

The biochemical and histoanatomical response of some woody species to anthropic impact in Suceava County, Romania

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Abstract: The use of extractive mining technologies in various areas of Suceava, Romania (Călimani Mountains - sulfur, Tarnița-Ostra - copper and barite, and Crucea-Botușana - uranium) has resulted in the accumulation of surplus chemicals with direct action on the ecosystem. The aim of this study was to analyze the possible damage on some woody species (*Picea abies* L., *Populus tremula* L., *Salix alba* L., *Betula verrucosa* Ehrh., and *Larix decidua* Mill.) and their adaptive and phytoremediation capacity through the biomarkers of oxidative stress and histoanatomical observations. Thus, we determined the total soluble protein concentration and superoxide dismutase, catalase, and peroxidase activities. Cross-sections of the leaves were also made. The results proved the influence of anthropogenic pollution in all studied areas, but the greatest antioxidant defense system reactivity (compared to the control, the unpolluted area of Putna) was detected under the action of uranium on all woody species from Crucea-Botușana. Structural changes, such as the thickening of epidermal cuticle, the thinning of hypodermic cell walls, and the reduction of the phloem, were also observed and attributed to anthropogenic pollution.

Key words: Anthropogenic pollution, superoxide dismutase, catalase, peroxidase, histoanatomical changes, plant

1. Introduction

The exposure of plants to climatic stresses, natural edaphic factors, and the massive changes in the environment due to human activities makes it necessary to select valuable genotypes of plants, capable of rapid acclimation to pollution and endowed with a robust capacity for phytoremediation of areas. The rapid and effective antioxidant defense system of plants is one of the main criteria for the selection of these genotypes endowed with the ability to maintain an appropriate balance between the formation of various reactive oxygen species (ROS) and detoxifying abilities of the vegetal cell. This is an essential mechanism for the survival of that species, as well as for the phytoremediation of the corresponding area.

The modulatory effect of excess sulfur (S) on the enzymes composing the antioxidant system has been reported in various scientific papers (Keller, 1985; Khan et al., 2008; Oniciuc et al., 2012). Furthermore, although the data concerning plant responses to environmental contamination with uranium (U) are few, it is known that this exposure can cause an imbalance in the oxidative status of the plant cells (Vanhoudt et al., 2009). In addition, metals like iron (Fe) and copper (Cu) induce Fenton (Fe^{2+}

+ $\text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\cdot$) and Haber-Weiss ($\text{O}_2^{\cdot-} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^\cdot$) reactions, generating ROS. Other metals with redox capacity (cadmium - Cd, zinc - Zn, and mercury - Hg) are able to cause metabolic imbalances, destroying the antioxidant potential of the cell through the inhibition of oxidative stress enzymes and increasing the production of oxygen radicals (Keunen et al., 2011).

Anthropogenic S mining activity carried out in the Călimani Mountains resulted in extremely low maintenance of soil pH due to the presence of S and its oxidation reactions (sulfates over the limit of intervention). These reactions have a direct impact on transforming toxic metals from the environment in mobile ions, absorbed by vegetation from the soil. Excess Fe, Mn, Cu, Zn, Cd, and Ni have been found in previous studies, but below the level of alert (Dițoiu and Oșean, 2007).

As shown by some scientific data, emanations, liquids, solids, and gases, and especially complex polymetallic sulfides (Popa and Barbu, 2001; Lucaciu et al., 2004; Dițoiu and Oșean, 2007; Ionce, 2010), induced various degrees of injury to the vegetation that were directly correlated with distance from the source of pollution, direct visibility, and the proportion of spruce in stand composition and

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inversely correlated with altitude and the proportion of beech stand composition (Popa and Barbu, 2001). According to the data, strong interference was detected (especially around sources of pollution) and *Picea abies* L. proved to be a very sensitive species, while the least damaged was beech, probably due to a lower density.

Studies concerning heavy metal distribution in water, soil, and different plants (Petrescu and Bilal, 2006a, 2007; Petrescu et al., 2010) showed that *Abies alba* and *Picea excelsa* are excellent bioaccumulators of radionuclides in water and sediment pollution, being able to stop their migration. Other data (Oniciuc et al., 2012) attest to the influence of these radioactive elements on the metabolism of some gymnosperms and angiosperms and their involvement (with direct modulatory effect) in soil microbiome metabolism.

Research directions of this paper complement previous lines of study (Tutu et al., 2009, 2011, 2012; Oniciuc et al., 2012) and aim to analyze the activity of enzymes that are part of the foliar antioxidant system (superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX)), as they have a crucial role in the adaptability of species to environmental pollution. We also analyzed some histoanatomical aspects of the leaves in order to identify possible pollution-induced abnormalities at that level.

2. Materials and methods

2.1. Study areas and description of sampling sites

The object of our work was the analysis of some woody species from different areas of Suceava County, Romania.

This county is situated in northeastern Romania (47.58°N, 25.76°E) and has a surface of 8553.5 km². We analyzed woody species from an unpolluted area (Putna) and from areas under the influence of anthropogenic pollution (Călimani Mountains - S exploitation, Târnița-Ostra - Cu and barite ores, and Crucea-Botușana - U mining) (Figure 1).

The Putna area is situated in the northern part of Suceava County and represents an important tourist region, being included in the UNESCO patrimony. The mineral resources from this area are not significant and are represented only by mineral springs (Cocerhan, 2012).

The Călimani Mountains are situated in the southwestern area of Suceava County, the mining area occupying a surface of 374.8 ha. Mining activity started in 1969, ended in 1997, and affects the natural environment through SO₂, H₂S, and H₂SO₄ emissions. The negative impact appears to be direct and also indirect, through rainfall. This region is crossed by numerous watercourses that produce considerable soil erosion. Combined with the exfiltration of the decantation of Lake Dumitreleu, the induced effect was the extension of the affected area (Gorea et al., 2014). An environmental report issued by SC BIOSOL PSI SRL Ploiești in 2011 (16/25.03.2011, <http://biosol.ro/>) showed that the crossing waters had a strongly acidic pH (2.5–3) and an elevated sulfates concentration (573.5–1310 mg/L, while the maximum accepted value is 300 mg/L).

The Târnița-Ostra area is situated in the southern region of Suceava County. On 1 January 2007, all the activity at the copper and barite processing factory from



Figure 1. Scaled map of Suceava County: a- Control area, b- Călimani Mountains, c- Târnița-Ostra, d- Crucea-Botușana.

Târnița was stopped. In 2008, safety and consolidation works started at Târnicioara Lake, one of the most important environmental risk areas, occupying a surface area of 28.5 ha. In 2012, the authorities initiated closure and ecological works at the Târnița-Ostra mine, which are still not finalized.

Crucea-Botușana is a very large mining area in the south of Suceava County and represents the largest U reserve in Romania. In addition to that, the pollution is amplified by the presence of other elements like thorium, vanadium, strontium, and lead. In the waters from the Crucea area, more than 85% of the U was present as carbonate complexes and 17% as hydroxide complexes. The concentration of U in the soil reaches 7.26 mg/g (Petrescu et al., 2010).

2.2. Sample collection

Investigations were carried out in leaf material harvested once a week for three consecutive weeks in May 2013. Plant samples were taken from five sites in the proximity of each mine. In each location, three woody species 3–5 years of age were randomly marked; all the samples were collected from the same individuals. In the control area, the samples were collected the same manner, the reference site being Putna Monastery.

The plant material was identified by specialists from the Department of Biology of Alexandru Ioan Cuza University, Iasi, and was represented by the species *Picea abies* L., *Populus tremula* L., *Salix alba* L., *Larix decidua* Mill., and *Betula verrucosa* Ehrh. After collection, the plants were transported and maintained at a low temperature until biochemical determinations. For the histoanatomical study, the plant material was preserved in 70% v/v ethyl alcohol. Voucher specimens (BC-B 1–20) were deposited in the biochemistry laboratory of the same department.

2.3. Biochemical investigations

2.3.1. SOD (E.C. 1.15.1.1)

SOD was measured according to the method of Winterbourn et al. (1975) with minor modifications (Artenie et al., 2008). Enzyme activity was determined thanks to the capacity of this enzyme to inhibit nitroblue tetrazolium reduction by superoxide anions generated after riboflavin photoreduction. The decrease of the absorbance was read at 560 nm using a Shimadzu UV-VIS 1700 spectrophotometer (Japan).

2.3.2. CAT (E.C. 1.11.1.6)

CAT activity was determined using the method of Sinha (Artenie et al., 2008). The method is based on colorimetric determination (at $\lambda = 570$ nm) of chromic acetate obtained through reduction of potassium dichromate in acid medium by the hydrogen peroxide that remains after enzyme inactivation.

2.3.3. POX (E.C. 1.11.1.X)

POX activity was measured according to the method of Gudkova and Degtiari (Artenie et al., 2008). POX catalyzes the reaction between o-dianisidine and hydrogen peroxide and the formation of a colored product with an absorption maximum at $\lambda = 540$ nm.

2.3.4. Total soluble protein

The total soluble protein content, expressed as mg/mL, was determined using Bradford's method with bovine serum albumin as the standard (Bradford, 1976; Cojocaru, 2009; Cojocaru et al., 2009).

2.4. Statistical analysis

All the investigations were made in triplicate. The results are expressed as means $\pm$ standard deviations. The differences between the control and the samples obtained from polluted areas were compared with the Student t-test using standard statistical packages (Microsoft Excel). The results were considered significant if the P-value was less than 0.05.

2.5. Histoanatomical investigations

Cross-sections were made from the leaves with the help of a botanical razor. The sections were later stained with iodine green and carmine red and analyzed with a Novex microscope (Euromex, the Netherlands). The samples were subsequently photographed with a Sony Cybershot DSC-W730 camera (Japan).

The reagents used for all determinations were of analytical purity and were purchased from Sigma Aldrich (Germany, USA) and Merck (Germany). For all the solutions, ultrapure Milli-Q water was used.

3. Results

3.1. Biochemical determinations

According to the experimental results (Figure 2), SOD effectively acts as a regulator of oxidative stress, especially in the case of coniferous species from the Călimani Mountains. *Larix decidua* Mill. displayed an activity of 10.349 ± 0.441 U/mg protein ($P < 0.001$) and *Picea abies* L. of 9.564 ± 0.363 U/mg protein. In terms of the reaction of the foliar antioxidant system to the presence of superoxide in angiosperm species, *Salix alba* L. had the most activity (8.771 ± 0.491 U/mg protein, $P < 0.001$), followed by *Populus tremula* L. (8.034 ± 0.538 U/mg protein) and *Betula verrucosa* Ehrh. (7.612 ± 0.446 U/mg protein, $P < 0.05$). This response, regarded as an adaptation of the analyzed species to the human factor, confirms the idea that SOD is an effective biochemical indicator of S pollution.

As seen in Figure 3, CAT activity in the Călimani Mountains exhibited a significantly higher amplitude in species of angiosperms than in conifers, as follows: 24.239 ± 4.732 U/mg protein ($P < 0.05$) in *Salix alba* L., 21.359 ± 3.423 U/mg protein ($P < 0.05$) in *Betula verrucosa* Ehrh., and

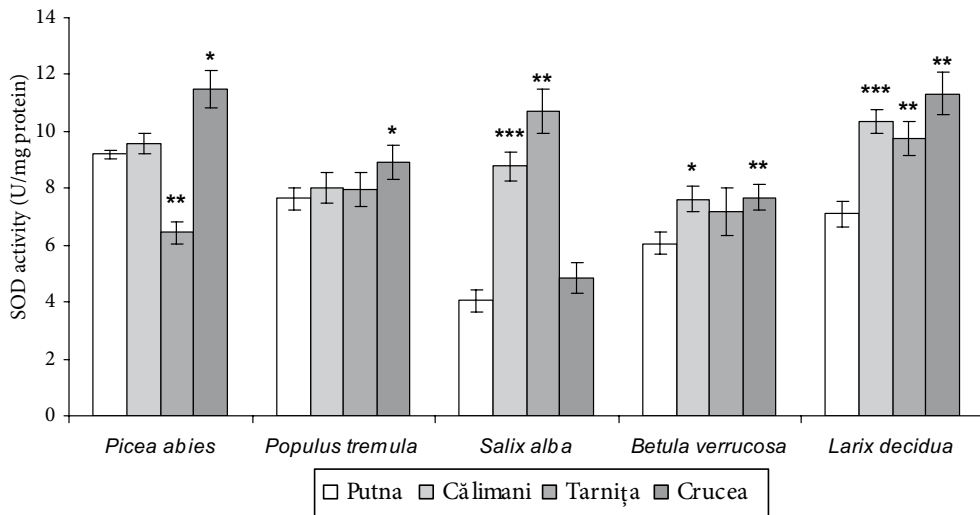


Figure 2. Superoxide dismutase activity in leaf material of some woody species collected from different areas of Suceava County (*P < 0.05, **P < 0.01, ***P < 0.001). The results are expressed as means of three parallel determinations $\pm$ standard deviations.

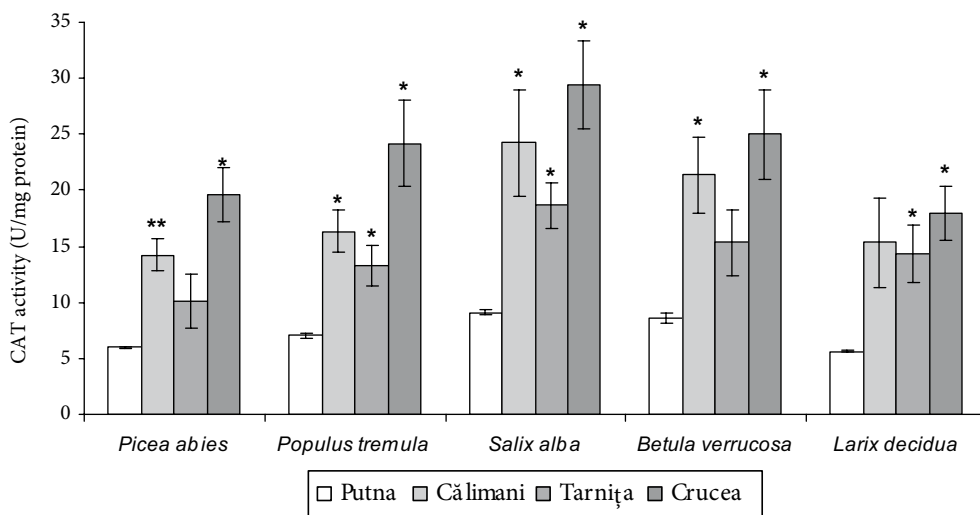


Figure 3. Catalase activity in leaf material of some woody species collected from different areas of Suceava County (*P < 0.05, **P < 0.01). The results are expressed as means of three parallel determinations $\pm$ standard deviations.

16.349 ± 1.892 U/mg protein ($P < 0.05$) in *Populus tremula* L. This suggests that the S present in the environment has the ability to increase plant photorespiration mediated by glycolate oxidase and/or to activate enzyme systems, such as xanthine oxidase coupled with SOD.

Increased POX activity was observed in *Salix alba* L., *Populus tremula* L., and *Picea abies* L. from Călimani (2.213 ± 0.311 U/mg protein with $P < 0.01$, 1.756 ± 0.189 U/mg protein with $P < 0.01$, and 1.256 ± 0.201 U/mg protein, respectively) (Figure 4). This suggests the importance of the enzyme in general plant metabolism and its involvement in processes other than the reduction of peroxide levels in

the leaves of the mentioned species. In the leaves of *Larix decidua* Mill. and *Betula verrucosa* Ehrh. (0.447 ± 0.072 U/mg protein with $P < 0.05$, and respectively 0.556 ± 0.139 U/mg protein), the enzyme profile was different, with POX activity levels being significantly lower, suggesting only limited peroxidative participation due to anthropogenic interference. Regarding POX activity in the leaves of larch and birch, we can consider that POX, in the presence of excess S in the environment, limits the activity of oxide reductase on small amounts of H_2O_2 .

SOD activity in a U-polluted area (Crucea) was calculated as follows: *Salix alba* L., *Populus tremula* L., and

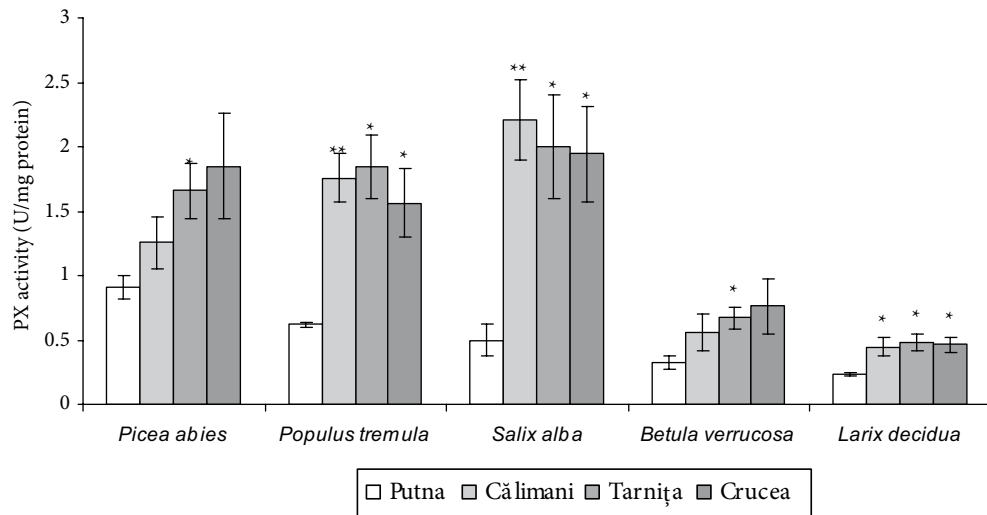


Figure 4. Peroxidase activity in leaf material of some woody species collected from different areas of Suceava County (* $P < 0.05$, ** $P < 0.01$). The results are expressed as means of three parallel determinations $\pm$ standard deviations.

Betula verrucosa Ehrh. expressed SOD activity of 4.851 ± 0.525 U/mg protein, 8.908 ± 0.587 U/mg protein ($P < 0.05$), and 7.685 ± 0.421 U/mg protein ($P < 0.01$), respectively, while conifers exhibited higher levels of enzyme activity compared to angiosperms, with infinitesimal differences between them (11.471 ± 0.647 U/mg protein with $P < 0.05$ in *Picea abies* L. and 11.337 ± 0.753 U/mg protein with $P < 0.01$ in *Larix decidua* Mill.) and significant differences compared to species of the control site (Putna).

A high accumulation of hydrogen peroxide was also detected in the leaves of *Salix alba* L., *Betula verrucosa* Ehrh., and *Populus tremula* L. from Crucea, reflected by the higher levels of CAT activity (29.347 ± 3.918 U/mg protein, 24.986 ± 4.041 U/mg protein, and 24.169 ± 3.864 U/mg protein, $P < 0.05$, respectively) than in *Picea abies* L. (19.647 ± 2.413 U/mg protein, $P < 0.01$). This shows an intense photorespiration in the presence of U in angiosperms, compared to the studied gymnosperms. In *Larix decidua* Mill., CAT activity was limited to just 17.894 ± 2.415 U/mg protein ($P < 0.05$).

The barite presence and the metal ions coming from polymetallic sulfide transformation (found in excess in Tarnița) have created an excessive accumulation of superoxide. This accumulation was demonstrated by a significantly reduced activity of SOD in *Picea abies* L. (6.436 ± 0.394 U/mg protein) compared to the control group (9.195 ± 0.166 U/mg protein).

SOD sensitivity to environmental factors was detected in *Betula verrucosa* Ehrh. (7.177 ± 0.811 U/mg protein) and *Populus tremula* L. (7.943 ± 0.581 U/mg protein). The threshold of its activity was slightly higher than that detected in the control area at the same species. *Larix decidua* Mill. (9.757 ± 0.615 U/mg protein, $P <$

0.01) and *Salix alba* L. (10.696 ± 0.783 U/mg protein, $P < 0.01$) exhibited an increase of SOD activity, suggesting the higher adaptability of these two species to excessive polymetallic interference in the environment. This statement is also supported by the activities of the other two oxide reductases studied in this paper.

Photorespiration mediated by the massive presence of polymetallic ions from the Tarnița site proved to have a lower amplitude than that under the influence of S in Călimani and U in Crucea-Botușana. The level of CAT activity in samples taken from the site contaminated with polymetals (Tarnița) was strongly increased in *Picea abies* L. (10.116 ± 2.42 U/mg protein), *Populus tremula* L. (13.267 ± 1.875 U/mg protein, $P < 0.05$), and *Betula verrucosa* Ehrh. (15.316 ± 2.919 U/mg protein), twice increased in *Salix alba* L. (18.648 ± 2.061 U/mg protein, $P < 0.05$), and higher in *Larix decidua* Mill. (14.316 ± 2.513 U/mg protein, $P < 0.05$) as compared to the control group. Fe, shown to be in excess in Tarnița soil, is able to double the activity of CAT according to some authors (Kampfenkel et al., 1995).

The behavior of POX was the same as in the case of CAT. In Tarnița, *Salix alba* L. had the highest level of POX activity (2.003 ± 0.402 U/mg protein, $P < 0.05$), followed by *Populus tremula* L. (1.846 ± 0.249 U/mg protein, $P < 0.05$) and *Picea abies* L. (1.661 ± 0.214 U/mg protein, $P < 0.05$). It appears that, in the case of *Betula verrucosa* Ehrh., under the influence of interfering polymetallic ions in the environment, the rate of synthesis, degradation, or covalent modification of the enzyme changed. Its activity level was only 0.673 ± 0.082 U/mg protein ($P < 0.05$), while it was 0.479 ± 0.065 U/mg protein ($P < 0.05$) in *Larix decidua* Mill. leaves.

3.2. Histoanatomical observations

A microscopic analysis of the studied species was made and the most significant leaf sections are described below. The *Picea abies* L. leaf section had a bifacial-heterofacial structure, while in cross-section the outline was irregular. The epidermis showed isodiametric or slightly high cells, with all walls thickened, the external one being cutinized and waxed. This fact draws attention, as data from the scientific literature (Turunen et al., 1997) revealed an increase of inconsistent areas in conifer needles exposed to SO₂ pollution, as exposure to acid rain resulted in the destruction of epicuticular wax structures by hydrolysis of alkyl esters. The hypodermis was bilayered in angles and monolayered, sclerenchymatous, and composed of cells with thickened walls otherwise (Figure 5a). The mesophyll was homogeneous, with elongated polygonal cells, perpendicular to the central cylinder, with two opposed resiniferous channels. Each channel was surrounded by a layer of sclerenchymatous cells in direct contact with the hypodermis. The central cylinder was on the outside of an endoderm of the first type (with Casparian points in the side walls of the cells), with the cells strongly elongated tangentially, a monolayered pericycle consisting of slightly elongated cells, and two phloem-xylem vessels (Figure 5b). At the periphery of the phloem were several (5-7) sclerenchymatous cells (Figure 5c).

In *Populus tremula* L., the contour of the cross-section through the petiole was elliptical, of different thickness at the two poles (Figures 6a and 6b). The epidermis had thick-walled cells, the external one being covered by an extremely fine cuticle. Below the epidermis was an area of collenchyma tissue, thicker on one side (4-5 layers). In samples collected from Tarnița, in the fundamental

parenchyma outside the petiole, many cells were disorganized, resulting in numerous cavities of different shapes and sizes. The collenchyma cell walls were not as thick. Transport vessels, of different sizes, were grouped into three centers, all having a lot of periphloem, sclerenchyma cells, and wood arranged in the front (Figure 6b).

The fundamental parenchyma was of meatic type; air gaps could be observed here and there under the area of hypodermic collenchyma cells (Figure 6c). Some cells contained calcium oxalate. In the fundamental parenchyma there were many collateral vascular bundles, each containing xylem and phloem, grouped into four centers, each with 4-7 bundles arranged with the wood face-to-face. All vascular bundles, of different sizes, had a very thick cord of sclerenchymatous fibers at the phloem periphery. Most vascular bundles were separated in between by single or multilayered parenchymatous rays. In the middle of each center of leading rays, there was a parenchymal tissue with extremely small cells, some of them with slightly thickened and lignified walls. Most vascular bundles presented both primary (rows of vessels separated by wooden cellulosic parenchyma) and secondary (irregularly dispersed vessels, separated by libriform fibers) structures. In the composition of the phloem predominate elements of Liberian parenchyma were adjacent to the sclerenchyma bands.

In sections of the *Populus tremula* L. leaf lamina, the median nervure protruded in both sides of the lamina, containing 3 phloem-xylem bundles of different sizes, 2 with xylem facing the adaxial side and one very small, opposite to them, with xylem facing the abaxial side (Figure 7a). All the vascular bundles presented well-developed periphloem sclerenchyma, surrounding the

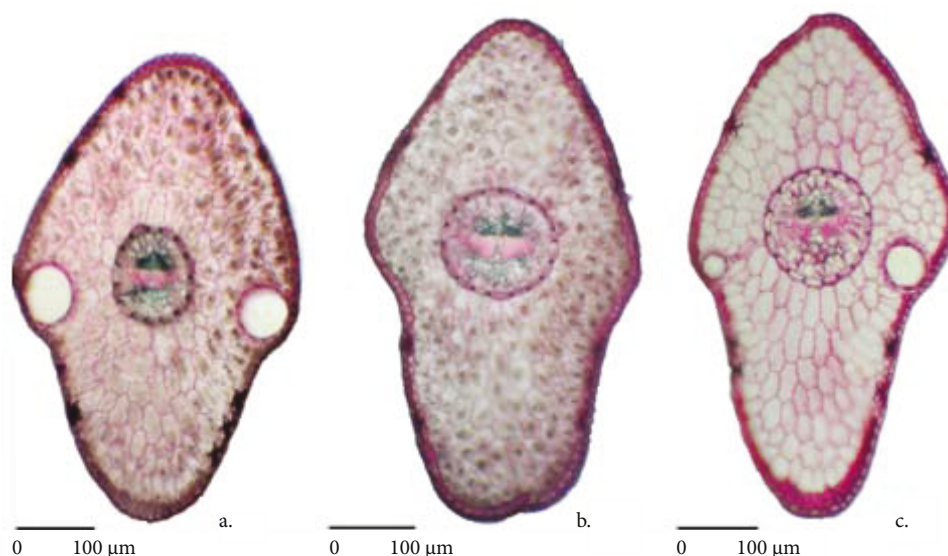


Figure 5. Cross-sections through the leaf of *Picea abies* L.: a- Control, b- Călimani, c- Tarnița.

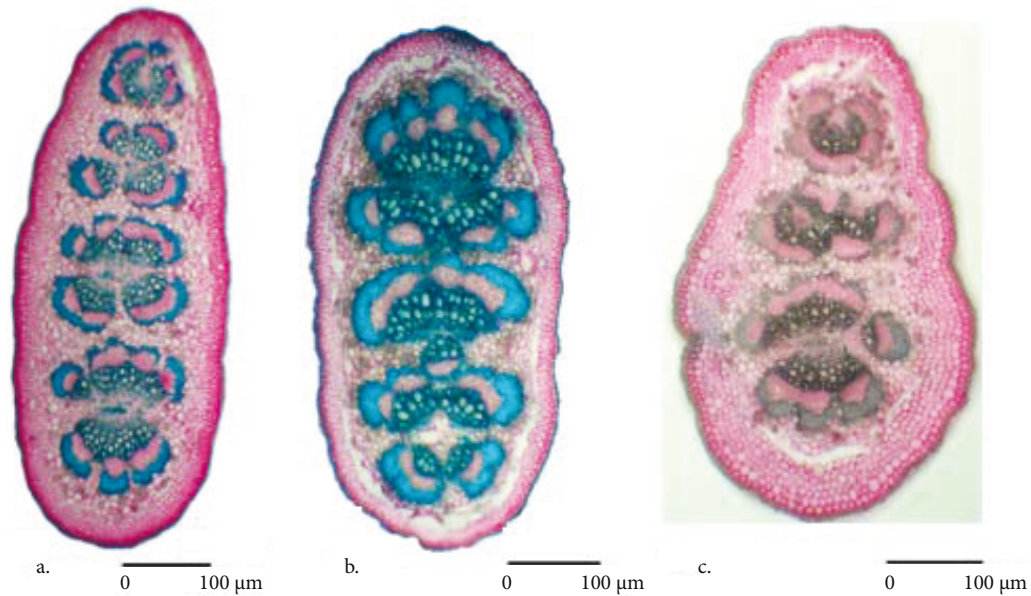


Figure 6. Cross-sections through the petiole of *Populus tremula* L.: a- Control, b- Tarnița, c- Crucea.

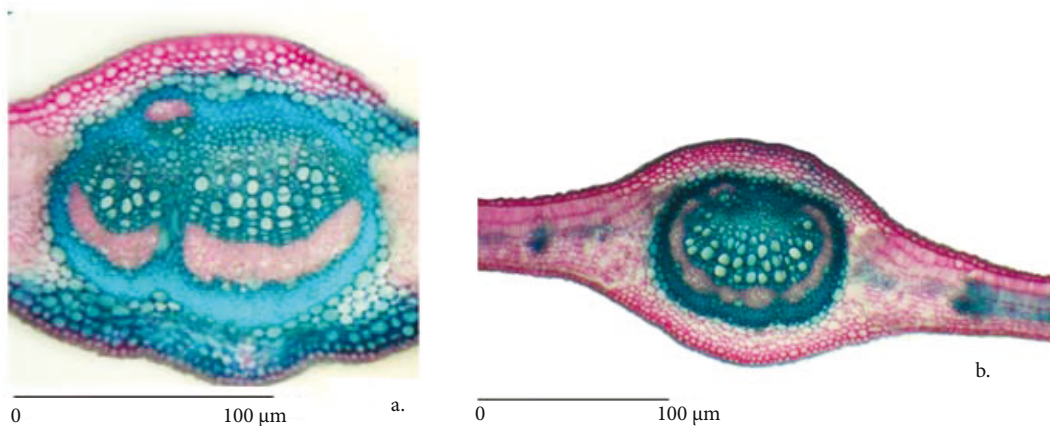


Figure 7. Cross-sections through the leaf lamina of *Populus tremula* L.: a- Control, b- Tarnița.

whole group of the bundles of the median nervure (Figure 7b). In hypodermic position, we observed multilayered collenchyma on the adaxial face and moderate sclerified and lignified parenchyma on the abaxial. The mesophyll was differentiated into bilayered palisade tissue with very high cells on the adaxial face and multilayered tissue with gaps on the abaxial one. Conductive tissues were represented by several different size bundles; the largest one presented one cord of sclerenchyma in both poles.

In the leaf lamina of *Salix alba* L. (control, Figure 8a), the strong median nervure protruded on the adaxial face and less so on the abaxial one. Epidermis cells were isodiametric and covered by a cuticle. From place to place there were visible short tector hairs, localized especially on the abaxial face of the median nervure (Figure 8b).

The mesophyll was differentiated into three-layered palisade tissue on the adaxial face, occupying 70% of the thickness of assimilating parenchyma and bi- or three-layered tissue on the abaxial face, consisting of square or short rectangular cells with very small air spaces between them. In some places, the parenchymal tissue appeared to be formed of 4 layers of cells, and the gapped one from a single layer of tangentially elongated cells (Figure 8b). From place to place in the upper epidermis, some cells visibly protruded, forming a pedestal bearing one short tector hair (Figure 8c).

The outline of the cross-section through the petiole of *Betula verrucosa* Ehrh. was circular, with a small groove on the adaxial side. The epidermis presented isodiametric cells with a thick external wall and covered by a cuticle.

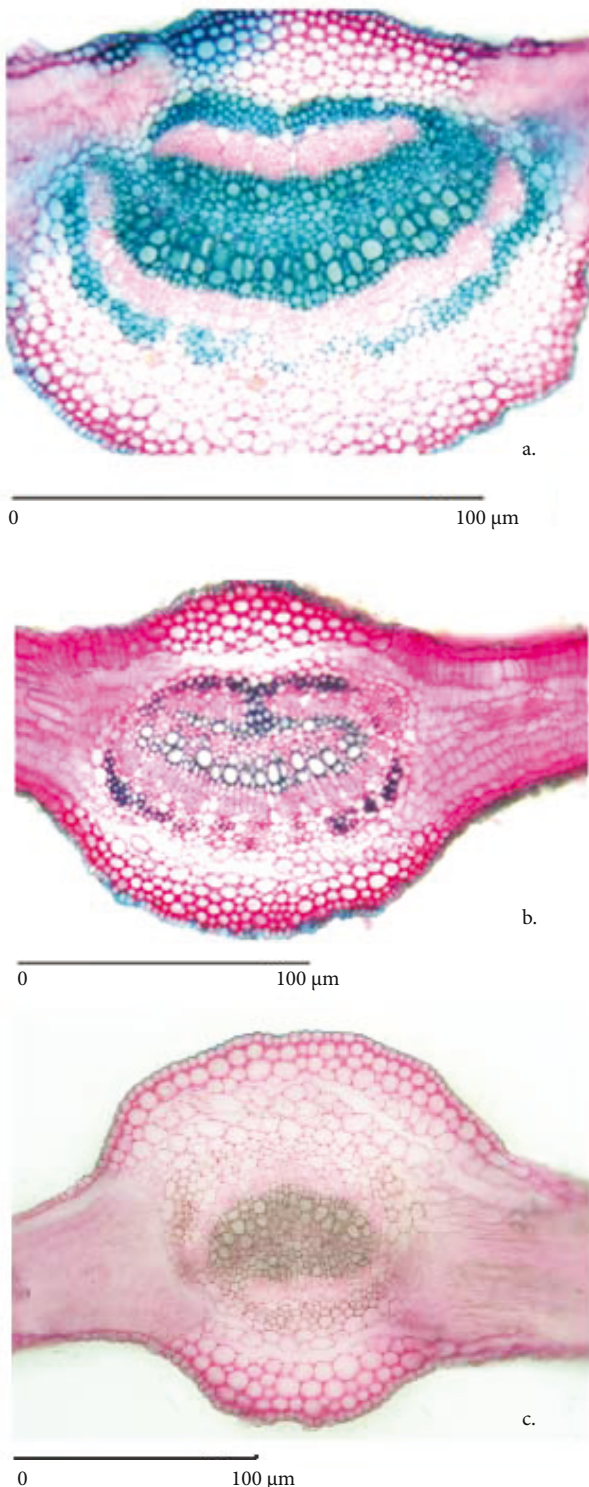


Figure 8. Cross-sections through the leaf lamina of *Salix alba* L.: a- Control, b- Călimani, c- Tarnița.

Collenchymatous tissue of different thicknesses was observed under the epidermis, on the circumference of the petiole (Figure 9a).

In *Betula verrucosa* Ehrh. samples collected from Călimani, at the level of the petiole, the cuticle was thicker, the collenchyma had the same thickness around the circumference of the stem, and the oxalic cells from the fundamental parenchyma were more numerous (Figure 9b).

The fundamental parenchyma had some cells with calcium oxalate. The transporting tissue formed a phloem-xylem median bundle and the wood was arc-shaped, with the concavity towards the adaxial side. In the composition of the phloem, we observed cells of oxalic parenchyma. In the samples from Călimani, small changes could be seen concerning the oxalic cells. On the outskirts of the phloem there was a thick ring of mechanical fibers, but they had relatively thin and poorly lignified walls (Figure 9c).

In the cross-section of the leaf lamina of *Betula verrucosa* Ehrh., the median nervure protruded slightly on both sides of the leaf. At this level, the epidermal cells were isodiametric, having their external wall thicker and cutinized. Under both epidermises, collenchyma tissue could be found. In the parenchyma surrounding the vascular bundles, large oxalic cells were visible. The conducting tissue formed a large phloem-xylem bundle, around which no distinct elements of sclerenchyma could be observed. Some phloem cells were oxalic. The mesophyll was differentiated into monolayered palisade tissue (bilayered sometimes, but with shorter cells) on the adaxial face and thick gapped tissue, with large or very large air spaces between them on the abaxial face (Figure 10a).

In *Betula verrucosa* Ehrh. samples collected from Călimani, at the level of the leaf lamina, the vascular bundle in the periphery exhibited no sclerenchyma elements. Next to the phloem, as in adjacent parenchyma, oxalic cells were visible; this was also observed in the parenchyma between the lateral nervures (Figure 10b). In the samples collected from Tarnița, the gapped tissue had fewer large air spaces than the palisade tissue (Figure 10c).

In *Larix decidua* Mill. (control), the cross-section outline of the leaf lamina was narrow, ribbon-shaped, and slightly convex in the central cylinder area. The epidermis had cells with slightly thicker outer walls than others. Sclerenchymatous hypodermis was visible on both sides, in the middle, as well as at the two poles of the section, being made up of very small cells with thickened walls (Figure 11a). At the edge of the section, there were two narrow secreting channels in direct contact with the hypodermis. The mesophyll was divided into two- or three-layered palisade tissue with relatively short cells on the adaxial side and gapped, multilayered tissue with isodiametric or slightly elongated cells (next to the central cylinder) on the abaxial face (Figure 11b). Hence, the limb had a bifacial-heterofacial structure. The central cylinder

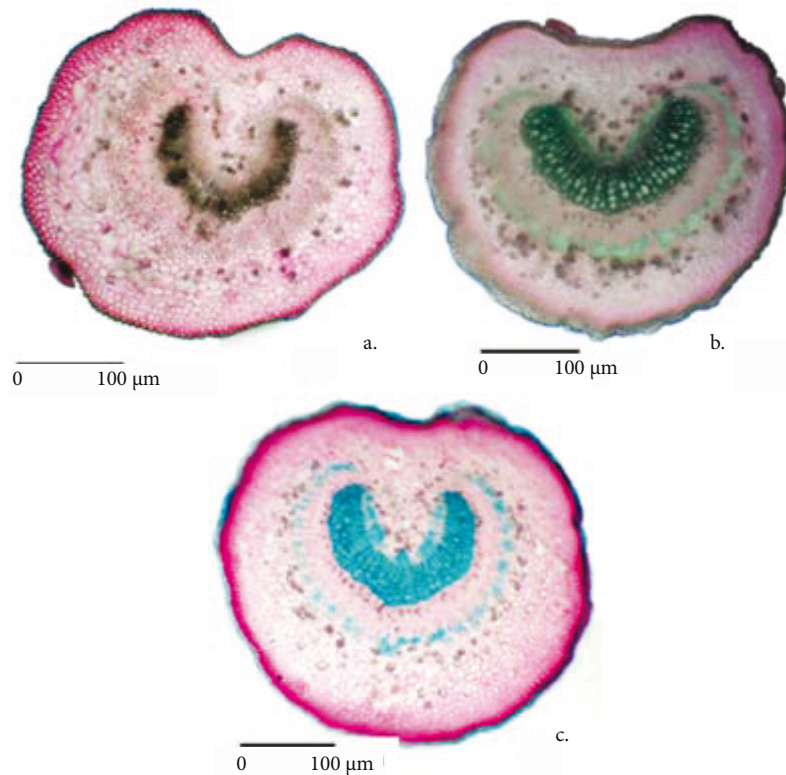


Figure 9. Cross-sections through the petiole of *Betula verrucosa* Ehrh.: a- Control, b- Călimani, c- Tarnița.

had a Casparian-type endodermis, composed of very large cells, almost isodiametric. The tracheid parenchyma was limited to a single-cell layer, the rest of the stele being occupied by the two phloem-xylem bundles separated by a monolayered bundle of sclerenchyma cells. The phloem pole had a thickened cord of sclerenchyma cells (up to 15 cells) (Figure 11c).

4. Discussion

4.1. Biochemical observations

In interpreting the data, it must be taken into account that the stress conditions specific to high mountains (increased intensity of UV radiation, well-defined seasonal and temperature differences, low CO_2 concentration, short growing season) lead to the accumulation of free radicals in plant cells. The pollution caused by the human factor (Dițoiu and Oșean, 2007) acts in tandem with it, enhancing the oxidative stress.

Plants exposed to S pollution accumulate more H_2O_2 in leaves because of the accumulation of superoxide anions due to the oxidation of SO_3^{2-} and HSO_3^- by free radical mechanisms. They require the participation of cytochrome c (Renuga and Paliwal, 1995) and, if no reaction spontaneously occurs, the presence of SOD (Khan and Malhotra, 1982). The presence of the enzyme in high

concentrations in leaves is synonymous with increased plant resistance to excess environmental S. SOD activity significantly inhibits SO_3^{2-} photooxidation (Malhotra and Khan, 1984).

The importance of the involvement of CAT and SOD in plant protection was demonstrated by Yiu and Tseng (2005), who showed that overproduction of these enzymes in cytosol and chloroplasts reduced the rate of visible foliar lesions by 3-4 times. The increase in the activity of one of the two oxide reductases does not necessarily increase the plant's resistance to stress, probably due to the balanced interaction between the two protective enzymes in the antioxidant system of the leaf. In our experiment, this was confirmed only for coniferous species. In the angiosperm species, the profile of CAT activity was further amplified in regards to that of SOD, compared to the species of gymnosperms, as can easily be seen in the results shown in Figure 3.

Inside the plant cell, SOD is the first line of defense against ROS. With different localizations (cytosol (Cu/Zn SOD), plastids (Fe SOD), and mitochondria (Mn SOD)), the enzyme has different expressions not only in the plant cell but depending also on the site of U action. For example, Vanhoudt et al. (2009) showed that SOD from cytosol provides a weak antioxidant action and

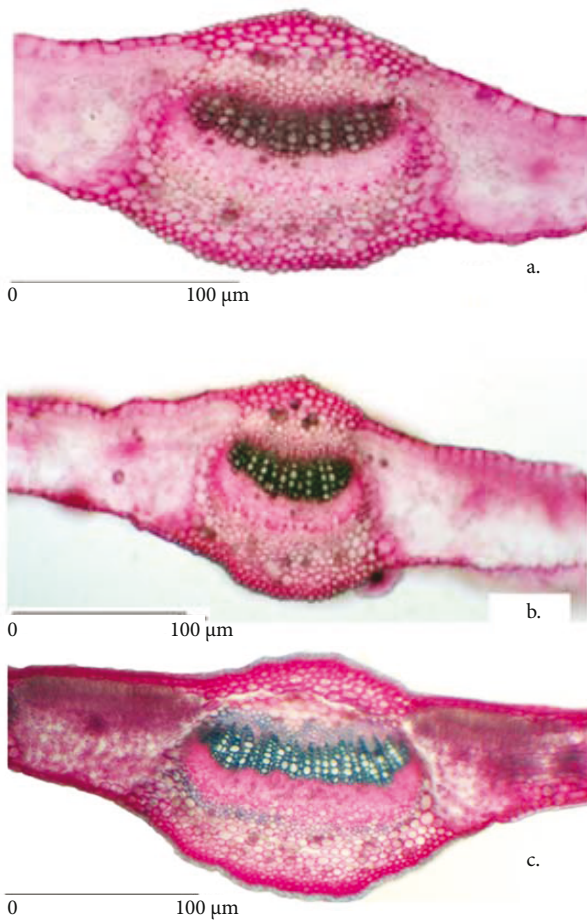


Figure 10. Cross-sections through the leaf lamina of *Betula verrucosa* Ehrh.: a- Control, b- Călimani, c- Tarnița.

that in roots plastids exhibits an improved antioxidant defense, which is not the case of leaf plastids; meanwhile, mitochondrial SOD easily copes with exposure to U. Our investigation focused on total SOD activity and is possible that one of the isoforms was strongly activated, while others were inhibited, which also explains the variation of SOD activity between the exposed species.

In assessing CAT activity in *Picea abies* L. and *Larix decidua* Mill. from Călimani (14.237 ± 1.44 with $P < 0.01$, respectively, and 15.319 ± 3.998 U/mg protein), we can take into account the protective role of cuticle and waxes from the coniferous leaves. Those are limiting factors for the absorption of SO_2 in the mesophyll, where it would be associated with oxidative damage and stimulation of enzyme activity, compared to the control (Putna area). In support of these affirmations are the data obtained from other authors regarding the reaction to stress of other species (Youssef et al., 2013).

Although some of the data indicate that CAT, POX, and SOD may be used as early indicators of oxidative stress

for *Picea abies* L. (Richardson et al., 1990), this species exhibits an adaptive property to the perpetual pollution of the environment, as demonstrated through the experimental tests of this paper, since both POX and SOD showed higher levels compared to the same species in the control site. The only exception was the sample collected from Tarnița (copper and barite ores), which exhibited less SOD activity (6.436 ± 0.394 U/mg protein). Data from the scientific literature support these findings, suggesting that spruce is a less suitable bioindicator of copper pollution (Serbula et al., 2014).

The obtained data regarding POX activity are in accordance with those in the scientific literature, which show that a classic response to higher concentrations of S in the atmosphere is increased POX associated with elevated levels of glutamate dehydrogenase and glutamine synthetase in *Picea abies* L. (Norway spruce) (Krupa and Arndt, 1990). In addition, our results also confirm the birch tree's higher sensitivity to S pollution as compared to the pine tree (Khan and Malhotra, 1982) in terms of POX activity. According to the experimental data, upon exposure to various types of stress (S, U, and other metals), antioxidant defense mechanisms were intensely affected in the leaves of all studied species. At U exposure, CAT was crucial in maintaining redox balance during oxidative stress generated under its action. In this regard, the data from the literature confirms not only that different varieties of U are able to cause oxidative stress in plants, but also that its toxicity depends strongly on the specific characteristics of the site where the plants are found (Saenen et al., 2013).

Previous data regarding the effects of chronic radioactive material (U) exposure on plant cells and the foliar antioxidant defense system found an overproduction of POX and SOD (Vandehove et al., 2006). When environmental U concentrations exceed $1000 \mu\text{M}$, the activity of these enzymes decreases. This is a significant biochemical indication that the species can be considered one of the most advanced genotypes in terms of U ecotoxicity adaptation. The results of this study confirm the data in question, with some exceptions discussed below. It is clear from the data plotted in Figures 3 and 4 that both CAT and POX showed high levels of antioxidant activity simultaneously (19.647 ± 2.413 with $P < 0.05$, and respectively 1.846 ± 0.409 U/mg protein) in the leaf material collected from *Picea abies* L. in Crucea, which suggests that it is possible for POX to have a totally different role than the decomposition of hydrogen peroxide, because it acts in the medium under the presence of a small amount of H_2O_2 , while CAT activity is intensified by the excess of peroxide. Scientific arguments strengthen this hypothesis, since POX is known for its multifunctional role in plants, like phenolic oxidation, lignification, and recovery of cell membrane damage under stress (Gülen et al., 2008; Chkhubianishvili

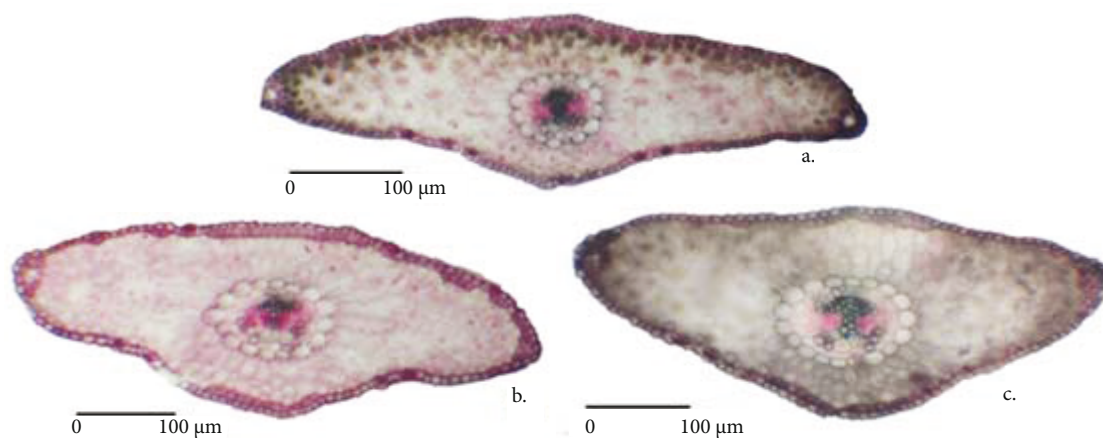


Figure 11. Cross-sections through the leaf lamina of *Larix decidua* Mill.: a- Control, b- Călimani, c- Târnița.

et al., 2011). It is then possible to consider *Picea abies* L. as an effective bioindicator reflecting the quality of the environment exposed to U, which is also supported by the discovery of an overproduction of superoxide anions, whose decomposition is mediated by SOD.

It is possible that in the case of conifers, the particular importance of CAT activity on the foliar level could be due to the rhizosphere attenuating the oxidative stress in leaves. This statement is based on the fact that the species of gymnosperms are known to develop rich symbiotic networks at the root level, with an increased efficiency in translocation of heavy metals, the maximum interaction at this level being among soil, plant roots, and U (Gobran and Huang, 2011).

It should be taken into account that the enzymes are proteins and that a significant increase in leaf soluble protein concentration due to the accumulation of U in the soil was previously recorded (Shtangeeva et al., 2006). Vanhoudt et al. (2009) stated that the level of antioxidant enzyme activity at the cellular level, activated by the presence of U in the environment, does not decrease when compared to the soluble proteins. These observations can be used also as significant elements that show the degree of soil pollution.

Antioxidant responses to pollution in the mining area of Târnița are varied, but they confirm that the toxicity of metals and barite in excess resulting from anthropogenic activities is capable of causing severe imbalances in vegetable bodies.

An important approach that can help us understand plant responses is the evidence that SOD is an enzyme with a major role in plant tolerance to metal stress (Tsang et al., 1991). The toxicity of the metal ions that act synergistically is attenuated compared with cases where they act alone (Bertrand and Poirier, 2005). Therefore, the noticeable diminished activity of this enzyme in *Picea abies* L. may be due to the presence of excess metal ions like Fe, Cu, and

Zn and of smaller quantities of Cd, Pb, As, and Ni. These elements are found in soil due to exploitation (Dițoiu and Oșean, 2007; Stumbea and Chicoș, 2012) and are able to act on the active center of the enzyme.

In analyzing the obtained results, it is necessary to take into account that the effect of metal stress on plants depends on the ratio between the intensity of stress and the capacity of the soil to act as a buffer. The soil's acidification enhances the absorption capacity of metal ions by increasing their solubility, hence increasing their toxicity on plant cells (Reddy et al., 1995). Regardless of depth, the polymetallic type of ores from Târnița contain pyrite, chalcopyrite, sphalerite, galena, and barite, and small quantities of arsenopyrite and pyrrhotite (Stumbea and Chicoș, 2012). Oxidation of pyrite is considered one of the greatest sources of acidity in the soil, and with the decreasing of pH, the soil accumulates soluble sulfates (Reddy et al., 1995).

According to data obtained by Dițoiu and Oșean (2007), as well as Ulea and Lipșa (2009) and Erzberger et al. (2012), the soil in the Călimani Mountains has been found to be predominantly acidic (pH between 2 and 3.75); in addition to other metal ions that exceed the threshold of intervention or which are found in small amounts (Mn, Zn, Cu, Ni), Fe also exceeded the warning limit. This can explain the higher values of SOD, CAT, and POX activity compared to the species in the control area.

The mechanism of POX action in plants exposed to high concentrations of heavy metals has not been fully elucidated. No matter how high the amplitude of enzyme activity is, it is necessary to take into account when justifying the level of POX that its activity was found to be significant at much smaller metal concentrations than those inducing visible environmental toxicity (MacFarlane and Burchett, 2001).

It is possible that the lower POX activity in *Betula verrucosa* Ehrh. and *Larix decidua* Mill. leaves, compared

to the other plants from all areas, may be attributed to the decomposition of the hydrogen peroxide, the biosynthesis of lignin, hormonal regulation, or the natural sensitivity to environmental factors that varies from species to species.

4.2. Histoanatomical observations

In *Picea abies* L. leaves collected from Tarnița (Figure 5c), the hypodermis had cells with much thinner walls in comparison with the control samples (with the exception of the adaxial and abaxial poles). This can be regarded as a consequence of pollution.

Turunen et al. (1997) argued that soluble and insoluble particles of heavy metals can accumulate on the surface of aerial organs of the plant or penetrate their barrier. A minor proportion of metal cations can be picked up via the stomata and cuticles. They are able to erode epicuticular waxes, block the epistomatal chambers, and thereby prevent the stomata from closing. These actions depend on the relationship between the tubes and amorphous deposits of wax, but also on the distribution of functional groups of the wax molecules. For example, alkanes and alkyl esters may increase hydrophobicity, while fat hydroxy acids reduce it. The microorganisms within the structure of the phyllosphere that are able to change the hydrophilic/hydrophobic nature of the waxes and the degree of microroughness play an important role, which is reflected in the degree of transport of cations through the cuticle (Turunen et al., 1997). It is also specified that massive S pollution of the environment is able to affect the structure of the cuticle layer of conifer needles and of the leaf lamina of *Betula verrucosa* Ehrh. much more than heavy metals.

Investigations carried out on the behavior of the plant organs in the presence of radionuclides in the environment certify that their absorption through the leaves is one of the ways in which the plant accumulates them (along with root uptake from the soil). The properties of the leaf epidermis and cuticle structure may have an effect on the foliar uptake of radionuclides (Koranda and Robison, 1978). In the petiole of *Populus tremula* L. collected from Crucea, transport vessels of different sizes were grouped into three centers; in each of them, the wood was located one in front of the other. Periphloem sclerenchyma was less developed, with some bundles being mono- or bilayered (Figure 6c).

It was found that the presence of U in the environment increased the rate of uptake of heavy metals in *Betula* sp. at leaf level, as follows: Cd > Mn > Zn > Pb > Cu > Ni > Fe (Wisłocka et al., 2006). The phenomenon of sensitization of the leaf to metals was also found in other species. Petrescu and Bilal (2006b) indicated heavy metal pollution in the area of the Crucea-Botușana mine. In this context, the emergence of morphological and anatomical changes at leaf level may not be due to radionuclides, but rather to heavy metals.

In the sample collected from *Populus tremula* L. (Tarnița), cells in the epidermis were covered by a thick outer wall with an extremely thick cuticle, visible especially on the upper side; this may indicate the reactivity of the plant to the anthropogenic pollution in the area (Figure 7b). Günthardt-Goerg and Vollenweider (2007), studying issues related to heavy metals in the environment, indicated the tendency of metal accumulation in older leaves, with a preference for lower leaf tissues. The metals are observed within the chloroplast or the vacuole and are able to accelerate cellular senescence via nuclei condensation, reducing the size of the chloroplasts and necrotizing the inferior epidermis of *Populus tremula* L.

In *Salix alba* L. samples collected from Călimani, the mesophyll was differentiated into bilayered palisade tissue on the adaxial face and gapped multilayered tissue on the abaxial, in comparison with the control (Figure 8b). Scientific data generally showed the presence of bilayered palisade parenchyma in different species of the genus *Salix* (Khalili et al., 2010).

In *Salix alba* L. samples collected from Tarnița, between the lateral nervures, the epidermal cells were larger, some slightly elongated tangentially, all with thick external walls and covered by a thin cuticle. The mesophyll was differentiated into palisade tissue, typically bilayered, with relatively short cells on the adaxial face and gapped, multilayered tissue, with some cells slightly elongated perpendicular to the epidermis towards the abaxial face.

In *Larix decidua* Mill. samples collected from Tarnița, the central cylinder had very little phloem (maybe due to pollution) and the two bundles were separated by a bilayered beam of sclerenchyma cells (Figure 11c).

The analysis of the biochemical and histoanatomical properties of the species from different polluted and unpolluted areas of Suceava County, Romania, led to these final conclusions: the range of antioxidant responses to pollution conditions from the Călimani Mountains, Tarnița, and Crucea-Botușana was different, depending on the type of polluting interference in the environment. The greatest antioxidant defense system reactivity was detected under U action in all studied species in the Crucea-Botușana area, except *Salix alba* for SOD and POX and *Populus tremula* for POX. The activity of SOD, CAT, and POX was positively stimulated by all kinds of pollution, the level of enzymatic activity surpassing that of the leaf material harvested from the species in the control area. The only exception was that the metal ions resulting from polymetallic sulfide alteration in Tarnița were able to inhibit the activity of SOD in *Picea abies* L. The occurrence

of structural changes in the analyzed samples, such as the thickening of the epidermal cuticle, the thinning of hypodermic cell walls, and the reduction of the phloem at the level of the vascular bundle, can be attributed to anthropogenic pollution.

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