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LIGNIN CONTENT IN *PICEA OMORIKA* NEEDLES

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Abstract

*Picea omorika ((*Pančić) Purkyně) is endemic to the Drina River valley in western Serbia and eastern Bosnia and Herzegovina near Višegrad. It was originally discovered near the village of Zaovine on the Tara Mountain in 1875, and named by the Serbian botanist Josif Pančić. Lignin in cell walls provides a mechanical support as well as a plant protection and resistance from the chemical and biological stress. In coniferous trees, coniferyl alcohol is a basic substrate for lignin production. In this study we analyzed lignin content in the needles of three half-sib *P. omorika* lines. 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. 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), as well as 3) by measuring absorbance of LTGA dissolved in 0.5 M NaOH at 280 nm. 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.
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. 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. As lignin make a part of plant antioxidant capacity, the results presented may indicate that among the studied P. omorika lines, B5 may have the highest protective capacity.
Key words: cell wall, *Picea omorika* (Pančić) Purkyně, lignin.

Introduction

Picea omorika (Pančić) Purkyně is a Balkan endemic coniferous species and Tertiary relict of the European flora, taxonomically close to the European spruce *Picea abies* (L.) Karst. The habitat of this species is rather narrow (approx. 10000 km2), occupying exclusively the middle and upper courses around the river Drina (JOVANOVIĆ, 1970). It was originally discovered near the village of Zaovine on the Tara Mountain in 1875, and named by the Serbian botanist Josif Pančić.
Lignin, a complex phenolic polymer, is important for mechanical support, water transport, and defense in vascular plants (LEWIS et al., 1999). In coniferous trees, coniferyl alcohol is a basic substrate for lignin production. The insolubility and complexity of the lignin polymer makes it resistant to degradation by most microorganisms. Therefore, lignin serves an important function in plant defense. In addition to being highly heterogeneous as a polymer, lignins can vary within a given cell wall (AGARWAL and ATALLA, 1986). Lignin heterogeneity is regulated during secondary cell-wall deposition, giving rise to layers of lignin that can differ in average monomer composition. Thus within a given cell wall, lignin subunit composition and overall quantity may vary depending on location in the wall, developmental state of the cell and tissue, and the influence of environmental stress. Lignin also varies in its composition and quantity between different cell types and between tissues within the same plant (CAMPBELL and SEDEROFF, 1996). Variation of lignin structure among different lignin samples could result in error in calculation of lignin content. There is no absolute reliable method for the determination of lignin content.

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In this study we analyzed lignin content in the needles of three half-sib *P. omorika* lines in three different ways. As lignin is known to share a considerable part of the mechanism of resistance to the external stress in plants, our aim was to find out whether there is difference in lignin among various omorika lines, and if so, to establish which line has the highest content of the referred compounds. Besides, such studies contribute to the selection of the omorika genotypes more successful in response to the polluted and urban environments.

Material and methods

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 needles were collected at approximately the same height of the trees. Needles were collected in august 2003. Plants used for the experiments were healthy, without any exogenous infection detected. All needle samples were taken from the trees between 9 and 11 AM, and immediately stored in the liquid nitrogen.
The following omorika lines were used in the experiments: A ("borealis") – branching similar to the branching in spruce, broad tree crown; B ("semidichotomus") - characterized by a spontaneously appearing double treetop, without visible biotic or abiotic causes; C ("serbica") - type of branching characteristic of the typical habitat of omorika, narrow pyramidal crown.
Dry needles (72 h at 80º) were ground into a fine powder. To obtain cell walls, 400 mg of needle powder was homogenized for 5-10 min in 10 ml 80% methanol. The homogenate was stirred for 1 h at room temperature and centrifuged for 5 min at 1500 g. The pellet was reextracted twice with 10 ml 80% methanol. The pellet was subjected to the following washing steps (STRACK et al, 1988, CHEN et al, 2000): 1x (1M NaCL, 0.5% Triton X-100), 2x distilled water, 2x 100% methanol, 2x 100% acetone (each step in 20 ml, 30 min). After drying the extractive-free cell wall material (1 g) was subjected to the treatment with 2% (w/v) cellulase and 0.25% (w/v) pectinase in 20 ml Na-phosphate buffer. Samples were incubated with gentle shaking for 48 h at 30º, centrifuged at 16000 g for 10 min, the supernatant discarded, and the pellet ("fraction I") washed by stirring and centrifugation with buffer three times, then with distilled water three times, and dried in vacuum.
Fraction I (100 mg) prepared as above was incubated for 4 h at 95ºC with 6 ml of 2 M hydrochloric acid and 1.2 ml thioglycolic acid. The samples were cooled to room temperature, centrifuged, and the supernatant discarded. After washing the pellet with distilled water (three times), LTGA (lignin-thioglycolic acid complex) was extracted by strong shaking for 18 h in 6 ml of 0.5 M NaOH, at 30ºC. After centrifugation, the supernatant was kept and combined with the supernatant obtained from washing the pellet with 3 ml of 0.5 M NaOH. The combined alkali extracts were acidified with 1.8 ml of concentrated hydrochloric acid. LTGA precipitate formed after 4 h at 4ºC was recovered by centrifugation and washed twice with distilled water. The extractive-free cell wall material was subjected to alkaline hydrolysis by dissolving in 1 M warm (80ºC) NaOH and incubating for 17 h at room temperature. The resulting pellet, called "fraction II", was washed twice with distilled water, dried (24 h at 80ºC) and used for lignin quantification.
Lignin quantification was performed in three different ways: by acetyl bromide test using (1) 2 mg of "fraction II" cell walls or (2) 1 mg LTGA (MORRISON, 1972), as well as (3) by measuring absorbance of LTGA dissolved in 0.5 M NaOH at 280 nm (DEAN, 1997; CHEN et al., 2000). In acetyl bromide test standard curves were obtained with freshly prepared solutions of coniferyl alcohol and lignin concentration was expressed in µg of coniferyl alcohol equivalents mg^{-1} cell walls (1) or isolated lignin (2). In procedure (3) standard curve was obtained with DHP synthesized from coniferyl alcohol, and lignin content was expressed in mg of DHP equivalents.

Results and discussion

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 1).

Table 1. . Lignin concentration in three *P. omorika* lines, determined by acetyl bromide test using extract-free cell walls (a) and isolated lignin (b), and by absorption maximum of isolated lignin (c). * Statistically significant difference in relation to C2 line.

Tabela 1. Koncentracija lignina u četinama *P. omorike* izmerena acetil bromidnim testom koristeći izolovan ćelijski zid (a) i izolovan lignin (b), i merenjem maksimuma apsorpcije izolovanog lignina (c). Označena statistički značajna razlika je u odnosu na C2 liniju.

Lignin content	*P. omorika* lines A3 ("borealis") Branching similar to branching in spruce, broad tree crown	 B5 ("semidihotomy") False double treetop	 C2 ("serbica") Typical omorika, narrow pyramidal crown
μg coniferyl alcohol equivalents mg^{-1}CW DW	10.63±0.18*	11.71±0.36*	10.09±0.18
μg coniferyl alcohol equivalents mg^{-1} isolated lignin	58.20±0.54*	68.66±1.62*	49.37±0.72
mg isolated lignin equivalents mg^{-1} DHP	0.416±0.002*	0.443±0.004*	0.402±0.002

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 1).

Since obtained extractive free cell wall made about 58 % of the needle dry weight, obtained lignin content corresponded to about 95-115 μmol lignin monomers/ g needle dry weight, measured by acetyl bromide test, which is similar value to that found in *P. abies* L in the same season (POLLE et al., 1994).

Conclusion

The determination of lignin quantity in three different ways proved to be reasonable. As lignin make a part of plant antioxidant capacity, the results presented may indicate that among the studied *P. omorika* lines, B5 may have the highest protective capacity.

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