Acta Bot. Croat. 60 (2), 227–235, 2001

CODEN: ABCRA 25
ISSN 0365–0588
UDC 581.174

Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Peroxidase activity, soluble proteins and chlorophyll content in spruce (*Picea abies* L. Karst.) needles affected by cement dust

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The correlation between the peroxidase activity, chlorophyll and soluble protein content as well as the changes in vascular bundle structure in Norway spruce (*Picea abies* L. Karst.) needles affected by cement dust were studied. In spite of the absence of any yellowing symptoms, a significantly lower chlorophyll content was measured in spruce needles affected by cement dust. Observed sieve cells distortions in needle samples indicated that spruce trees grown near the cement factory were Mg deficient. Total guaiacol peroxidase activity in non-affected was significantly higher than in dust-affected needles, while soluble protein content was not significantly different.

Key words: *Picea abies*, cement dust, peroxidase activity, proteins, chlorophyll

Introduction

A considerable number of investigators have paid great attention to the effect of various phytotoxic pollutants such as SO_2, O_3, NO_x and heavy metals on cytological, histological, physiological and morphological changes in different conifer species (SOIKKELI 1981, RUETZE 1988, FINK 1989, 1993, TURUNEN and HUTTUNEN 1990, BARNES et al. 1995, KAINULAINEN et al. 1995, MIKKELSEN et al. 1995, WILD and SCHMITT 1995, PFIRRMANN et al. 1996, SCHULZ et al. 1996, ŠIFFEL et al. 1996, TAUSZ et al. 1996, TURUNEN 1996, PUECH and MEHNE-JAKOBS 1997, GIERTYCH et al. 1999, ROITTO et al. 1999). A comprehensive review given by FARMER (1992) pointed out that limited research attention has been paid to the impact of alkaline dust pollution arising from road traffic, open-cast mining and different industrial processes (e.g. cement factories). The probable reasons were the absence of any visible damage symptoms on the surrounding vegetation and the local character of such pollution.

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Different hierarchical levels (cell, organism, ecosystem) could be used for stress bioindication. Biochemical indicators are the most successful tools in the early diagnosis of air pollution stress (WILD and SCHMITT 1995, SCHULZ et al. 1996) as well as changes in needle chlorophyll content, which indicates an altered plant physiological state even before yellowing can be noticed.

As a consequence of air pollutant action, the formation of H_2O_2 and other reactive oxygen species occurred (KUZNIAK and URBANEK 2000). Peroxidases that participate in H_2O_2 detoxification process by utilizing different phenolic cosubstrates were used as stress bioindicators of some road trees (WONGKAEW et al. 1991) as well as for mapping areas of air pollution (KELLER 1974).

Our previous investigations into the impact of cement dust on spruce needles showed the deposition of callus in the hypodermal layer of cells making a coat around the needle mesophyll. This indicates that cement dust provokes the plant to provide a response to the stress situation.

In this study we have endeavoured to show the correlation between peroxidase activity, chlorophyll and soluble protein content as well as some histological characteristics in Norway spruce (*Picea* abies L. Karst) needles affected by cement dust.

Material and methods

The samples were taken from two localities. The first locality was in the immediate vicinity (less than 3 km) of a cement factory (Našice, Croatia) and the other one was at a distance of about 50 km (Osijek, Croatia). From each sample plot we selected five 15–50 years old Norway spruce (*Picea abies* L. Karst.) trees. Current – year needles were used as study material for every analysis.

For chlorophyll content determination, sampling was carried out monthly from July to December 2000. Material for total soluble protein and peroxidase activity analysis as well as for histological investigations was taken at the end of a six month period (in December 2000).

Pigments were extracted with absolute acetone and determined spectrophotometrically (LICHTENTHALER 1987). Proteins were extracted with 0.1 M Tris-HCl buffer, pH 8.0, containing polyvinylpyrrolidone – PVP (10 mg/ml buffer) and determined according to BRADFORD (1976), using bovine serum albumin (BSA) as a standard. Total guaiacol peroxidase 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 was started by adding 100 µl of protein extract. Total reaction mixture volume was 1.1 ml. In order to investigate the effect of medium pH values on peroxidase activity, reaction mixture pH was adjusted to 5.8, 6.4 and 7.0. For that purpose the crude protein extract obtained from control needles was used as the source of enzyme.

All measurements were done in triplicate and averaged. The data obtained were arranged in two groups and calculated by Students t-test and correlation coefficient (r). The results from each sample plot were assembled as one sample: sample 1 was from dust-affected trees near the cement factory and sample 2 from non-affected trees in Osijek.

For light microscope investigations segments about 2-mm-long were cut from the middle of each current – year needle and fixed for 24 hours at +4 °C in 6% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8). The specimens were then dehydrated in ethanol, n-propanol and n-butanol (two changes in each) and embedded in glycol methacrylate (Historesin, Leica). 3 μm thin sections were stained with 0.05% Toluidine blue O in benzoate buffer, pH 4.4 (FEDER and O'BRIEN 1968, O'BRIEN and MCCULLY 1981).

Results

Visual inspection revealed that the needles from the neighbourhood of the cement factory were covered with dust layers, but in spite of that they showed no symptoms of yellowing (Fig. 1A). It could be seen that very thick crusts of dust were partially plugging the stomata along the needle.

The results of chlorophyll content measurements during the whole investigation period showed a higher amount of chlorophyll *a* and total chlorophyll in the non-affected than in the dust-affected needles (Figs. 2A, 2C). The amount of chlorophyll *b* was slightly higher in affected needles during July while in August and subsequently it showed the same attitude as chlorophyll *a* (Fig. 2B). The seasonal dynamics of chlorophyll content was similar for both samples, with an evident vertical shift (Fig. 2). The correlation coefficient indicated a high correlation in chlorophyll *a* and total chlorophyll between affected and non-affected needles ($r_{Chl\,a} = 0.96$, $r_{Chl\,a+b} = 0.90$). The correlation coefficient for chlorophyll *b* was 0.79 calculated for the period from August to December. The mean content of chlorophylls in dust-affected and non-affected needles during the six month period are given in Tab. 1. The differences in chlorophyll *b* and total chlorophyll content appeared to be statistically significant while that for chlorophyll *a* was not.

Tab. 1. The mean values of chlorophyll content (in mg/g fresh weight) in dust-affected (sample 1) and non-affected (sample 2) needles during the six month investigation period. Chl *a* – chlorophyll *a*, Chl *b* – chlorophyll *b*, Chl *a+b* – total chlorophyll. P(t) – percent of similarity, NS – non significant.

	SAMPLE 1	SAMPLE 2	P(t)
Chl *a*	0.72 ± 0.13	0.87 ± 0.17	NS
Chl *b*	0.38 ± 0.03	0.49 ± 0.07	<1%
Chl *a+b*	1.10 ± 0.15	1.35 ± 0.22	<5%

Total soluble protein content was lower in dust-affected than in non-affected needles (Tab. 2) but the difference was not shown to be significant.

Total guaiacol peroxidase activity in non-affected was much higher than in dust-affected needles (Tab. 2). The experiment with different medium pH values showed that the increase of reaction mixture pH caused a decrease in peroxidase activity (Fig. 3).

The analysis of vascular bundle structure in histological sections of needles from affected trees revealed a certain injury that could be characterised as premature aging (Fig. 1B). The oldest sieve cells were distorted and some of them were totally crushed. Sieve cells in non-affected needles were characterised by a wide open lumen without distortions (Fig. 1C).

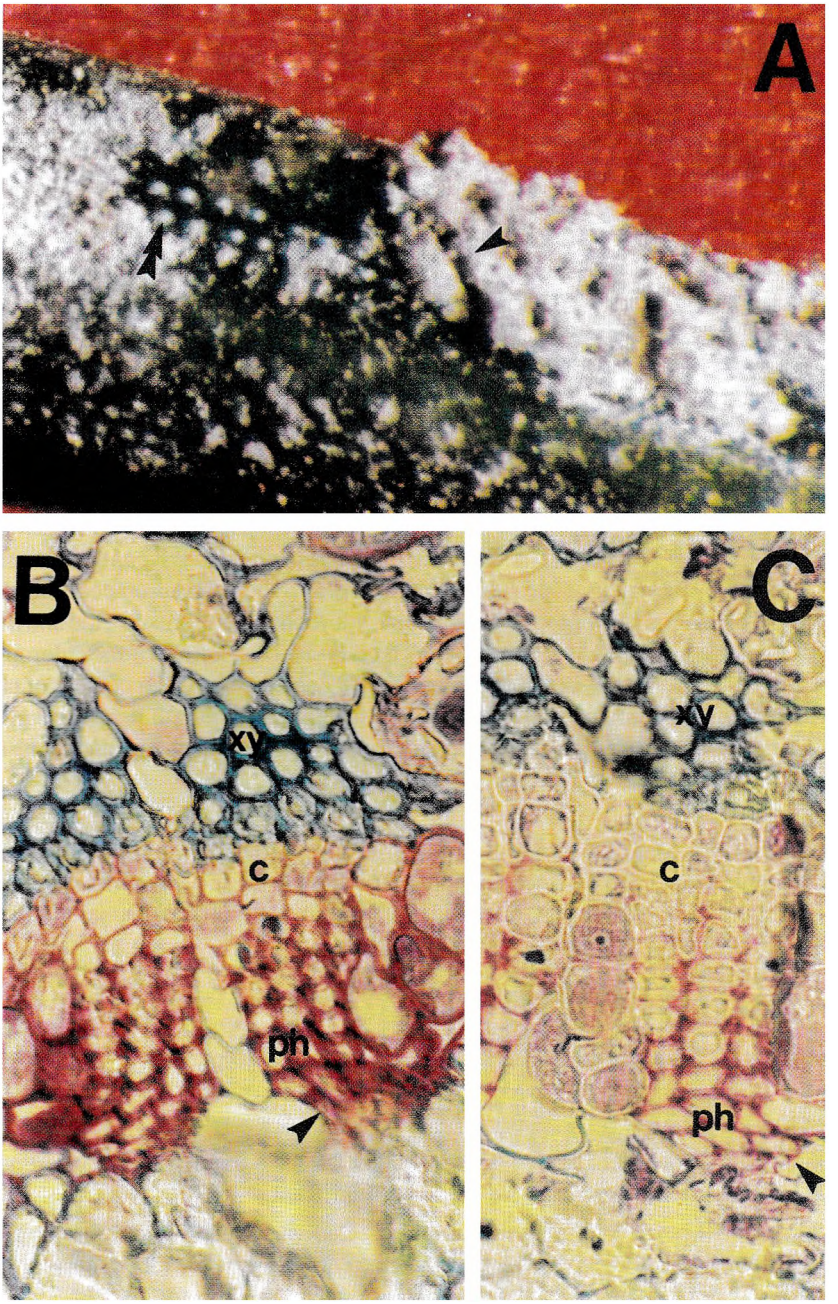

Fig. 1. A. Current-year spruce needles with visible cement dust layers (arrowhead) covering the stomata (double arrowhead). Magnitude 40x. B, C – the cross-sections of spruce needles; ph – phloem, xy – xylem, c – cambium. Magnitude 1000x. B – the oldest sieve cells of cement dust-affected needles appear to be distorted and some of them are totally crushed (arrowhead). C – sieve cells of control needles are characterised by a wide open lumen without distortions (arrowhead).

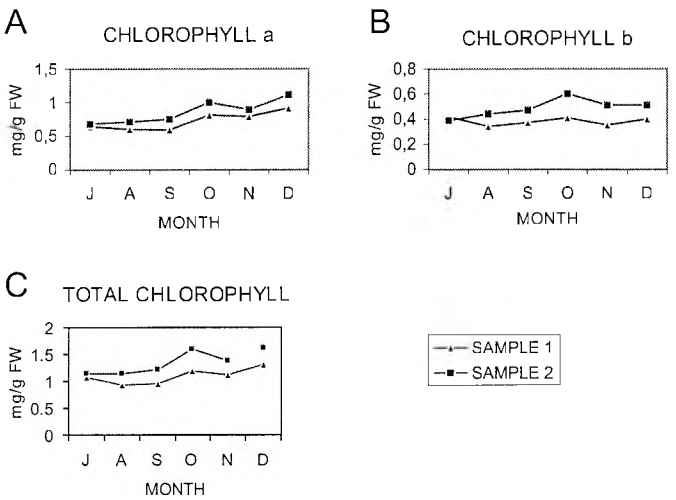

Fig. 2. The dynamics of chlorophyll content in current – year Norway spruce needles exposed to cement dust (sample 1) and non-affected control needles (sample 2) during the six month investigation period. FW – fresh weight.

Tab. 2. Total soluble protein (in mg/g fresh weight) and guaiacol peroxidase activity – POD ($min^{-1}mg^{-1}$ fresh weight) in dust-affected (sample 1) and non-affected (sample 2) spruce needles. P(t) – percent of similarity, NS – non significant.

	SAMPLE 1	SAMPLE 2	P(t)
PROTEINS	16.37 ± 4.57	18.60 ± 2.29	NS
POD	0.04 ± 0.01	0.15 ± 0.02	<0.1%

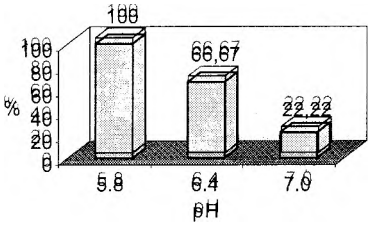

Fig. 3. The effect of experimental changes of the pH of the reaction mixture on total guaiacol peroxidase activity.

Discussion

In spite of the absence of symptoms of yellowing, a significantly lower chlorophyll content was measured in spruce needles affected by cement dust (Tab. 1). An increase of chlorophyll content (Fig. 2) in the period from October to December is in accordance with the results given by HERBINGER et al. (1999). They reported an increase in chlorophyll content during short days and a decrease during the action of low temperatures. In our investigation, the

expected decrease of chlorophyll content during November and December was absent probably because of the relatively high air temperatures in that period. According to the data given by the Hydro-meteorological Institute Zagreb, the average month temperatures during the year 2000 were 8.5 °C in Našice and 9.9 °C in Osijek in November and 2.5 °C in Našice and 3.5 °C in December. The average values in the period 1981–1997 for the same months, on the same locations were 5.4 °C, 4.9 °C, 0.2 °C and 1.6 °C, respectively. In dust-affected needles the dynamics of chlorophyll content was the same as that in non-affected needles but an evident shift in metabolism was observed (Fig. 2). The alkalization and high Ca content of the environment were shown to inhibit the assimilation of Mg, Mn and Fe from soil and thus led to a depression of the biosynthesis of photosynthetic pigments in spruce needles (MANDRE and TUULMETS 1997). Chlorophyll *b* content appeared to be more affected than chlorophyll *a* (Tab. 1). Chlorophyll *b* is synthesised from chlorophyll *a* by the action of a specific oxygenase enzyme (von WETTSTEIN et al. 1995). It seems that the reaction is bi-directional, involving chlorophyll *b* reductase activity (SUZUKI et al. 1997, TANAKA et al. 1998). So, one could speculate that chlorophyll *a* content is compensated form chlorophyll *b* pool in response to environmental changes.

The specific responses of spruce trees to different abiotic factors are best manifested at the tissue level. Mg-deficient needles are characterised by a breakdown of the phloem cells (FINK 1999, 1993, 1989). The sieve cell distortions in needle samples from Našice described (Fig. 1B) indicated that spruce trees grown in the vicinity of the cement factory were Mg deficient.

Results concerning total soluble protein contents presented here (Tab. 2) are similar to those given by other investigators (POLLE et al. 1992, SCHULZ et al. 1996) and there were no significant differences between dust-affected and non-affected needles.

It has been reported that elevated calcium concentration raises peroxidase activity in different plant systems (PENEL 1986, CONVERSO and FERNÁNDEZ 1996). Since cement dust contains high levels of calcium oxide (CaO) we expected that this should increase peroxidase activity in spruce needles. Notwithstanding our expectations, peroxidase activity in needles affected by cement dust was significantly lower than in the control needles from Osijek (Tab. 2). The basis for this probably inheres in another possible control mechanism of peroxidase activity – the pH effect. So, we showed that the raised pH of the reaction mixture influenced the total guaiacol peroxidase activity (Fig. 3). Even a small shift of pH value in the alkali direction (from 5.8 to 7.0) decreased the activity by about 80%, a proof that pH variation in a polluted environment may modify peroxidase activity.

The results obtained concerning peroxidase activity afford us a new view in the elucidation of the fact that vegetation covered with cement dust reveals no yellowing symptoms. Chlorophyll degradation is maintained by the action of peroxidases (HUFF 1982, JOHNSON-FLANAGAN and MCLACHLAN 1990, JOHNSON-FLANAGAN and SPENCER 1996) and chlorophyllases (DRAZKIEWICZ and KRUPA 1991, DRAZKIEWICZ 1994, MATILE et al. 1997, KRÄUTLER and MATILE 1999). The inactivation of both enzyme systems in the alkaline microenvironment produced by cement dust should prevent chlorophyll degradation in such a milieu.

Cement dust can be said to have a dual impact on spruce needles. A direct influence caused by deposition on needle surface and input through the stomata was evident in peroxidase activity decrease because of the alkaline microenvironment. An indirect impact

was visible in sieve cell distortion and a decreased amount of chlorophyll, indicating Mg deficiency. It might be considered that the precipitation of cement dust onto the ground could be the reason for calcium imbalance in the soil, which results in the mineral nutrition of spruce trees being disturbed.

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