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BRIEF COMMUNICATION

Phenolics, lignin content and peroxidase activity in *Picea omorika* lines

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Abstract

We analyzed low molecular mass phenolics, lignin content and both soluble and cell wall bound peroxidase activity in the needles of three *Picea omorika* (Pančić) Purkyne lines grown in the generative seed orchard. The highest values of the total phenol content as well as of catechine, caffeic acid, coniferyl alcohol, isoferulic acid and lignin concentration were detected in B5 line ("semidichotomy" line). The soluble guaiacol peroxidase activity was the highest in A3 line (line "borealis"). The highest activity of cell wall bound peroxidases was measured in B5 line, and it was in correlation with lignin content.

Additional key words: guaiacol peroxidase, omorika, pollution stress.

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Picea omorika (Pančić) Purkyne is a Balkan endemic coniferous species and a Tertiary relict of the European flora, taxonomically close to the European spruce *Picea abies* (L.) Karst. Omorika is more resistant to the air pollution and drought in comparison with other conifers (Král 2002). Cultivated omorika is used throughout Europe as a decorative species. An increase in low molecular mass phenol concentration was commonly observed after a plant exposure to stress conditions (Brignolas *et al.* 1995, Giertych and Karolewski 1993). On the other side, lignin in cell walls provides both a mechanical support as well as a plant protection from the chemical and biological stress (Lewis and Yamamoto 1990). Lignin production, starting from phenolic monomers, is considered to be catalyzed by peroxidase (POD; E.C. 1.11.1.7), but there are different opinions about which POD fraction, soluble or cell wall-bound, has a role in lignin synthesis in the cell walls (McDougal 1991, Polle *et al.* 1994). The studies on different genetic lines of omorika may contribute to the selection of the genotypes more successful in response to the polluted and urban environments. In this study we analyzed low molecular mass phenolics, lignin content and the activity of both soluble and cell wall-bound peroxidases in the needles of three half-sib *P. omorika* lines. This is the first study of low molecular mass phenolic compounds and lignin in *P. omorika* species. Low molecular mass phenolics, lignin and POD are known to have a considerable part in the mechanism of resistance to the external stress in plants. Our aim was to find out whether there is any difference in phenolics and lignin content among various omorika lines, and if so, to determine which line has the highest content of the referred compounds, as well as which POD fraction correlates in activity with lignin content in the needles.

The needles were obtained from 15-years old *Picea omorika* trees, grown in a generative seed orchard in Godovik (43°51' N, 20°02' E, 400 m a.s.l.), Serbia. The total phenol content was determined in 80 % methanol extract of the powdered needles, using the Folin-Ciocalteu reagent (Singleton and Rossi 1965). The separations of low molecular mass phenolics were

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Abbreviations: DHP - dehydrogenate polymer; EDTA - ethylenediaminotetraacetic acid; GC-MS - gas chromatography with mass spectrometry; HPLC - high performance liquid chromatography; LTGA - lignin-thioglycolic acid complex; POD - peroxidase; PVP - polyvinylpyrrolidone.
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performed on *Waters Breeze* chromatographic system (*Waters*, Milford, MA, USA) connected to *2465 Waters* electrochemical detector, equipped with a gold working and a hydrogen referent electrode. Signals were detected in direct scan mode at the constant potential of +0.6 V, the signal filter time was 0.2 s and the sensitivity was 100 nA for the full mV scale. Peak confirmation and reverse phase high performance liquid chromatography (RP HPLC) were conducted by *Hewlett Packard* (Palo Alto, CA, USA) diode-array detector adjusted to 210, 280 and 330 nm, with reference signal at 600 nm. The separations were performed on *Waters Symmetry C-18 RP* column 125 × 4 mm with 5 μm particle size (*Waters*, Milford, MA, USA). Mobile phases were 0.1 % phosphoric acid and acetonitrile. The extraction of lignin was performed according to the procedure of Strack *et al*. (1988) and Chen *et al*. (2000). The purified cell wall material was first obtained from the needles. Thioglycolic acid was used to isolate lignin from the extractive-free cell wall material (Chen *et al.* 2000). Cell wall-bound phenolics were obtained from the extractive-free cell wall material by alkaline hydrolysis in 1 M warm (80 ºC) NaOH during 17 h at room temperature. HPLC was used to determine low molecular mass phenolics and gas chromatography joint with mass spectrometry (GC-MS) for the detection of dimers. Mass spectra were recorded by a *Finnigan MAT 8230* (San Hose, CA, USA) spectrometer, using the method of chemical ionization. The pellet left after alkaline hydrolysis was used for lignin quantification. Since there is no absolute reliable method for the determination of lignin content, lignin quantification was performed in three different ways: by acetyl bromide test using: *1*) 2 mg of NaOH hydrolyzed cell walls or *2*) 1 mg lignin-thioglycolic acid complex (LTGA), according to the procedure of Morrison (1972), as well as *3*) by measuring absorbance of LTGA dissolved in 0.5 M NaOH at 280 nm, according to Dean (1997). In acetyl bromide test, standard curves were obtained using freshly prepared solutions of coniferyl alcohol. In procedure (*3*), a standard curve was obtained with dehydrogenate polymer (DHP) synthesized from coniferyl alcohol. Soluble and cell wall-bound peroxidase activities were determined according to the procedures of Otter and Polle (1994) and Takahama and Oniki (1994). Frozen needles were powdered in liquid nitrogen. After the extraction (using 0.1 M Tris-HCl buffer pH 7.6, containing 1 mM dithiotreitol, 1 mM EDTA, 0.2 % *Tween 80* and 2 % PVP, in 1:5 m/v ratio) and centrifugation of the homogenate, the supernatant was used for the measuring of the soluble peroxidase activity. After washing and centrifugation, the pellet (cell wall fraction) was used for the measuring of the activity of the cell wall-bound POD fraction. Each experimental variant was represented by four trees, which were separately treated in four replicates each. The statistical analysis of the results was performed using the Mann-Whitney ranking test, at the 0.05 level of significance.

There was a significant difference in the total soluble low-molecular mass phenol concentration among three *P. omorika* lines (Table 1). The lowest phenol concentration was found in C2 line, while the highest content was present in B5 line. The value of the total phenol concentration in omorika needles was comparable to that found in spruce needles (Karolewski and Giertych 1995). Among the low-molecular mass phenolic compounds, in omorika methanol extract, catechine was present in the highest concentration, followed by chlorogenic acid. There was a significant difference in catechine concentration among all three lines. Caffeic acid, coniferyl alcohol and isoferulic acid are present in higher concentration in A3 and B5 lines than in C2 line (Table 1). Catechine has been considered as a possible bioindicator of early stress in Scots pine, since its content in needles followed the external pollution (Härtling and Schulz 1998). It is worth noting that the content of catechine in omorika needles [100 - 240 μmol g^{-1}(f.m.)] was considerably higher in comparison with its concentration reported for pine [1.39 - 8.93 μmol g^{-1}(f.m.); Härtlig and Schulz 1998] and spruce [13.4 - 32 μmol g^{-1}(f.m.); Heller *et al.* 1990] trees of similar age. Such a high content of catechine may be beneficial in response to external stress. An interesting result is that isoferulic acid was found in omorika needles, while the concentration of ferulic acid was insignificant. Free ferulic acid has been proposed to have a protective role in plants against the external stress. Due to its phenolic nucleus and an extended side chain conjugation, it readily forms a resonance stabilized phenoxy radical that accounts for its potent antioxidant potential (Graf 1992). Isoferulic acid may have such a protective role in *P. omorika* species. On the other side, syringic and *p*-coumaric acid, which is not expected to considerably participate in production of coniferous lignin (Lewis and Yamamoto 1990), were found in low concentration in omorika needles (Table 1). The obtained results show that those phenolics that are considered to have a role in stress response or to be involved in lignin formation are present in considerably higher concentration in A3 and B5 line than in C2 line. Therefore, the obtained results may be a basis for the selection of the omorika lines that will be more resistant to the air pollution and urban conditions. Alkaline hydrolysis of the cell walls enables studying of the type and amount of the esterified and some of the etherified phenolics in the cell walls that make transversal connections among the wall polymers and in this way they give mechanical firmness to the whole wall structure. So far, grasses has been the most studied plant material in this respect. A HPLC analysis showed that alkaline hydrolyzate of *P. omorika* needle cell walls contains ferulic acid and coniferyl alcohol (Table 1), with no significant differences among the lines. A GC-MS analysis confirmed the presence of ferulic acid (molecular ion = 209) in the alkaline hydrolyzate of all three lines, while coniferyl alcohol (molecular ion = 181) was found in A3 and C2 line. Hydroxycinnamic acids are known as directly esterified or etherified to lignin surfaces (Lam *et al.* 1992). The detection of ferulic acid in alkaline

Table 1. Concentration of total soluble phenols [mmol(gallic acid equivalents) g^{-1}(f.m.)], individual soluble phenols contained in highest concentration in the extract, as measured by HPLC [μmol g^{-1}(f.m.)] and cell wall-bound monomeric phenols released from extract-free cell walls by alkali [nmol mg^{-1}(cell wall d.m.)] ([a]), in the needles of three *P. omorika* lines: A3 ("borealis"; branching similar to branching in spruce, broad tree crown), B5 ("semidichotomy", false double treetop), C2 ("serbica"; typical omorika, narrow pyramidal crown). Letters and corresponding numbers indicate fenogroup and related mother genotype, respectively (*, ** - statistically significant difference in relation to C2 and A3 line, respectively).

P. omorika	A3	B5	C2
Total soluble phenols	1.54 ± 0.04*	1.71 ± 0.04*	1.35 ± 0.04
Caffeic acid	0.50 ± 0.09*	0.33 ± 0.07*	0.08 ± 0.01
Syringic acid	5.46 ± 0.80	5.48 ± 1.45	2.73 ± 0.54
Coniferyl alcohol	8.66 ± 0.69*	9.86 ± 0.40*	5.38 ± 0.71
p-coumaric acid	1.73 ± 0.36	1.40 ± 0.13	1.58 ± 0.08
Isoferulic acid	6.08 ± 0.48	10.18 ± 0.19*,**	5.29 ± 0.73
Chlorogenic acid	14.22 ± 1.86	13.60 ± 1.27	16.37 ± 2.46
Protocatechuic acid	2.17 ± 0.06*	2.01 ± 0.26*	1.12 ± 0.28
Catechine	117.60 ± 5.60	218.38 ± 19.45*,**	165.90 ± 5.85**
Ferulic acid[a]	0.78 ± 0.22	0.75 ± 0.12	1.08 ± 0.20
Coniferyl alcohol[a]	0.42 ± 0.14	0.43 ± 0.17	0.61 ± 0.28

hydrolizate of omorika needles shows the presence of such a type of covalent cross-links among wall polymers. The detection of coniferyl alcohol in alkaline hydrolyzate of cell walls shows the presence of direct ether linkages between polysaccharides and lignin, including primary hydroxyls on terminal coniferyl alcohol units (Iiyama *et al.* 1994), since such bonds are quite labile to alkali. GC-MS analysis of the cell wall alkaline extracts has shown the presence of dehydroferulic acid dimers (molecular ion = 417) and ferulic acid-coniferyl alcohol dimers (molecular ion = 389). This is an evidence of ester and ether linkages between lignin and polysaccharides in studied cell walls (Fry 1983, Lam *et al.* 1992, Iiyama *et al.* 1994, Hatfield *et al.* 1999), formed not only by dehydroferulic acid dimers, but also by mixed dimers involving ferulic acid and coniferyl alcohol. An interesting fact that coniferyl alcohol trimers (molecular ion = 541) were also detected in the alkaline hydrolyzate of needle cell walls of A3 and B5 line, shows that this compound can itself, without ferulic acid, form weak bonds (esther or ether) with lignin. Coniferyl alcohol trimers may be an evidence of more extensive cross-links among wall polymers in *P. omorika* species. Similarly, a ferulic acid dehydrotrimer has been found in saponified maize bran insoluble fiber (Bunzel *et al.* 2003). These results reveal that polysaccharide chains can be more extensively cross-linked than previously recognized.

In coniferous trees, coniferyl alcohol is a basic substrate for lignin production. The determination of lignin quantity in three different ways proved to be reasonable. Acetyl bromide test uses cell wall as a starting material, which is the main reason why lignin content might be overestimated. On the other hand, lignin quantification using thioglycolic acid may underestimate lignin content, since predominantly β-O-4 bonds are observed by this method. There was a significant difference in lignin concentration among all three lines (Table 2). An acetyl bromide test of both isolated lignin and extract-free cell walls gave the same ratio of lignin concentration among the three lines. Lignin concentration was the highest in B5 line, according to both the acetyl bromide test and the maximum of lignin absorption in NaOH (Table 2). The ratio of lignin concentration among the lines conforms to the ratio of free coniferyl alcohol concentration in their methanol extract (Table 1).

Soluble guaiacol peroxidase activity was the highest in A3 line. The highest activity of cell wall bound peroxidases was found in B5 line (Table 2). The lowest activity of both POD fractions was found in C2 line. In response to the stress conditions, in plants, POD has a role in removing hydrogen- and organic peroxides and in the synthesizing of cell wall polymer lignin (Castillo 1986, Lewis and Yamamoto 1990, Bisbis *et al.* 2003/4, Bernardi *et al.* 2004). Polle *et al*. (1994) claimed that apoplastic peroxidases (including soluble and ionically bound to the cell walls) are responsible for lignin synthesis. According to McDougal (1991, 1992), predo-

Table 2. Lignin content and activity of free and cell wall-bound peroxidase fraction in the needles of three *Picea. omorika* lines. Lignin concentration was determined by three different methods (for details see text) (*, **, [++] - statistically significant difference in relation to C2, A3 and B5 line, respectively; [g, ca] - 92 mM guaiacol, 100 μM coniferyl alcohol as peroxidase substrates, respectively).

P. omorika	Lignin content			Peroxidase activity		
	[nmol(coniferyl alcohol equivalents) mg^{-1}(cell wall d.m.)]	[nmol(coniferyl alcohol equivalents) mg^{-1}(isolated lignin)]	[mg(isolated lignin equivalents) mg^{-1}(DHP)]	soluble [U g^{-1}(f.m.)]	cell wall-bound [U mg^{-1}(cell wall d.m.)]	
A3	59 ± 1*	323 ± 3*	0.416 ± 0.002*	67 ± 2[g *, ++]	0.043 ± 0.002[g *]	0.018 ± 0.001[ca *]
B5	65 ± 2*	381 ± 9*	0.443 ± 0.004*	62 ± 2[g *]	0.052 ± 0.002[g *,**]	0.024 ± 0.001[ca *,**]
C2	56 ± 1	274 ± 4	0.402 ± 0.002	44 ± 3[g]	0.037 ± 0.001[g]	0.015 ± 0.001[ca]

minantly cell wall bound peroxidases produce lignin in the cell walls. Our results show the accordance of lignin content with the activity of cell wall bound peroxidases in omorika needles. The line with the highest bound POD activity also shows the highest lignin content (Table 2).

In the previous study Kähkönen *et al*. (1999) screened a large number of plant species for the total phenolic concentration and their antioxidant activity. They showed that *Picea abies* contained the highest total phenolic amount. The authors suggested that a further study is necessary to determine the effect of intraspecies variation. Since phenolic concentration and type, as well as peroxidase activity and lignin content, make a part of plant antioxidant activity, the results presented may indicate that among the studied *P. omorika* lines, B5 may have the highest protective capacity concerning the referred metabolites.

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