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TEM INVESTIGATION OF TANNINS AND CHLOROPLAST STRUCTURE IN NEEDLES OF DAMAGED SILVER FIR TREES (*ABIES ALBA* MILL.)

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As part of investigations on the effect of air pollution on plant anatomy, we examined the condition of tannins and chloroplast structure in damaged fir trees (*Abies alba* Mill.) at Risnjak National Park. Two populations were chosen for needles sampling: one at the Risnjak site with trees having 20%, 45% and 85% damage, and the other at the Donja Dobra site (control locality) with relatively healthy trees having 5% to 10% damage. Current and previous-year needles were fixed with glutaraldehyde and osmium tetroxide, and then the condition or ultrastructure of tannins and chloroplasts were examined by transmission electron microscopy (TEM). The tannins were shown to be granular and arranged in thin or thick ribbons. The chloroplasts were first somewhat rounded and then round or irregular in shape, with reduced and swollen thylakoids, especially those of the grana, increased numbers of plastoglobuli, large lipid droplets/accumulations, and vesiculation of the cytoplasm. These symptoms were more frequent in previous-year than in current-year needles. All these alterations can be attributed to air pollution.

Key words: Tannins, chloroplasts, transmission electron microscopy, air pollution.

INTRODUCTION

This work continues our investigations of needles of damaged silver fir trees (*Abies alba* Mill.) from two sites in the Gorski Kotar region.

The Gorski Kotar region is the part of Croatia between the Mediterranean and continental area, widely investigated and well described by many researchers due to its air pollution, particularly sulphur dioxide pollution (e.g., Opalički, 1972; Spaić, 1972; Glavaš, 1987, 1992a,b; Durbešić and Kerovec, 1990; Prpić, 1990; Komlenović and Rastovski, 1992; Vrbek and Gašparac, 1992; Kušan et al., 1993; Bačić and Popović, 1998).

Two sites were chosen for the study: Risnjak, a natural forest in the national park, and Donja Dobra, the control locality, a planted site. Both forests are ~150 years old.

The Risnjak habitat is a polluted site, located at 700 m a.s.l., on a dolomite base. Damage to the silver fir trees is very evident; in a sequence of ten trees it ranged from 20% to 85% (Roša, 2001). The sulphur dioxide concentration during 2000 and 2001 was high, ranging from 41.3 to 47.5 $\mu g/m^3$ (Amantaurić, 1981; Miko et al., 2000). Spectrophotometric analysis of soil elements (Miko et al., 2000) indicated the presence of many metals (heavy metals Pb and Ni, micronutrients Zn, Mn, Mo, macronutrient Mg). There was much sulphur in the soil also. Lower content of chlorophyll *a* and *b* as well as total chlorophylls and carotenoids in the needles was also recorded there (Bačić et al., 2003). Microanalysis (EDAX) of element weight percentages also suggests element accumulation there in high amounts not characteristic for very young current-year needles (unpublished results). The hydrometeorological data show that Risnjak is a relatively cool habitat corresponding to its altitude.

The forest at the Donja Dobra site, the control locality, looked relatively healthy. It is situated at a

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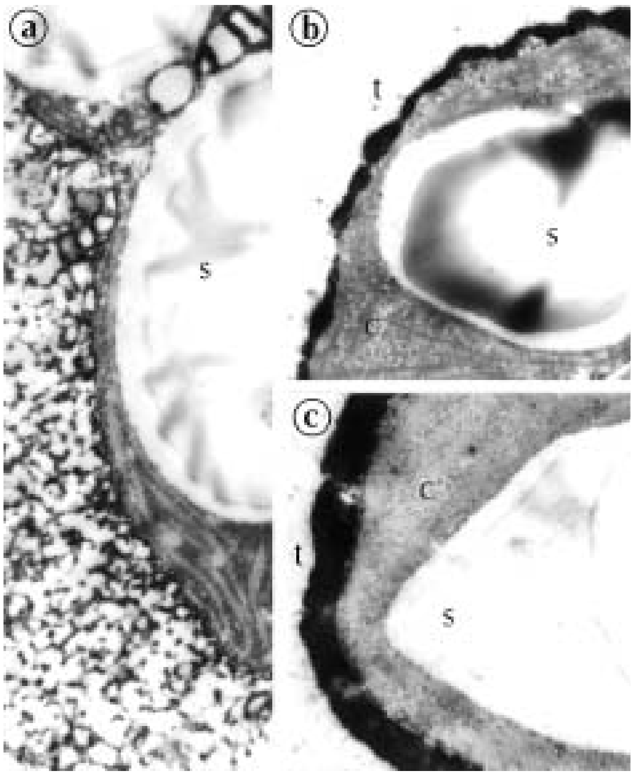

Fig. 1. Mesophyll cells with part of chloroplasts and central vacuole of silver fir (*Abies alba* Mill.) trees from the Risnjak habitat. (**a**) Previous-year needles (tree with 20% damage). Granulated and scattered tannin particles in central vacuole. × 15,000, (**b**) Current-year needles (tree with 45% damage). Tannin in form of thin ribbon at border of central vacuole. × 14,000, (**c**) Previous-year needles (tree with 85% damage). × 19,000. s – starch grains; t – tannin; c – chloroplasts.

lower altitude, 570 m a.s.l., also on a dolomite base. Here the estimated damage to ten silver fir trees varied from only 5% to 10% (Roša, 2001). The sulphur dioxide concentration during 2000 and 2001 was considerably lower than at the Risnjak site, ranging from 16.5 to 23.9 $\mu g/m^3$. Spectrophotometric data on soil elements (Miko et al., 2000) indicate the presence of Al, Na, K, Ba, Ti, V and others, but no heavy metals and sulphur (Bačić et al., 2003). The content of chlorophyll *a* and *b*, total chlorophyll and carotenoids in needles was higher here than at the Risnjak site, with no signs of defoliation or chlorosis. Microanalysis (EDAX) of element weight percentages indicates lower element accumulation and contamination, particularly in previous-year needles (unpublished results). Hydrometeorological data do not differ significantly from those of the Risnjak site.

All these data show Risnjak as the more polluted habitat, and Donja Dobra as comparatively healthy.

MATERIALS AND METHODS

At two sampling sites the needles were taken exclusively from fir trees (*Abies alba* Mill.) in which stages of damage had been determined earlier (Roša, 2001), and sampling was done about three times per year. At Risnjak the samples were taken mostly from the trees with three damage stages: relatively healthy trees with 20% damage, moderately healthy trees with 45% damage, and severely diseased trees with 85% damage, visibly due to pollution. At the Donja Dobra site, samples were taken from two categories of trees: almost healthy trees with only 5% to 10% damage, and trees without any visible signs of damage. current and previous-year needles were sampled from both populations. For ultrastructure studies, small pieces of tissue from 3–5 needles were fixed for 4 h in 2% glutaraldehyde in cacodylate buffer, pH 7.2. After rinsing in the same buffer, the material was postfixed for 2 h in 1% OsO_4. The fixed material was dehydrated in ethanol. Fixation, postfixation and dehydration procedures were performed at 1˚C. After dehydration the ethanol was replaced by propilene oxide and the tissue was embedded in Araldite and polymerized at 65˚C. Ultrathin sections were cut on an RMC MT 6000-XL ultramicrotome, then stained with uranyl acetate and lead citrate. The sections were examined with a Zeiss EM 10 A transmission electron microscope.

RESULTS AND DISCUSSION

In both habitats we observed various types of damage within the cells of needles from the trees with different percentages of damage, both in current and in previous-year needles.

Different stages of cell damage could be distinguished by light microscopy. Changes in the structure of tannins were noted in needles from the Risnjak habitat. In very young, nearly healthy-looking current-year needles from trees with 20% damage, we found granular tannins (Fig. 1a) in thin or thick ribbons around vacuoles (Fig. 1b,c). Thick ribbons of tannins predominated in needles from trees with moderate (45%) damage and particularly from trees with severe (85%) damage (Fig. 1c). In previous-year-needles from all trees with 20–85% damage, tannins were present exclusively in the form of thick ribbons (Fig. 1c).

In specimens from the control habitat of Donja Dobra, tannins occurred in the form of granules or scattered particles in nearly healthy-looking cur-

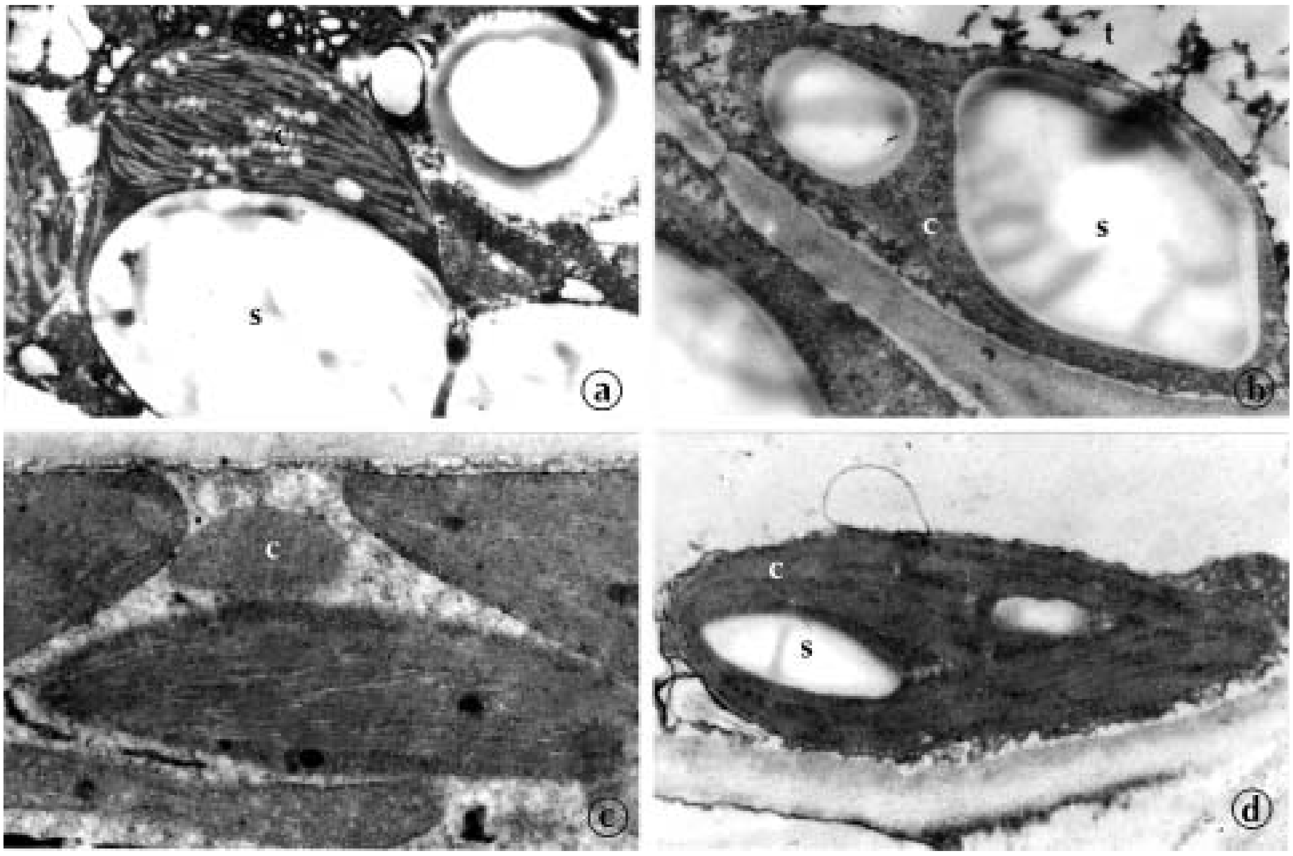

Fig. 2. Chloroplasts from mesophyll cells of silver fir (*Abies alba* Mill.) trees from Risnjak (**a,b**) and Donja Dobra (**c,d**) habitat. (**a**) Current-year needles (tree with 85% damage). Chloroplasts with swollen thylakoids, numerous mono-osmiophilic plastoglobuli and large starch grains. × 12,000, (**b**) Previous-year needles (tree with 45% damage). Chloroplasts with dense mono-osmiophilic stroma and large starch grains. × 18,000, (**c**) Previous-year needles (tree with 5% damage). Lens-shaped chloroplasts with dense stroma and intact grana structures. × 22,000, (**d**) Current-year needles (tree with 5% damage). Chloroplasts with typical grana structure and starch grains. × 22,000. c – chloroplasts; s – starch grains; t – tannin; p – plastoglobuli.

rent-year needles from 5% and 10% damaged trees, but in previous-year needles they were present as granules or as thin ribbons.

The observed changes are in accordance with findings recorded by Kukkola et al. (1997): in needles from nearly healthy trees, tannins appeared as granules or scattered particles; in needles from moderately damaged trees they occurred as thick ribbons, with cytoplasm appearing as a thin dark mass between the tannins and cell wall; in the third, severe stage, tannins and cytoplasm made a mixed mass. In this stage, the cell looked empty or else consisted of merely a wall and some tannins, and in this stage the mesophyll cells were collapsed. However, with good resolution the tannins in the central vacuole appeared in granular form throughout the year, or as a rather thick ribbon in the most damaged cells, or disappeared entirely (Soikkeli, 1980). In this stage the organelles were clearly distinguishable during every season (Soikkeli, 1980). Different stages of cell damage could also be seen in the same needles. All these observations point to alterations of tannin structure.

Although the presence of tannins in plant cells has been known widely, their function is still largely obscure. Synthesis and accumulation of tannins is described as a common biochemical symptom associated with plant diseases as a defense mechanism in plants (Beckman et al., 1972; Feeny, 1970; Kouki and Manetas, 2002; Maie et al., 2003.), but could also be a postmortem process (Davies, 1972). The occurrence of tannins could also be due to changes in osmotic potential as a result of air pollution (Behrens et al., 2003).

In current-year needles of nearly healthy trees from the Risnjak site with only 20% damage, the chloroplasts were intact and lens-shaped, and the grana thylakoids were large. In trees from that site with 45% and 85% damage, in current-year needles the chloroplasts were already somewhat rounded, with swollen, reduced thylakoids and large starch grains (Fig. 2a,b)

In previous-year needles of trees from Risnjak with 20% and 45% damage, we noted only somewhat rounded chloroplasts, with swollen thylakoids, particularly those of the grana; in the trees 85% damage the chloroplasts were round, with swollen thylakoids, no thylakoids within the grana, large starch grains, and curling of thylakoids in some chloroplasts (Fig. 2a).

In nearly healthy trees from the Donja Dobra site with 5% and 10% damage, the chloroplasts in current-year needles were lens-shaped, with intact grana structures (Fig. 2c,d), whereas in the previous-year needles from trees of the same damage class they were somewhat rounded, with slightly swollen thylakoids (Fig. 2a,b).

All the abnormalities were more frequent in needles from the Risnjak site, and they were more pronounced in previous-year than in current-year needles. These were probably the consequence of air pollution.

Similar and more accurate observations of chloroplasts were made by Godzik and Knabe (1973), Godzik and Sassen (1974), Soikkeli (1978), Soikkeli and Tuovinen (1979), Engelbrecht and Louw (1973) and Soikkeli (1980). They found that the envelopes of chloroplasts were damaged before any visible symptoms. In the chloroplasts were large starch grains, and the stroma and grana thylakoids were well developed. Large starch grains might be an indication of stress caused by $S0_2$ air pollution. Moreover, the stroma and cytoplasm were rich in polysomes. With time the chloroplasts became rounded, and the round form prevailed over lens-shaped ones (Mlodzinowski and Bialbok, 1977; Soikkeli, 1978; Soikkeli and Tuovinen, 1979). In addition, the appearance of swollen thylakoids caused by sulphur dioxide (Wellburn et al., 1972; Fischer et al., 1973; Soikkeli and Tuovinen, 1979) appeared to be reversible in unpolluted air. At a very late stage of cell damage, only undulating thylakoids were found by Soikkeli (1978, 1981a). At the same time, thylakoids and chlorophyll content were reduced. Another type of damage was curling of thylakoids. According to some authors, this curling could be a secondary effect caused by dehydration of stroma, or could be caused by both frost and pollutants (Soikkeli, 1981a,b; Soikkeli and Tuovinen, 1979). On the other hand, Engelbrecht and Louw (1973) and Soikkeli and Tuovinen (1979) observed reduction of grana thylakoids, and Godzik and Knabe (1973) reported the same finding but in apparently healthy material. The accelerating effect of grana reduction was reported by Masuch et al. (1973).

Granulation of stroma as fibrillar or crystalloidal structures has often been analyzed in relation to air pollutants (Fischer et al., 1973; Soikkeli, 1981a), probably as the effects of pollution on stroma, such as inhibition of many enzymes involved in CO_2 fixation, and as a secondary effect due to the dehydration of stroma.

According to the cited authors, in the chloroplasts there were increased numbers of plastoglobuli, particularly electron-transparent ones, and large lipid droplets, as well as vesiculation of cytoplasm and an increase in polysomes; these are often called general stress symptoms. In our case, the changes, especially the vesiculation of cytoplasm in needles from the Risnjak site, are probably induced by SO_2 pollution and deposition of acidic moisture.

These observations are confirmed by findings of increased plastoglobuli (Soikkeli, 1981a,b), particularly in needles collected from the sulphur-polluted area. Accumulation of lipid-like materials as a sign of disturbed lipid metabolism, and decreases in polysomes as a sign of reduced protein synthesis, have been evaluated and described by many authors (Fischer et al., 1973; Soikkeli, 1981a).

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