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THE EFFECT OF DISTURBED MINERAL NUTRITION ON *PICEA OMORIKA* PANČIĆ NEEDLES – A SINGLE CASE STUDY

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A widespread conifer forest decline in Europe and North America has been expressed through various combinations of symptoms. In our study, unspecific biochemical indicators and histological findings were compared with visually assessed damage symptoms in order to get a detailed diagnosis of a single declining *Picea omorika* Pančić tree. By measuring the content of photosynthetic pigments and proteins as well as total guaiacol peroxidase activity we confirmed an altered physiological state of the investigated tree. Histological analysis showed specific patterns of decline for Mg and K deficient trees.

Key words: *Picea omorika*, photosynthetic pigments, proteins, peroxidase activity, needle histology

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Sveprisutno propadanje šuma u Europi i sjevernoj Americi manifestira se kao kombinacija različitih simptoma. U provedenom istraživanju usporedili smo nespecifične biokemijske indikatore i histološke nalaze s vizualnom procjenom oštećenja, a s ciljem postavljanja detaljne dijagnoze propadanja jednog stabla Pančićeve omorike (*Picea omorika* Pančić). Određivanjem sadržaja fotosintetskih pigmenta, proteina i aktivnosti ukupnih gvajacilnih peroksidaza potvrđena je promjena fiziološkog statusa biljke. Histološke analize pokazale su specifičnosti karakteristične za stabla deficijentna magnezijem i kalijem.

Ključne riječi: *Picea omorika*, fotosintetski pigmenti, proteini, aktivnost peroksidaza, histologija iglice

INTRODUCTION

Conifer forest decline in Europe and North America has attracted the attention of a number of investigators for about twenty years. It has been demonstrated that decline is primarily caused by abiotic factors such as air pollution and disturbances

in mineral nutrition (MCLAUGHLIN, 1985; SCHULZE, 1989). Different diagnostic approaches have been applied in order to establish distinctions among the actions of various damage factors. Most investigators have used quantitative biochemical analysis for that purpose (BARNES *et al.*, 1995, KAINULAINEN *et al.*, 1995; MIKKELSEN *et al.*, 1995; WILD & SCHMITT, 1995; PFIRRMANN *et al.*, 1996; SCHULZ *et al.*, 1996; ŠIFFEL *et al.*, 1996; TAUSZ *et al.*, 1996; KRONFUSS *et al.*, 1998; GIERTYCH *et al.*, 1999; ROITTO *et al.*, 1999; ORMROD *et al.*, 1999). For instance, SOLBERG *et al.* (1998) demonstrated that Mg deficient Norway spruce (*P. abies* L. Karst.) needles showed yellowing symptoms accompanied with significantly lower chlorophyll content in comparison with green needles. PUECH & MEHNE-JAKOBS (1997) found that the Mg uptake of spruce trees was influenced by their nitrogen source. The Mg deficient trees in their investigation had a lower chlorophyll content and specifically altered needle histology. Unspecific answers to a variety of stress factors proved to be the biggest problem in that kind of approach. On the other hand, the qualitative approach to structural and ultrastructural levels of needle functioning and integrity were found to give much more specific results (SOIKKELI, 1981; RUETZE *et al.*, 1988; FINK, 1989; 1993; PUECH & MEHNE-JAKOBS, 1997). In a number of controlled experiments FINK (1993) showed that the target cells react differentially to specific influences, e.g. Mg and K deficient spruce needles had characteristically changed vascular bundle histology, and the main target cells of gaseous pollutants were the mesophyll cells.

We have observed the growth of a solitary planted *Picea omorika* Pančić tree for almost twenty years. In the last two years progressive yellowing was noticed on one side of the tree and we had a reasonable suspicion that part of the root had penetrated into the cloaca as the yellowing side was orientated towards it. This provided the impulse to attempt to integrate the quantitative and qualitative approach, in order to find a detailed diagnosis for this declining tree.

MATERIAL AND METHODS

Study site

The material for this study was collected from a single *Picea omorika* Pančić tree grown in Vrbovsko (Gorski kotar, Croatia). The tree was grown on calcareous soil in the vicinity of the cloaca. The sudden decline of the needles suggested that the root system of the tree had reached the cloaca. This would bring about a deranged $\text{NO}_3^-/\text{NH}_4^+$ ratio and in this way influenced the conditions of mineral nutrition. The surrounding Norway spruce trees grown on the same soil at some distance from the cloaca showed no damaging symptoms.

Sampling and laboratory procedures

The current-year needles were harvested from lateral shoots of the *Picea omorika* Pančić tree. Needles were arranged in two samples. Sample 1 consisted of damaged needles from lateral shoots away from the apical shoot. Lateral shoots near the apical shoot of the same branch contained undamaged green needles, arranged in

sample 2. Both types of samples were taken from three different positions on the same tree and processed separately. Sampling was done in July 2000.

Photosynthetic pigments were extracted twice with ice-cold absolute acetone and determined by a spectrophotometric method (LICHTENTHALER, 1987).

Soluble proteins were extracted in 1 M sucrose and 0,056 M 2-mercaptoethanol in 0,2 M Tris-HCl buffer, pH 8,5 (WETTER & DYCK, 1983). Protein concentration was determined according to BRADFORD (1976), using bovine serum albumin (BSA) as a standard. The amount of dry weight was determined by drying at 105 °C during 24 hours.

Total guaiacol peroxidase activity was determined spectrophotometrically by measuring the increase in absorbency at 470 nm. The reaction mixture contained 5 mM guaiacol and 5 mM H₂O₂ in 0,2 M phosphate buffer, pH 5,8 (SIEGEL & GALSTON, 1967). The reaction was started by adding 100 µl of protein extract.

All measurements were done in triplicate and averaged. The results were worked out by Students t-test modified for small samples.

For light microscopy, needles were cut in small pieces and fixed for 24 hours in 6% glutaraldehyde in 0,05 M phosphate buffer, pH 6,8. 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 0 in benzoate buffer, pH 4,4 (FEDER & O'BRIEN, 1968; O'BRIEN & MCCULLY, 1981).

RESULTS AND DISCUSSION

Visual inspection of needles from sample 1 showed yellowing symptoms. Some needles of the same sample exhibited yellowing followed by brown discoloration. The chlorosis and later on browning started from the needle tip toward the base. An identical pattern of needle discoloration has been reported in Norway spruce trees with experimentally induced Mg and K deficiency (FINK, 1993). The analysis of photosynthetic pigments confirmed the macroscopic symptoms (Fig. 1). Total chlorophyll concentration was four times higher in green needles – sample 2 ($0,60 \pm 0,14$ mg/g FW) than in yellow ones – sample 1 ($0,15 \pm 0,02$ mg/g FW). Differences in chlorophyll a, chlorophyll b and total chlorophyll concentration were statistically significant between samples 1 and 2 ($P(t) < 5\%$). A reduced chlorophyll concentration was observed in current-year and one-year old needles of Norway spruce in the NH₄⁺ – dominated treatment (PUECH & MEHNE-JAKOBS, 1997). Also, the reduced Mg content was found to be positively correlated with chlorophyll content in Scots Pine (*Pinus sylvestris* L.) and Norway spruce needles (NYMAN, 1992; MANDRE & TUULMETS, 1997; SOLBERG *et al.*, 1998). The chlorophyll a : b ratio was higher in sample 1 than in sample 2 (Fig. 2), which means that chlorophyll b was more affected than chlorophyll a. However, there was no statistically significant difference ($P(t) > 5\%$) for the same ratio. MEHNE-JAKOBS (1994, 1996) reported an increased chlorophyll a : b ratio in needles of Norway spruce as a response to Mg deficiency. The amount of

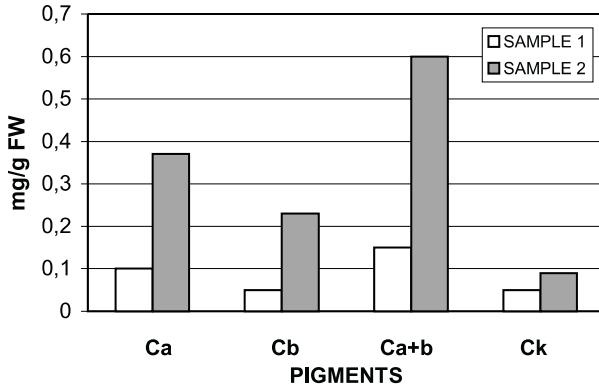


Fig. 1. The average content of chlorophyll a (Ca), chlorophyll b (Cb), total chlorophyll (Ca+b) and total carotenoids (Ck) in damaged (SAMPLE 1) and green (SAMPLE 2) needles, expressed as mg/g fresh weight (FW).

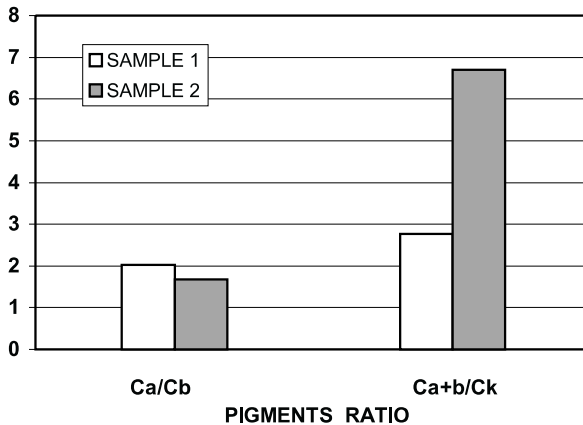


Fig. 2. The chlorophyll a : b ratio (Ca/Cb) and total chlorophyll : total carotenoids ratio (Ca+b/Ck) in damaged (SAMPLE 1) and green (SAMPLE 2) needles.

total carotenoids was about twice as low in sample 1 ($0,05 \pm 0,01$ mg/g FW) as in sample 2 ($0,09 \pm 0,01$ mg/g FW) (Fig. 1). Total chlorophyll : total carotenoids ratio was 2,41 times higher in sample 2 than in sample 1 (Fig. 2), which means that the carotenoids were less affected. Both values, the total carotenoid content and total chlorophyll : total carotenoid ratio showed significant differences ($P(t) < 5\%$) between these two samples. The role of carotenoids in protecting the photosynthetic apparatus against harmful effects of light and O_2 is well established (SIEFERMANN-HARMS, 1987). It could be possible that the chlorophyll degradation was speeded up by the effect of light and reactive oxygen species as the protecting function of carotenoids was disturbed. Although the mineral nutrient disturbances are almost always con-

nected with the yellowing of conifer needles, there was no confirmation of any specific pattern of photosynthetic pigments concentration that could be uniquely attributed to a certain type of mineral deficiency (SOLBERG *et al.*, 1998).

The dry weight differences between samples 1 and 2 were not statistically significant, but the protein content was about three times higher in the damaged needles of sample 1 (Tab. 1). An increased protein and free amino acid content could be a suitable indicator of the needle N-content (NYMAN, 1992). The increase of total soluble protein content in Norway spruce needles is also highly correlated with direct uptake of gaseous NO_x (SCHULZ *et al.*, 1996), as well as induction of nitrate reductase activity (von BALLMOOS *et al.*, 1998).

There was no detectable total guaiacol peroxidase activity in green needles (sample 2). The induction of peroxidase activity has been recorded in sample 1 needles (Tab. 1). Peroxidases are known to decompose H_2O_2 by oxidation of various phenolic cosubstrates and may be considered as general indicator for oxidative stress caused by SO_2 or heavy metals (ROITTO *et al.*, 1999). Besides unspecific peroxidases

Tab. 1. The mean values of dry weight (DW), protein content and peroxidase activity (POD) in damaged (SAMPLE 1) and green (SAMPLE 2) needles; ND – not detectable. Statistical significances : P(t) < 5% – significant, NS – nonsignificant.

	SAMPLE 1	SAMPLE 2	P(t)
DW (%)	36,38 ± 1,23	39,74 ± 2,09	NS
PROTEINS (mg/g DW)	14,64 ± 3,25	4,60 ± 0,02	< 5%
POD ($\Delta\text{A}_{470} \text{ min}^{-1} \text{ mg}^{-1} \text{ FW}$)	0,083 ± 0,01	ND	< 5%

several others much more specific enzymes (catalase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase and glutathione reductase) participate in the H_2O_2 scavenging pathway (POLLE *et al.*, 1990). However, OSSWALD *et al.* (1992) found no difference in guaiacol peroxidase activity of healthy and damaged Norway spruce needles. On the other hand, it has been demonstrated that peroxidase catalyses degradation of thylakoid – bound pigments in the presence of H_2O_2 and 2,4 – dichlorophenol in canola (*Brassica napus* cv. Westar) seeds (JOHNSON-FLANAGAN & MCLACHLAN, 1990). So, it could be possible that induced peroxidase activity in sample 1 needles was a cause of chlorophyll degradation rather than just an unspecific symptom indicating an oxidative stress situation.

Observations at the level of light microscopy showed a striking difference between green and damaged needles (Plates I–III). Green needles of sample 2 were characterized by an intact epidermis, turgent mesophyll cells and the intact vascular bundle (Plate I, Figs A and B). Cambial cells were organized in 3–4 rows. Younger sieve cells appeared to have a heterogeneous dark content, while the older were filled with brighter content or looked empty. Only the oldest sieve cells were

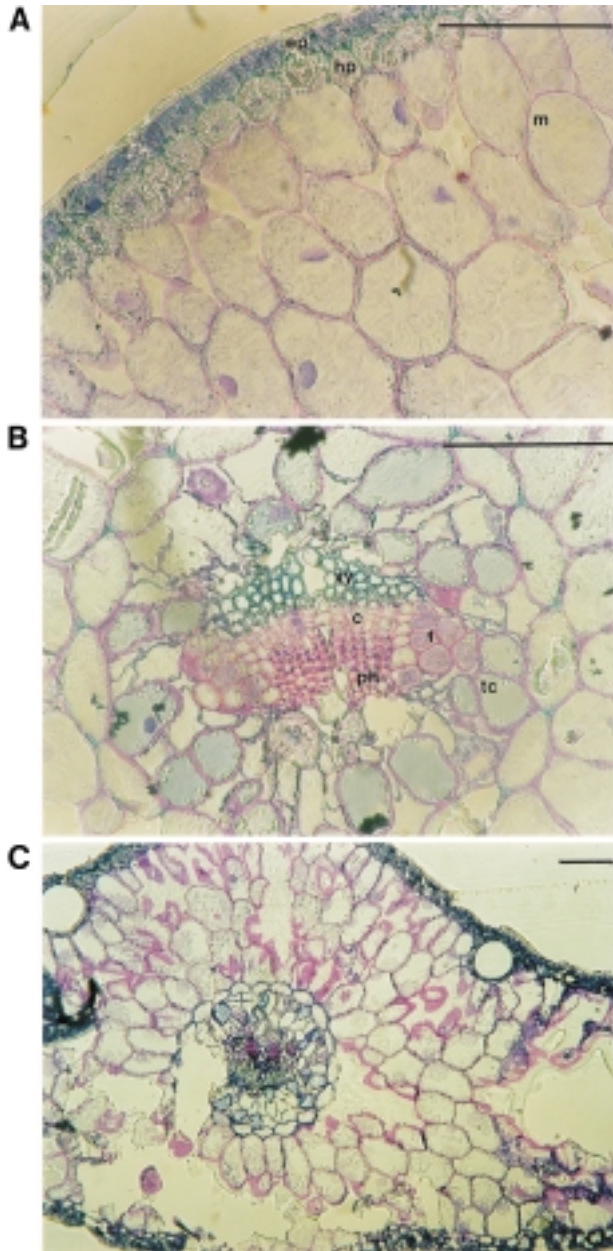


PLATE I. Cross-sections through the current-year needles from lateral shoots of *Picea omorika* Pančić. Bar = 100 μm. A. Part of green needle with epidermis (ep), hypodermis (hp) and turgescient mesophyll cells (m). B. Intact vascular bundle in green needles: ph – phloem, xy – xylem, c – cambium, tc – transfusion cells. C. Yellow needle with necrotic mesophyll and disturbed vascular bundle.

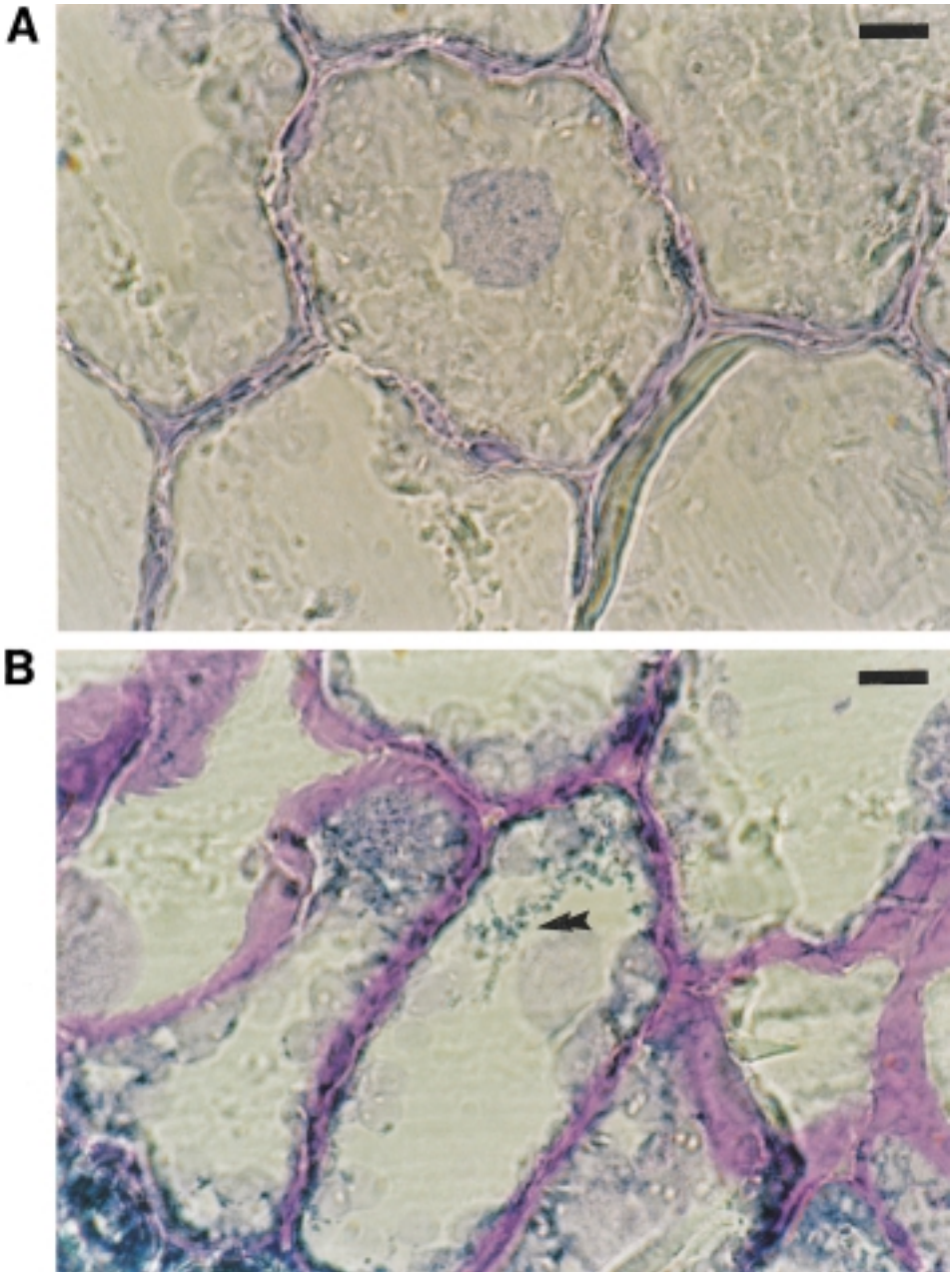


PLATE II. The mesophyll cells of sampled *Picea omorika* Pančić needles. Bar = 10 μ m.
A. Green needle. B. Yellow needles contain tannin deposits (arrow).

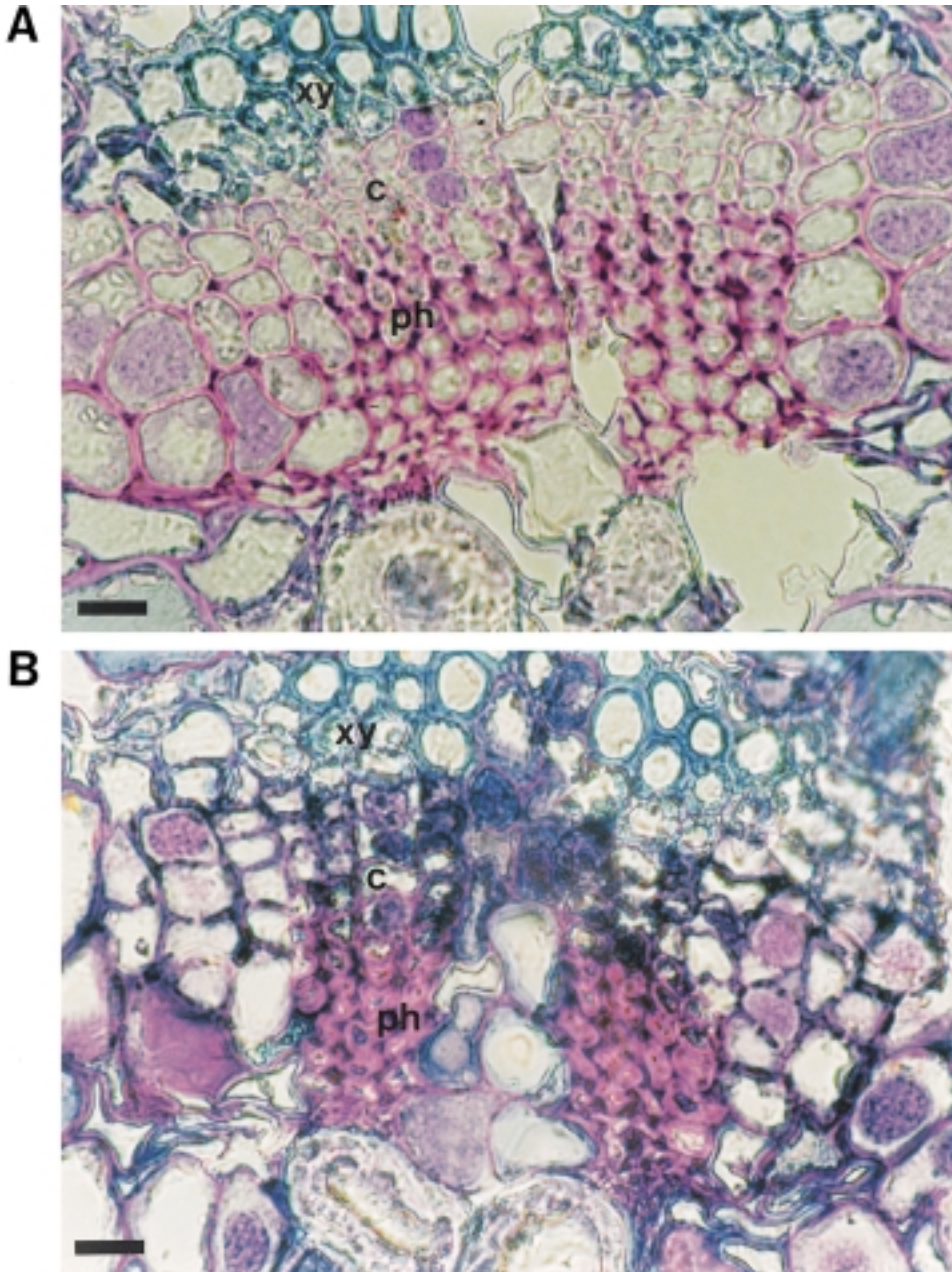


PLATE III. Cross-sections through conducting elements: ph – phloem, xy – xylem, c – cambium. Bar = 10 µm.

A. Green needle. B. Yellow needle

distorted (Plate I, Fig. A). The mesophyll in the yellow needles was highly necrotic (Plate I, Fig. C). In contrast to green needles (Plate II, Fig. A), some mesophyll cells of damaged needles contained grain structured tannin deposits (Plate II, Fig. B). The amount and structural changes of tannins were used for distinguishing different stages of mesophyll cell injury in conifer needles (SOIKKELI, 1981; RUETZE *et al.*, 1988). GIERTYCH *et al.* (1999) demonstrated significant negative correlation between K and total phenolics content in black pine (*Pinus nigra* Arn.) needles. A further characteristic of damaged needles was the totally collapsed phloem in vascular bundle, with hypertrophied cambial cells (Plate III, Fig. B). The symptoms of disturbances in the structure of vascular bundle described are specific for Mg deficient needles. Needles with K deficiency exhibit the same symptoms followed by mesophyll necrosis (FINK, 1989; 1993; PUECH & MEHNE-JAKOBS, 1997).

CONCLUSION

The results of total soluble protein measurement confirmed high nitrogen input from the cloaca. Modified conditions of mineral nutrition caused the Mg and K deficiency of investigated *Picea omorika* Pančić tree. Visual symptoms were in accord with the measured biochemical indicators. Histological analysis confirmed the specific pattern of decline for Mg and K deficient trees. Thus we were able to establish that the use of qualitative histological methods as screening option accomplished with quantitative biochemical analysis gives a specific insight into single situation. Therefore, such approach could be routinely used for diagnostic purposes in conifer pathology.

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SAŽETAK

Utjecaj promijenjene mineralne ishrane na iglice Pančićeve omorike (*Picea omorika* Pančić) – istraživanje pojedinačnog slučaja

H. Lepeduš, V. Cesar & E. Has-Schön

Propadanje šuma četinjača u Europi i sjevernoj Americi prvenstveno je uzrokovano abiotičkim faktorima, kao što su aeropolucija ili promijenjeni uvjeti mineralne ishrane. U pokušaju preciznog dijagnosticanja takvih stresnih čimbenika, primjenjivana su dva, u osnovi različita, pristupa: kvantitativna biokemijska analiza (BARNES

et al., 1995; KAINULAINEN *et al.*, 1995; MIKKELSEN *et al.*, 1995; WILD & SCHMITT, 1995; PFIRRMANN *et al.*, 1996; SCHULZ *et al.*, 1996; ŠIFFEL *et al.*, 1996; TAUSZ *et al.*, 1996; KRONFUSS *et al.*, 1998; GIERTYCH *et al.*, 1999; RIOTTO *et al.*, 1999; ORMROD *et al.*, 1999), te histološka i histokemijska analiza, koja kvalitativno determinira ciljne stanice za određene čimbenike (SOIKKELI, 1981; RUETZE, 1988; FINK, 1989; 1993; PUECH & MEHNE-JAKOBS, 1997).

U provedenom istraživanju primijenjena su oba pristupa, s ciljem uspostavljanja detaljne dijagnoze propadanja jednog stabla Pančićeve omorike (*Picea omorika* Pančić). Kao materijal su korištene iglice lateralnih izbojaka s istog stabla. Lateralni izbojci koji su bili bliže apikalnom izbojku sadržavali su zelene iglice, a oni koji su bili udaljeniji od apikalnog izbojka žute i smeđe iglice. Sadržaj fotosintetskih pigmenta bio je signifikantno niži u oštećenim iglicama, a koncentracija proteina otprilike tri puta veća. Također, u oštećenim iglicama detektirana je stanovita peroksidazna aktivnost, koje nije bilo u zelenim iglicama. Histološkom analizom utvrđen je potpuni kolaps sitastih stanica floema u provodnim elementima oštećenih iglica, kao i nekroza mezofila. Dobiveni rezultati upućuju na poremećaj u opskrbi biljke magnezijem i kalijem. Također, istraživanje je pokazalo da je integriranjem i kombiniranjem kvantitativnih biokemijskih i kvalitativnih histoloških metoda moguće rutinski odrediti uzrok(e) propadanja stabala četinjača, čak i u pojedinačnim slučajevima.