STRUCTURAL CHANGES IN SILVER FIR NEEDLES IN RESPONSE TO AIR POLLUTION

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Abstract. In this paper the structure of the two years old silver fir leaves from polluted and clean sites was comparatively investigated. Under the action of heavy metals (especially Pb, Cu and Zn), important accumulations of tannin and polyphenolic compounds were noticed in epidermis and hypodermis cells and in assimilatory tissues. The transfusion parenchyma was severely affected in some needles of plants growing in polluted areas. With some exceptions, the epidermis preserves its integrity. The conductive tissues, especially the phloem, are affected by the pollutants. These features suggest a massive translocation of heavy metals through the vascular bundles. The micromorphological investigations show different degrees of degradation of wax tubules from the epistomatal ring. The amorphous wax could be observed on the needles from polluted sites.

Keywords: anatomy, heavy metals, leaf, pollution, *Abies alba* Mill.

INTRODUCTION

European silver fir (*Abies alba* Mill.) is an important tree in Central and Eastern European forests. It is a mountain tree but sometimes it occupies lowlands, especially in the northern parts of its distribution. In Europe, silver fir occurs in smaller and bigger groups following the distribution of mountain ranges. The geographical distribution of silver fir is limited mainly to European mountains: the Alps, Carpathians, Apennines and Pyrenee.

The area of silver fir forests and its percentage of the forests decreased significantly during the last 200 years in most European countries. Reasons for this decline are related to the human impact, through deforestation, over-exploitation, promotion of faster growing tree species, clear-cut forestry, improper management and air pollution [20]. Symptoms are manifested by needle browning, early defoliation and stand mortality [12].

The emission of air pollutants from the various industrial and social activities has a large impact upon the vegetal organisms growing in these areas. The main sectors emitting air pollutants are road transport, power and heat production sectors, industry and agriculture [20].

Histo-anatomical studies and investigations regarding the modifications that occurred in tree's leaves under the effect of air pollutants are numerous [1, 5, 9, 13, 16, 18, 19]. Reports on histological investigations regarding the response of conifer leaves to the pollution with heavy metals are very scarce [11]. Micromorphological changes in leaf epicuticular wax under the influence of air pollutants were investigated in different *Pinaceae* species [15, 17] and also in *Abies alba* [2, 3]. Generally, air pollutants (ozone, heavy metals and acid rains) induced different degrees of injuries and an accelerated structural degradation of the epicuticular wax [17]. The reaction of different species to the altered environmental conditions is strongly correlated with their structural and functional features. Studies show that under the action of pollutants, plants develop different morphological and anatomical changes [9, 11, 16].

Heavy metals are enriched in the environment by various human activities. They are transported by air and water and ultimately reaching the soil and the sediments, where they become bound [8]. Their presence induces morphological and structural changes in the plants organs; poorly mobile elements (such Cr, Cu, Pb) directly injure the roots and only indirectly the leaves; mobile elements (Cd, Ni, Zn) can affect foliage directly and thus cause more visible symptoms [11].

In this paper the structural and micromorphological changes from *Abies alba* leaves collected from polluted sites (from the adjacent aria of the Ceahlău National Park, NE Romania) and from the park area were investigated. The simultaneous effects of some heavy metals were followed.

MATERIALS AND METHODS

1. Material sampling

The vegetal material consists of two years leaves of *Abies alba,* which have been collected from Ceahlău National Park (Romania, Neamț Country) and its adjacent area (Fig. 1) in July 2008. The control sample

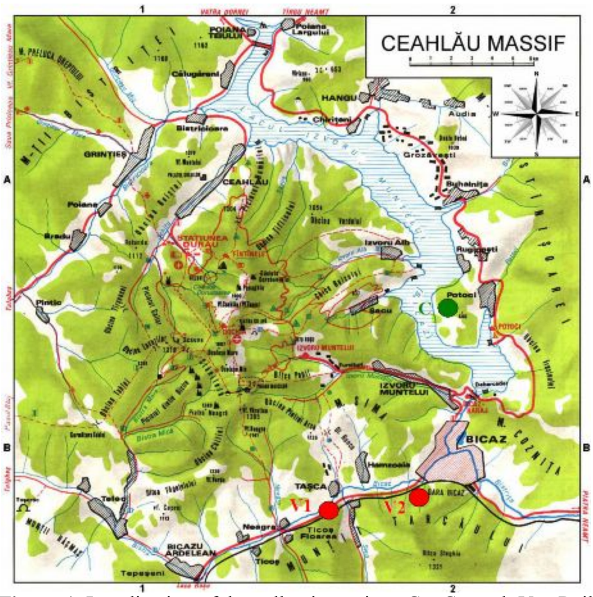

Figure 1. Localization of the collection points: C – Control, V_1 - Rail station Tasca , V_2 - carrying station of a cement factory (image source: http://www.ceahlau.neamt.ro/).

(M) was collected from the protected area of the park, whereas the test samples were taken from the Taşca railway station (V_1) and from the neighboring carrying station of a cement factory (V_2).

The parameters regarding air pollutants present in the area were obtained from Neamț Environmental Protection Agency (Table 1).

Table 1. Concentrations of air pollutants in the investigated areas (source Annual Report of Neamț Environmental Protection Agency).

Pollutant type / Colection point	Dust sus-pendsions (mg/m^3)	NO_2 (µg/m^3)	SO_2 (µg/m^3)	Pb (ppm)	Cd (ppm)	Zn (ppm)	Cr (ppm)	Cu (ppm)	Co (ppm)
Control (protected area) (M)	0.021	5.651	3.719	45.31	0.214	210.05	4.05	27.02	3.59
Rail station Tasca (V_1)	0.121	17.152	5.164	104.11	0.798	887.06	21.31	122.90	20.85
Carrying station (V_2)	0.281	20.974	6.833	138.30	2.817	1673.72	78.84	193.07	41.91
Allowed limits (Council Directive 96/62/EC/1996)	-	200	2.5	100	2	800	-	140	-

2. Histo-anatomical investigations

The leaves were fixed in formalin/ethanol/acetic acid/water (FEA; 4:69:5:22 volume to volume) and conserved in 70% ethanol. Free hand sections were performed using a razor blade. The sections were observed without coloration, in order to show the phenolic and tannin deposits from different tissues, colored with ruthenium red and iodine green or with vanillin (10% in ethanol)/ HCl for phenolic compounds identification. The observations were made in normal or polarized light. The photos were taken with an Olympus E-330 photo camera, using an Olympus BX51 research microscope.

3. SEM investigations

The median region of 10 leaves from each side was cut, dried in open air for 24 hours [3, 17], mounted on double sided adhesive carbon discs and sputter-coated with gold/palladium (using K500X Emitech). The investigations were done using Tescan Vega II SBH Scanning Electron Microscope at an acceleration voltage of 27.88 kV.

RESULTS

Histo-anatomical investigations

The leaves of silver fir tree from polluted sites show moderately visible injuries. They appear as brown spots along the needles; the affected leaves are no more than 20 percent from the total.

The histo-anatomical features of the control leaves are similar with those described in the literature (Fig. 2a). The epidermis consists of a single layer of cells. The cell walls are thick and strongly lignified. The hypodermis is also unilayered, discontinuous especially in the vicinity of the stomata. The mesophyll is differentiated in palisade parenchyma (bi-layered under the upper epidermis) and spongy parenchyma (under the lower epidermis). In all assimilatory cells, and especially in spongy parenchyma cells, numerousness crystals of calcium oxalate are visible (Fig. 2b). They are located in the exterior of the cell wall. The extracellular localization of the calcium oxalate crystals in *Pinaceae* leaves was noticed by Fink (1991) [7] in *Picea abies*.

In case of the needles from polluted areas, the sections were carried out through the visibly injured regions. The epidermis cells and the stomata are sometimes covered by solid deposits (Fig. 4d). In many samples the epidermis cells, as well as the stomata cells (guard and subsidiary cells) are full of tannin (especially from V_2), whereas in other samples they exhibit thick external walls and a wide lumen. Rarely, crushed cells are visible (in the region of the mid vein, in lower epidermis – top of the leaf from V_2) (Fig. 3e). The hypodermis is not especially affected; occasionally, the lumen is also filled with tannin (Fig. 2e, 2f). The injuries that occurred in mesophyll cells are extended, affecting both palisade and spongy parenchyma (Fig. 2c, 2e, 2f, 4c). They begin with the protoplast alteration and the increase in thickness of the cellular walls (Fig. 3c, 3d). The injured areas from the assimilatory tissues contain elevated amounts of phenolic compounds (Fig. 3a) (histochemical identification with alcoholic solution of vanillin/ HCl was performed – Fig. 4a, 4b). In other areas, variable deposits of tanin were observed (Fig. 2e, f).

The frequency of the calcium oxalate crystals is increased in the needles from polluted sites (Fig. 2d). They are visible inside the cells, along the walls and precipitated outside the mesophyll, connected to the external parts of the cell walls or in the intercellular spaces.

The central cylinder, especially the transfusion parenchyma, shows the highest degree of alterations (Fig. 2e). Numerousness cells (more from their abaxial part) are crushed and large lacunas appear in their place. The phloem from vascular bundles in the V_1 and V_2 needles is also changed in appearance: the walls become sinuous and slightly thicker, the cell lumen becomes narrow (Fig. 2e, 3a). In other leaves (Fig. 3b) from V_2 samples, a visible hypertrophy of the phloem could be noticed. These are the results of an intense cambial activity on the phloemic part, followed by an increase of the number of sieve cells layers and the modification in the phloem shape. However, this kind of modification is not common for the injured needles from polluted sites. Sometime, in V_2 samples strongly damaged leaf, probably consecutively of insect attack with mesophyll cells fulfilled with tannin and large areas of necrosis in assimilatory tissues could be observed (Fig. 3f).

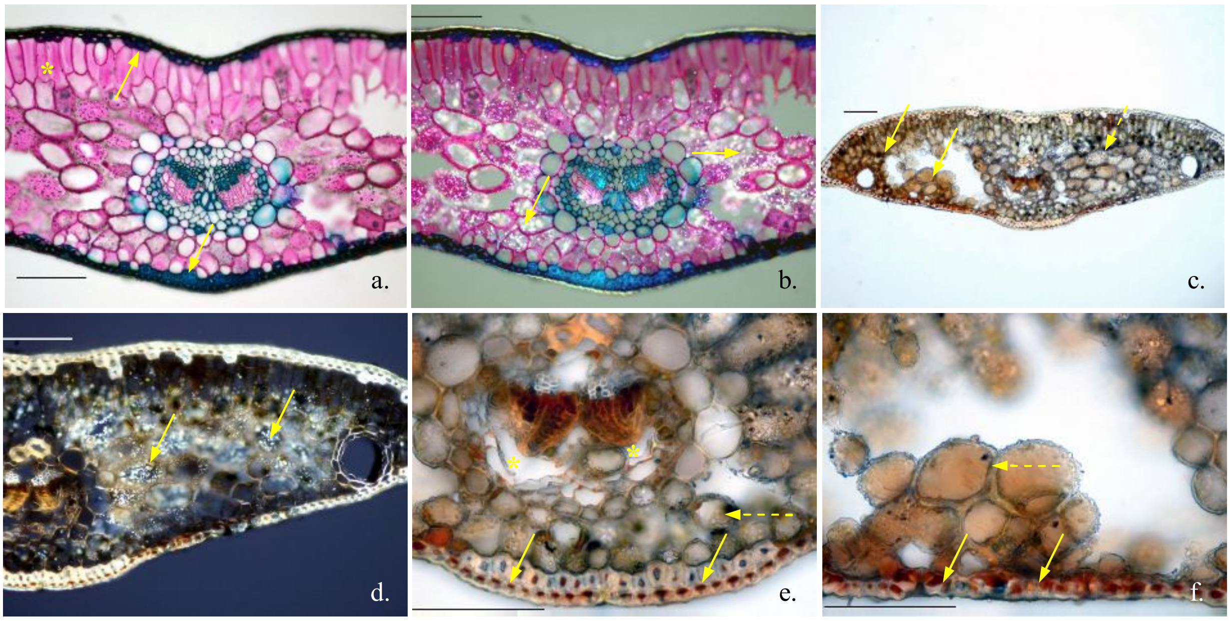

Figure 2. **a** – Crossection through the median part of a needle from control area. Intact structure could be observed; the hypodermis discontinuous (arrow) both under the lower and the upper epidermis, the palisade parenchyma is unilayered. **b –** The same section view in polarized light. Calcium oxalate crystals could be observed linked by the external walls of the cells, especially in spongy parenchyma (arrows). **c** – Crossection through the median part of an injured needle from V_1 samples. Tannin accumulation (arrow) and polyphenolic compounds (green arrow) both in palisade and spongy parenchyma are indicated. **d** – The same section (higher magnification and polarized light). Numerousness calcium oxalate crystals (arrows) could be observed inside and outside of the cells. **e -** Crossection through the median part of an injured needle from V_1 samples. Lower epidermis and hypodermis cells are fulfilled with tannin (arrows); in spongy parenchyma cells dark spots – polyphenolic compounds (dotted arrow) could be observed; the central cylinder structure is altered – the transfusion tissue is collapsed (star) and the phloem from vascular bundles is hypertrophied. **f –** Detail from the lateral inferior part of the needle – all epidermis cells (included stomata) and hypodermis cells are fulfilled with tannin (arrows); the cell walls of the necrotic spongy parenchyma cells are very thick (dotted arrow) (scale bars = 100 µm).

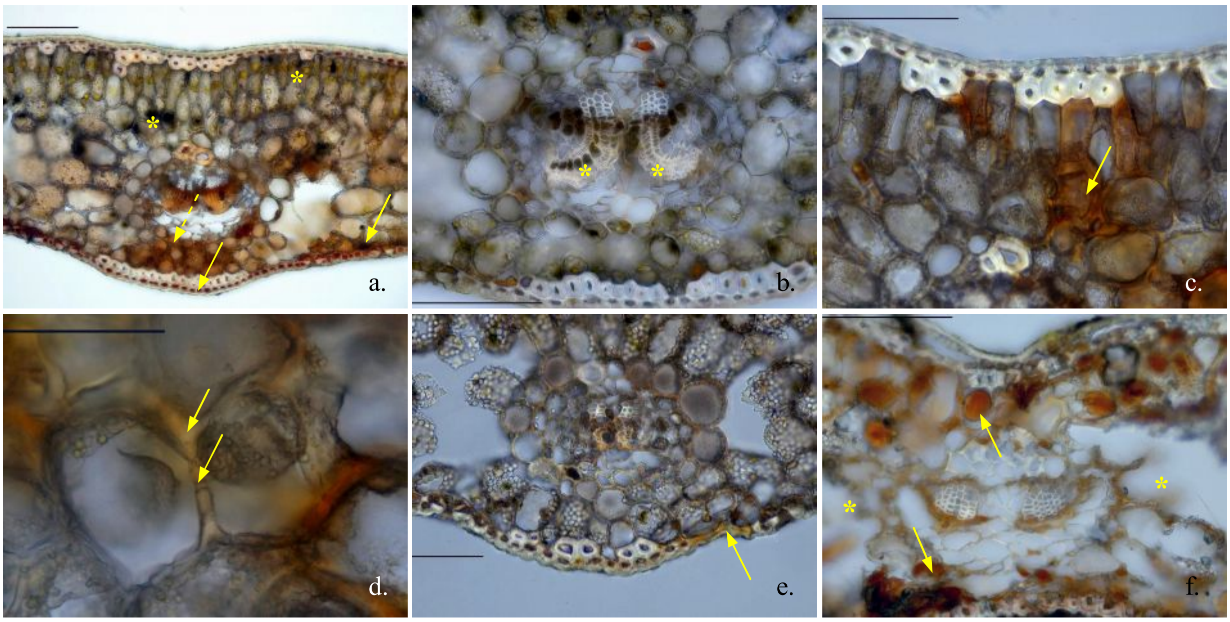

Figure 3. Crossections through the median part of a needle from V_2 area. **a** –Epidermis and hypodermis cells contains tannin (arrows), the spongy parenchyma cells located under the central cylinder are necrotic, with tannin in the walls (dotted arrow) and in some palisade parenchyma cells polyphenolic compounds (star) could be observed. **b -** Particular modification in the phloem shape from a vascular bundle (star). **c, d -** Very thick cell walls in assimilatory tissue (arrows). **e –** Crossection through the top of a needle. Crushed cells could be observed in lower epidermis (arrow). **f** – Strongly damaged leaf, probably consecutively of insect attack – mesophyll cells fulfilled with tannin (arrows) and large areas of necrosis in assimilatory tissues (stars). (a-c, e, f - scale bars = 100 µm, d – scale bar = 50 µm).

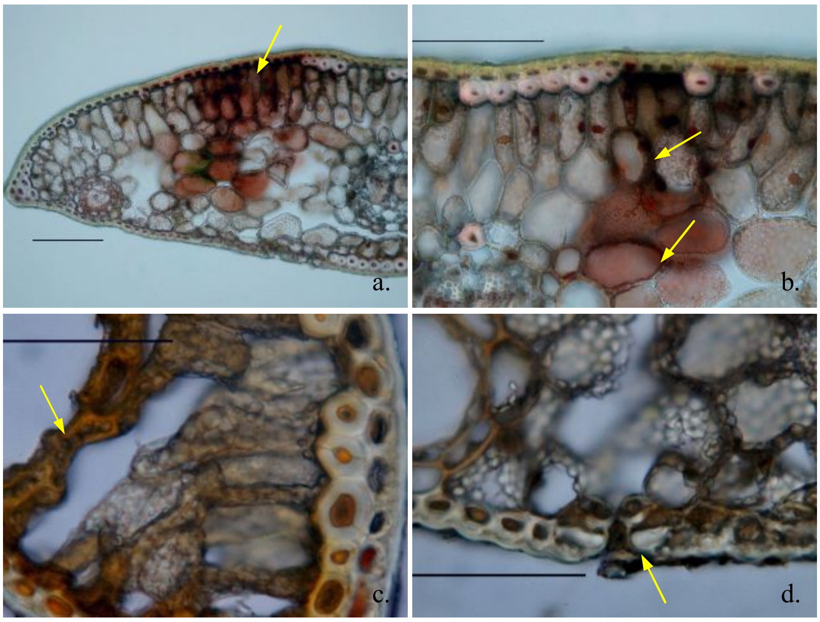

Figure 4. Crossections through the median part of a needle from V_2 area: **a, b –** phenolic compounds identification alcoholic solution of vanillin/ HCl (arrows). **c** – Crushed and necrotic cells from assimilatory tissue (arrow). **d** - Stomata covered by solid deposits (arrow). (a, b - scale bars = 100 µm, c, d - bars = 50 µm)

Micromorphological investigations

The SEM observations on the silver fir tree stomata (on two years needles from clean area) are almost completely occluded by anastomosed wax tubes, covered by small and uniformly disposed granules (Fig. 5a, 5b, 5c). On the needles from polluted sites, the majority of stomata have the stomatal antechamber covered with a compact crust of amorphous wax (Fig. 5d, 5e, 5f).

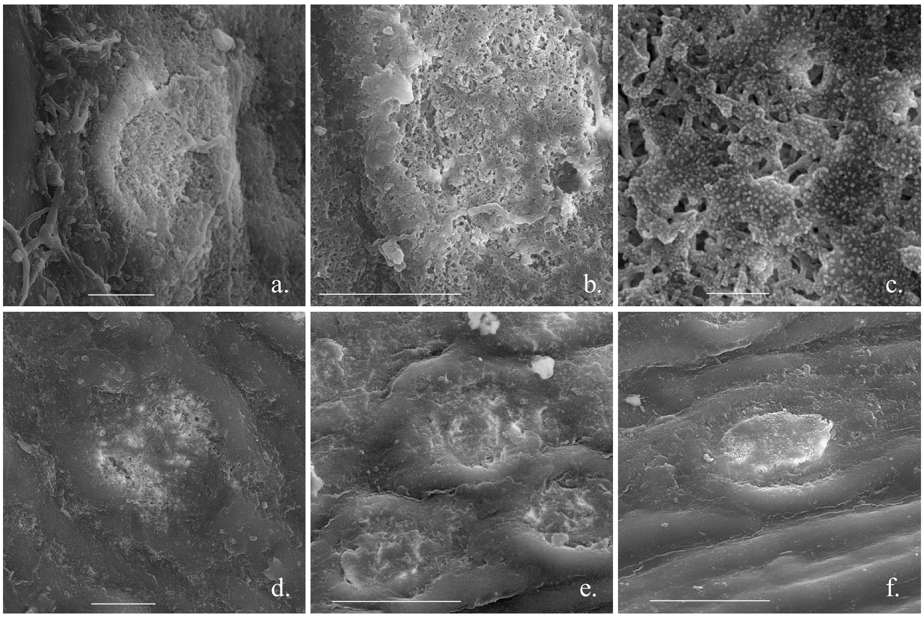

Figure 5. SEM micrographs illustrating the structure of the epicuticular wax from the stomata region. **a -** Healthy epistomatal wax - needle from control area. **b -** Detail showing partially fused wax tubules, normally for a 2 years old needle. **c -** Anastomosed wax tubes, covered by small and uniformly disposed granules. **d –** Small crust over the central part of the stomatal pore – needle from V_1 sample. **e –** Compact crust of epicuticular wax over the stomata - needle from V_1 sample. **f –** Almost complete obturated stomatal pore - needle from V_2 sample (Scale bar – a, b =20 µm, c =2 µm, d =20 µm, e, f =50 µm).

DISCUSSIONS

In the investigated areas the pollution with heavy metals is moderate. The concentrations found exceed the accepted thresholds especially for lead, zinc and copper. The presence of these heavy metals disturbs the normal plant metabolism, but the response is determined by many factors: the internal concentration of the pollutant, the level of tolerance for the pollutant or its toxic derivatives, developmental (ontogenetic) stage, edaphic and climatic factors [8, 11]. Coniferous trees, especially the silver fir *Abies alba,* are pollution-sensitive species [2, 14].

Until now, only ultrastructural investigations regarding the influence of the air pollutants (including heavy metals) were performed for silver fir tree [2, 3].

Regarding the influence of different pollutants upon the plant structure, two types of investigations are usually performed: analyses of the individuals from polluted sites (reported to a control one) or from controlled experiments where the concentrations and the modality of the administration of the tested substances are very well know. Our investigations belong to the first type of studies.

Structural changes in the silver fir tree leaves (two years age) affected especially the central cylinder and the assimilatory tissues. The transfusion tissue is frequently collapsed in the vicinity of the phloem from the vascular bundles. In a review, Gunthardt-Goerg and Vollenweider (2007) [11] notice that the toxic metals enter in leaves through the stomata and show an accumulation in gradient connected to the vein system.

The increase of cell walls thickness from assimilatory tissue is a common response of the cells to the stress caused by air pollutants, including heavy metals [11]. Necrosis involving thickening and pigmentations of the cell walls of palisade parenchyma were described by Alvarez et al., 1998 for *Abies religiosa* needles exposed to ozone. In *Abies alba* needles, wall thickening involve both palisade and spongy parenchyma. This symptom is correlated with important tannin accumulation in the affected cells.

Plants can synthesize and accumulate a comprehensive spectrum of phenolics in response to physiological stimuli and stress [6]; in analyzed samples, dark deposits of polyphenolic compounds could be observed into the cells of assimilatory tissues. Frequently, on the same section, both phenolic compounds and tannin deposit could be observed. The distribution pattern of these zones is randomly. The intense damages that occur in conducting and in assimilatory tissues are related with the pollution-sensitivity of silver fir. The capacity of some species to isolate and store the pollutants in relatively inactive tissues, away from more active and vital leaf tissues represents a detoxification response and implicitly a protective mechanism [4, 11]. This kind of reaction was not observed in silver fir needles.

The presence of heavy metals in the assimilatory tissues of the silver fir needles interfere with other elements from the normal metabolic pathways, such as calcium. This could explain high frequency of calcium oxalate crystals (also present in non-affected leaves) both in the cells and in inter-cellular spaces. As conifers generally seems to keep the calcium in the apoplast, outside the cells (in contrast with angiosperms, which precipitate the calcium oxalate into the vacuole) [7], the massive presence of the crystals inside the cells could represent an uncontrolled penetration of calcium into the cells and the reaction of detoxification. Other investigations [4, 19] shows that oxalate may also be involved in the detoxification of other hazardous metals such as lead, cadmium, and copper. In these cases the metal is incorporated into the oxalate crystals.

The exposure to the air pollutants caused changes in the cuticular wax. This is represented especially by an accelerate fusion and degradation of tubular wax structures which cover the stomatal pores. This reaction was observed in Norway spruce needles [10, 17] and in silver fir needles [2, 3]. The transformation of tubular wax into an amorphous one is normally caused by aging, but in polluted areas this process is more rapid. In the analyzed samples, the amorphous crust covers only partially the stomatal pores; Bacic et al. (2005) [3] find completely obtured stomata in polluted areas from Croatia. The environmental conditions (such as temperature) and different components of the air pollutants (organic compounds, acid rain) could influence the degree of injuries that occur at this level [11, 18].

The air pollutants from investigated area induced specific structural modifications in silver fir needles: localized thickness of assimilatory cells walls, accumulation of tannin and phenolic compounds in spongy and palisade parenchyma cells, sometime also in hypodermis; the phloem is frequently affected in needles with visible injuries. There are not histological and micromorphological differences between the samples from V_1 comparatively wit the samples from V_2. This aspect was expected, because the pollutants concentrations are similar in both investigated areas.

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