen granal and stroma thylakoids (Fig. 14). Occasional disintegration of the chloroplast envelope, the membranes of the ER or the tonoplast, could be associated to tannin-induced deformations after their leaching from the vacuole.



FIGS 1-8. Light micrographs of blade cross-sections stained with toluidene blue O, illustrating leaf anatomy. FIG. 1: Normal leaf. FIG. 2: Normal leaf, high magnification, adaxial surface and palisade. FIG. 3: Leaf treated with deionized water. FIG. 4: Leaf treated with deionized water, high magnification, adaxial surface and palisade. FIG. 5: Leaf after the end of the pH 4.0 treatment. FIG. 6: Leaf after the end of the pH 4.0 treatment, high magnification, adaxial surface and palisade. FIG. 7: Leaf after the end of the pH 2.5 treatment. FIG. 8: Leaf after the end of the pH 2.5 treatment, high magnification, adaxial surface and palisade, ep = epidermal cell, pal = palisade, chl = chloroplast, od = oil droplet, N = nucleus.

FIGS 9-14. Electron micrographs of mesophyll cell ultrastructure. FIG. 9: Normal leaf. Palisade cells, 2nd layer. FIG. 10: Normal leaf. Chloroplast and a part of the vacuole. Plastoglobuli of various densities can be observed in chloroplast stroma. FIGS 11 and 12: Chloroplasts from leaves after pH 2.5 treatment. Plastoglobuli and starch grains are obvious. FIG. 13: The vacuole of a similar cell. Numerous dark, oil droplets are visible. FIG. 14: Detail of a granum after pH 2.5 treatment. Stroma



thylakoids, slightly swollen (upper half of the picture). CW = cell wall, Nu = nucleus, Cl = chloroplast, Va = vacuole, Mi = mitochondrion, sg = starch grain, cr = crystal, ER = endoplasmic reticulum, pg = plastoglobuli, od = oil droplets, Ta = tannins.

DISCUSSION

As expected, leaves treated with deionized water, present statistically significant difference in leaf area. Their blade expands more than in leaves of all other groups. This is due to the elimination of the environmental stress of aridity eventually leading to a less xeromorphic structure.

The structure of leaves subjected to pH 4.0 simulant could simply be observed in the leaves of plants growing in a somehow warmer and/or more arid Mediterranean area. Chloroplasts, mitochondria and microbodies were absolutely similar to those observed in the cells of normal leaves.

Acid rain at pH 2.5 resulted in an extremely xeromorphic leaf structure. Secondary metabolites –particularly tannins– are accumulated as a result of stressful conditions. The same is true for the increased number and size of plastoglobuli. Both of these structural features could mainly be due to the severe osmotic stress caused by the acid rain simulant.

Stomatal frequency does not appear to vary among leaf groups. This is probably due to the fact that leaves develop their stomatal complexes at an early developmental stage and the number of stomata cannot alter on a mature leaf even if this leaf is exposed to stress. Yet some signs of response of the guard cells and subsidiary cells to stressful conditions, previously described (Christodoulakis, 1993), were observed at the ultrastructural level on the stomatal complexes of the leaves from *groups 3* and *4*. This observation will be further investigated.

Evaluation of our data leads to the conclusion that leaves of *Laurus nobilis*, besides being resistant to air pollutants, have a "strong temper" against acid rain and remain productive, at least for the period of two months that our experiment lasted. Although the atmospheric conditions in Athens metropolitan area have been dramatically improved due to the improvement of the means of mass transportation and the total adoption of catalytic technology in internal combustion engines, the use of *Laurus nobilis* –globally and wherever possible– for environmental restorations or reforestations, would be of great help for improving the atmosphere of "uncertainty" we managed to develop on our planet.

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