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BIOCHEMICAL AND ANATOMICAL CHANGES OF SPRUCE NEEDLES EXPOSED TO URBAN DUST POLLUTION

H. LEPEDUŠ and V. CESAR

Department of Biology, Faculty of Education, University of J. J. Strossmayer
L. Jägera 9, HR-31000 Osijek, Croatia; E-mail: hlepedus@yahoo.com

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The effect of extensive urban dust pollution, caused mainly by road traffic, on some biochemical and structural characteristics of current-year Norway spruce (*Picea abies* L. Karst.) needles was investigated. Two categories of needle samples were formed according to the data about the pollution levels obtained from the Croatian National Institute of Public Health: less and more affected. Apoplastic guaiacol peroxidases were used as the molecular stress markers. Peroxidase activity was doubled in more affected needles compared with the less affected ones. Also, the electrophoretic pattern of samples extracted from more affected needles revealed the expression of additional isozyme band, which could be attributed to the activation of detoxifying mechanisms. Anatomy of more affected needles was changed as well. Necrosis of needle mesophyll usually connected with the stomata was the most outstanding character. Also, distortions of sieve cells were present in the same needle samples indicating possible disturbances in mineral nutrition.

The obtained results showed that needles of Norway spruce trees that are exposed to the higher pollution level undergoes to both structural and biochemical changes. Besides of the described changes, the investigated spruce trees are able to survive in more polluted environment as well.

Key words: dust pollution, guaiacol peroxidases, needle anatomy, *Picea abies*

INTRODUCTION

The industry and the road traffic are considered to be the main sources of air pollutants in urban areas. Basically, the two types of the pollutants are present in such areas: dust and gaseous pollutants. The size of urban road dust particles can vary between 3 and 100 μm, while dust emitted from the motor vehicle exhausts range from 3 to 30 μm in diameter (Thompson *et al.* 1984). Besides of different size, the chemistry of dust can be very variable (Farmer 1993): the most of urban dusts are alkaline and may contain a number of metals, which could have a toxic influence to the surrounding vegetation. The gaseous pollutants that are released from the car engine (NO_x, SO_2) could act directly on the vegetation either by entering inside the leaves through the stomata or by producing the acid mists upon the moisturising. As has been pointed out by

Fink (1999) such field mixtures are showing considerable variability in concentration and relative portion of the individual components. Also, described influences are often accompanied with the changes in soil chemistry. The additive and opposite effects expressed in the field are able to make unclear the biochemical and structural changes of spruce needles that were observed in controlled experiments with the single component or controlled mixtures.

The changes of guaiacol peroxidases were shown to be very sensitive tool in detection of some external influences such as the gaseous pollutants and heavy metals (Peters *et al*. 1989, Van Assche and Clijsters 1990, Pfanz *et al*. 1993, Roitto *et al*. 1999, Soukoupova *et al*. 2002). Peroxidases are heme-containing glycoproteins that catalyse oxidation of various phenolic compounds (Hiraga *et al*. 2001) and are involved in numerous processes: changes of cell walls (lignification, suberization), auxin catabolism, wound healing and defence against pathogen infection. Also, peroxidases are the part of the mechanism for SO_2 detoxification (Pfanz *et al*. 1993). Our previous investigation on the influence of the cement dust on spruce needles (Cesar and Lepeduš 2001) showed that the total activity of guaiacol peroxidases was decreased because of highly alkaline microenvironment. Besides a very high sensitivity of peroxidases as well as other biochemical and molecular markers, it is not possible to distinguish effects of various stress factors. On the other hand, the changes in needle structure appeared to be quite specific in respect to the type of stress influence. Fink (1989, 1993) reported that each type of stress influences changed target cells specifically. For example, the target cells for gaseous pollutants were mesophyll cells, while in the conditions of disturbed mineral nutrition the vascular bundle histology was specifically altered.

The aim of our study was to compare the activity and isozymes pattern of apoplastic guaiacol peroxidases as well as the changes in the anatomy between needles of cultivated spruce trees grown under different levels of urban dust pollution in the city of Osijek (Croatia).

MATERIALS AND METHODS

The material for study was collected from ten about 25-year-old cultivated Norway spruce (*Picea abies* L. Karst.) trees grown in the city of Osijek, Croatia (Fig. 1). Two categories of needle samples were formed according the data on pollution levels for chosen locations obtained from the Croatian National Institute of Public Health: less (control) and more affected (Table 1). More polluted location had about 30% higher dustfall amount. Also, the Pb and Cd content in the dustfall were doubled in comparison with the less polluted location. The pH values of the dustfall were around 8 for both locations,

Table 1
The data about the pollution levels in April 2001, obtained from the Croatian National Institute of Public Health. The measured parameters were: the amount of dustfall (mg m^{-2} per day), pH value of the dustfall, Pb content in the dustfall (μg m^{-2} per day) and Cd content in the dustfall (μg m^{-2} per day).

Location	Amount of dustfall	pH	Pb	Cd
Less effected	112.00	8.29	8.00	1.33
More effected	158.00	8.04	15.00	2.70

indicating alkaline character of urban pollution. There was no available data about gaseous pollutants for chosen locations. The sampling was done in April 2001. Current-year needles were harvested from the middle crown of investigated spruce trees.

Fig. 1. The map of Croatia and the surrounding countries. The location of the city of Osijek is marked

Protein extracts for analysis of apoplastic peroxidases were prepared slightly modified as described by Polle *et al*. (1990). Needles were cut, weight and briefly washed with 0.16 M mannitol in order to remove cytoplasmic peroxidases released upon the cutting of the needles. Then, needles were blotted and infiltrated at –70 kPa in periods of 30 seconds with the extraction buffer (0.1 M Tris-HCl buffer, pH = 8.0). Protein extract was centrifuged, filtered and used for activity measurements and electrophoresis.

The guaiacol peroxidases activity was determined spectrophotometrically by measuring the absorbance increase at 470 nm. The reaction mixture contained 5 mM guaiacol and 5 mM H_2O_2 in 0.2 M phosphate buffer, pH = 5.8 (Siegel and Galston 1967). The reaction has been started by adding 200 µl of protein extract to 800 µl of reaction mixture.

The isoperoxidase pattern analysis was conducted using the anionic discontinuous vertical native polyacrylamide gel electrophoresis (PAGE). The 1 mm thick stacking (2.5%) and separating (14%) polyacrylamide gels were prepared. The 1.5 M Tris-glycine (pH = 8.3) buffer system (without the SDS) described by Laemmli (1970) was used. The electrophoresis was carried out at +4 °C for five hours (2 hours at 20 mA of constant current followed by 3 hours at 150 V of constant voltage). The isoperoxidase bands were visualised by immersing the gel in the same reaction mixture used for peroxidase activity determination.

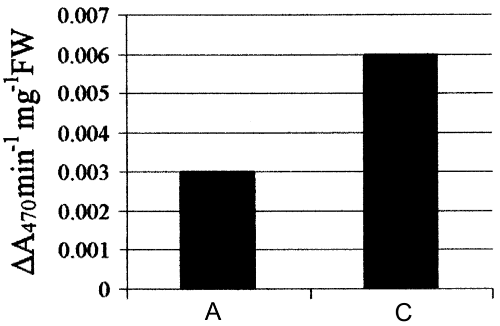
The data obtained by the quantitative measurements were arranged in two statistical samples: one for less and the other for more affected needles. Each sample contained five Norway spruce trees (N = 5). The statistical evaluation was done using the *t*-test modified for small sample (Pavlic 1977).

Needle anatomy was investigated by light microscopy. Small pieces of the tissue were cut from the middle of each needle and fixed for 24 hours in 6% glutaraldehyde in 0.05 M phosphate buffer, pH = 6.8. Then, specimens were dehydrated in 2-methoxyethanol, ethanol, n-propanol and n-butanol (two changes in each) and embedded in methacrylate resin (Historesin, Leica). Three µm thin sections were stained with 0.05% Toluidine blue 0 in benzoate buffer, pH = 4.4 (Feder and O'Brien 1968, O'Brien and McCully 1981).

RESULTS AND DISCUSSION

The visual examination of investigated spruce needles revealed the yellow spots in both sample types. The appearance could be described as irregular. However, the samples of more affected needles had bigger spots, sometimes joined together. Also, the dust deposits that partially cover the stomata of more affected needles were noticed. The review on the effects of dust in vegetation given by Farmer (1993) revealed that besides blocked stomata some other effects were observed: reduced diffusive resistance, increased absorption of insolation, increased leaf temperature and reduced growth. Visual symptoms of spruce yellowing were carefully evaluated by Fink (1989, 1993), in controlled experiments and in field investigations. According to those investigations the discoloration caused by magnesium deficiency started from the needle tip, while the irregular appearance of the chlorotic spots and bands that mottled the needle could be attributed to the ozone impact. However, the chlorotic spots of the spruce needles in the field described by Fink (1993) revealed the same pattern as in our investigation, but different to any symptoms observed in the controlled experiments.

The values of apoplastic peroxidase activity are shown in Figure 2. Activity was doubled in the samples of more affected needles (0.006±0.001 ΔA_{470} min^{-1} mg^{-1} f.w.) compared with the control samples (0.003±0.001 ΔA_{470} min^{-1} mg^{-1} f.w.). As reviewed by Kuzniak and Urbanek (2000), the hydrogen peroxide as well as other reactive oxygen species (ROS) are generated in response to different biotic and abiotic environmental factors. The positive correlation was shown for the activity of apoplastic peroxidases and the content of some elements (S, Ni and Cu) in Scots pine (*Pinus sylvestris*) needles (Roitto *et al.* 1999). Also, some gaseous air pollutants, such as O_3 and SO_2, stimulated the extracellular peroxidase activity in different plant species (Pfanz *et al.* 1993, Peters *et al.* 1989). The peroxidase activity measured in more affected needles was significantly higher (P(t) < 1%). This could be considered as the activation of



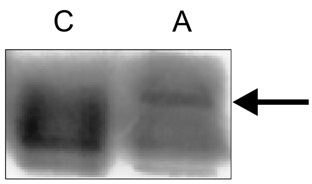


Fig. 2. (Left side) The mean values of apoplastic guaiacol peroxidases (ΔA470 min^{-1} mg^{-1} fresh weight) in current-year needles of Norway spruce (*Picea abies* L. Karst.) collected from the control (C) and more polluted (A) sampling sites

Fig. 3. (Right side) Electrophoretic pattern of apoplastic guaiacol peroxidases extracted from the needles collected from the control (C) and more polluted (A) sampling sites. Arrow is indicating the band that is present only in the more affected needles

detoxifying mechanisms upon the stronger stress conditions through the higher input of Pd and Cd at that sample plot (Table 1). Investigation on the Cd influence on peroxidase activity and electrophoretic pattern in spruce needles done by Radotic *et al.* (2000) are in accordance with our study. They reported the increase of the wall-bound peroxidase activity upon the Cd treatment, as well as the changes in the electrophoretic pattern. The induction of a new isoperoxidase band in sample of the more affected needles can be seen in the gel after the electrophoretic separation and staining on peroxidase activity (Fig. 3). Van Assche and Clijsters (1990) reported the induction of new isoperoxidase bands in response to heavy metals, which could be related to the disintegration of biomembranes due to the lipid peroxidation. Polle *et al.* (1991) showed changes in both pattern and activity of guaiacol peroxidases in spruce needles under high elevation stress. The appearance of new isoperoxidase band in the samples of affected needles (Fig. 3) could be related to the higher stress conditions, too. On the other hand, a natural variation in the isoperoxidase pattern is making unclear the interpretation of the differences in isoperoxidase pattern when cultivated spruce trees are investigated.

Microscopic evaluation of investigated needles showed that both sample types had changed anatomy in respect to the regular one. The most outstanding characteristic was the necrosis of needle mesophyll, which appeared in both sample types. However, in less affected needles necrosis was restricted to the single, exceptionally two mesophyll cells (Fig. 4A). Also, distortion of the oldest phloem sieve cells was present in the same needles (Fig. 4B). On the contrast, much bigger necrotic areas were present in more affected needles (Fig. 5A), usually connected with the stomata (Fig. 5B). There was no more possible to distinguish vacuole and cytoplasmic areas in the collapsed mesophyll cells (Fig. 5C). Disintegration of the tonoplast was described as the latest stage of the cellular decompartmentalisation, followed by the cell death (Fink 1999). The mesophyll necrosis in spruce needles was also reported by Ruetze *et al.* (1988) as the consequence of the action of gaseous pollutants. Further, cuticle of the more affected needles was severely damaged (Fig. 5D) in comparison with the less affected needles which possessed intact cuticle (Fig. 4A). Surface waxes were shown to be eroded by the alkaline dust influence, most probably due to saponification of the wax (Bermadinger *et al.* 1987). The vascular bundle anatomy in more affected needles was changed at the same way as in the less affected ones (Fig. 4B): the oldest sieve cells in the phloem were distorted. This symptom was shown to be quite specific for Mg-deficient conifer needles (Fink 1993, Puech and Mehne-Jakobs 1997, Lepeduš *et al.* 2001).

It can be concluded that higher extent of the urban dust pollution provoked the more intensive physiological answer and structural changes of current-year spruce needles in comparison with less polluted conditions. Pollu-

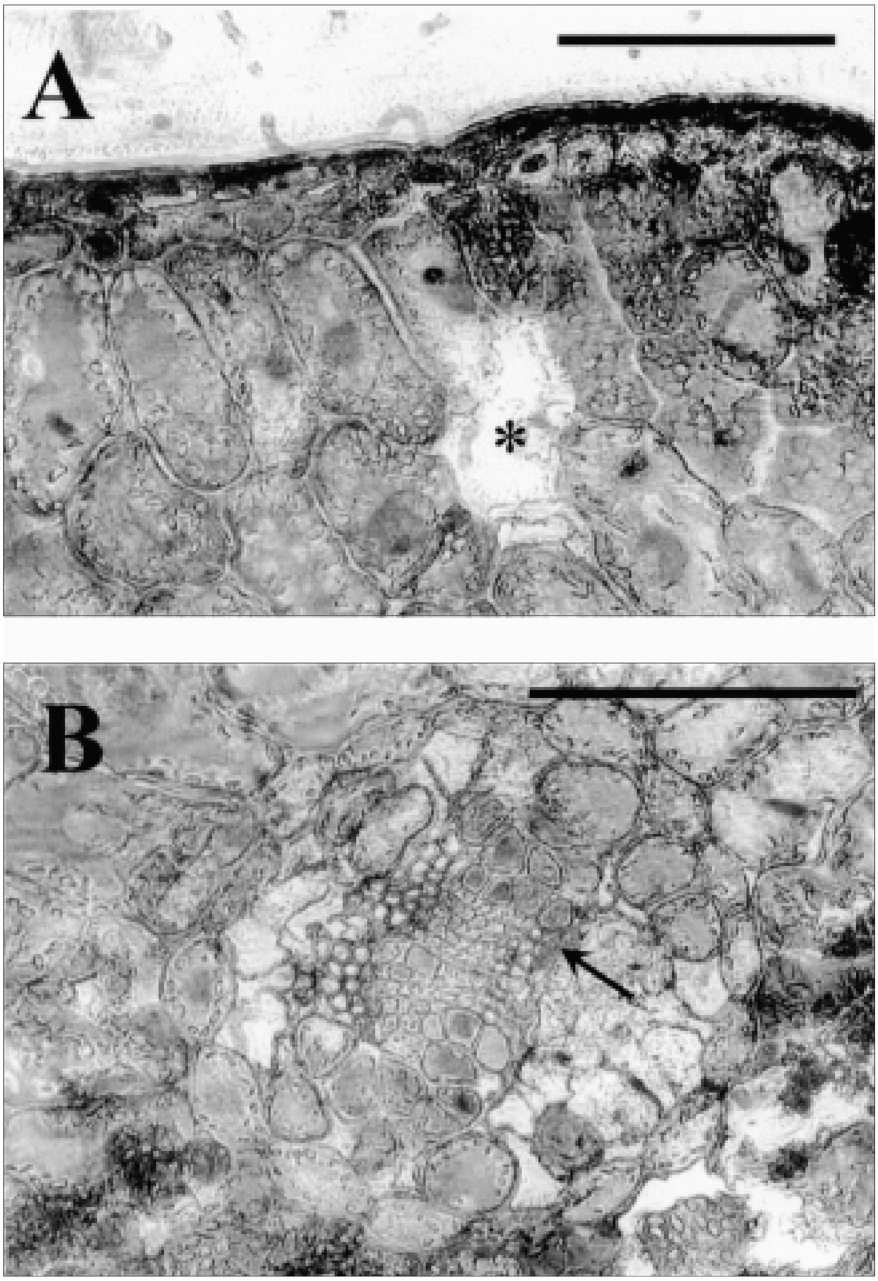


Fig. 4. Cross sections through the current-year needles of Norway spruce (*Picea abies* L. Karst.) trees collected from the control (loss polluted) sampling site. Bar = 100 µm. – A: Part of the needle with necrotic area restricted to the single mesophyll cell is assigned with the asterisk. – B: Vascular bundle with distorted the oldest sieve cells (arrow)

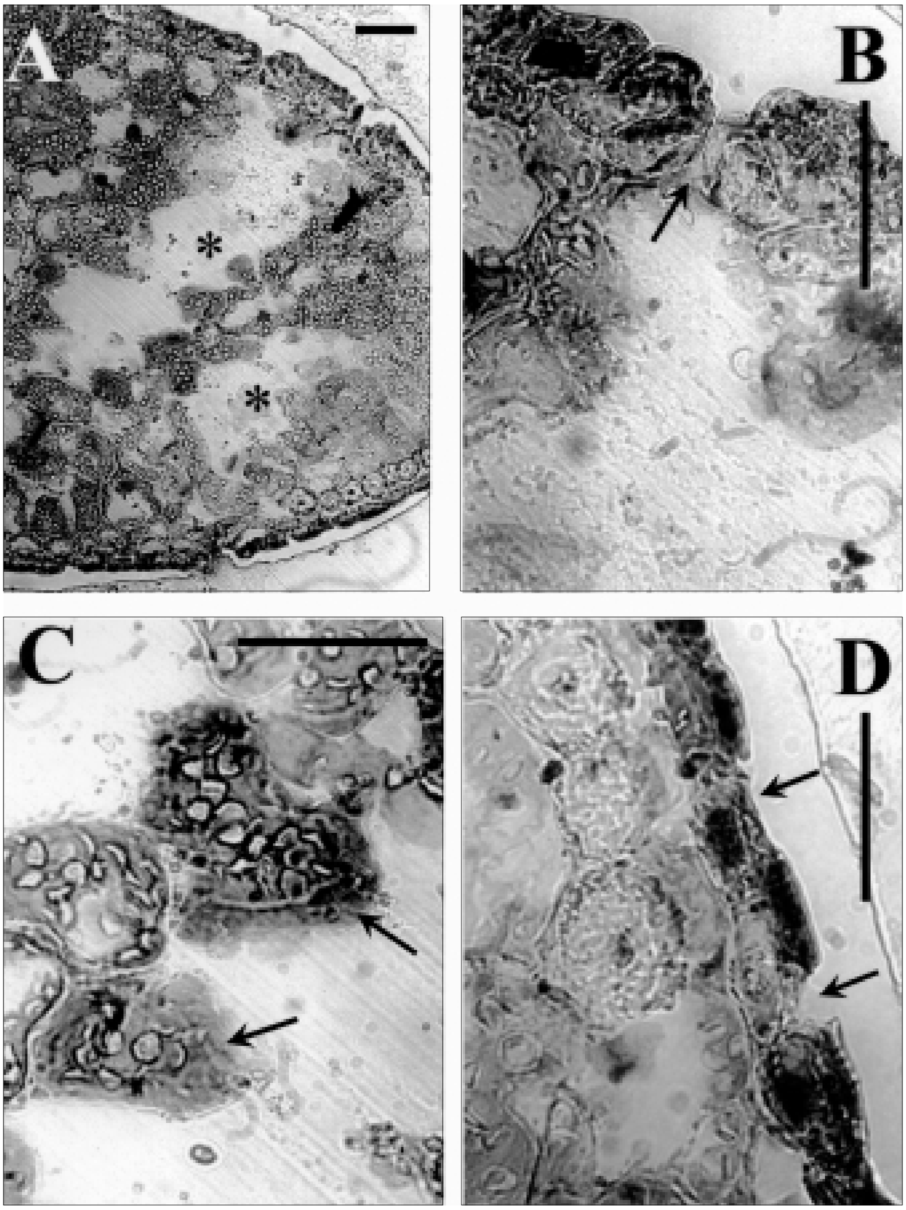


Fig. 5. Cross section through the current-year needles of Norway spruce (*Picea abies* L. Karst.) trees collected from the more polluted sampling site. Bar = 50 µm. – A: The part of the mesophyll with large necrotic area (asterisk). – B: A detail of the mesophyll revealing that necrosis originates from the stoma (arrow). – C: A detail of the necrotic area revealing the severely damaged cells without possibility to distinguish the vacuole and cytoplasm (arrow). – D: A detail of needle revealing the cuticle injures (arrows)

tion caused the appearance of chlorotic symptoms as well as changes in needle anatomy. Besides the air pollution influence, the vascular bundle anatomy revealed symptoms characteristic for disturbed mineral nutrition, which could be the additional cause of the chlorosis. The activation of defence mechanisms against the oxidative stress was observed through the increased apoplastic guaiacol peroxidase activity and the expression of new isoperoxidase form. In spite of the described structural changes, the investigated spruce trees are able to survive in more polluted environment as well.

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