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VARIABILITY OF PEROXIDASE ACTIVITY AND HEAVY METAL CONTENT IN THE NEEDLES OF AUSTRIAN PINE (Pinus nigra Arnold) HALF-SIB LINES

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Enzyme activity of peroxidases of total proteins and heavy metal contents were studied in the needles of eight different half-sib lines of Austrian pine. The samples were taken from a fourteen-year-old seedling seed orchard on Jelova Gora (near Užice). The results of this study show the pronounced variability of the concentration of total proteins, heavy metal concentration and enzyme activity of peroxidases in the study Austrian pine half-sib lines. This infers that proteins have different levels of expression in individual Austrian pine lines. Also the enzyme of peroxidases shows different activity depending on the genetic constitution of the trees of the analysed half-sib lines, because based on the seed orchard size and the planting pattern, it can be assumed that the effect of

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external factors is reduced to a minimum. The peroxidases present as soluble in the apoplast or with covalent bond to cell wall, have an important role in lignification, suberisation, healing of injuries, defence against pathogens and atmospheric pollutants.

Key words: peroxidase, heavy metals, needle, austrian pine

INTRODUCTION

The influence of genetic variability on some enzymes and the occurrence of isoenzymes which are specific for some varieties of plant species have been known from earlier studies (SCHMIDTLING & HIPKINS, 1998; LEINEMANN, 2000). Question is still posed how great the influence of genetic diversity is on these biomacromolecules in comparison with the effect of various environmental factors. In order to familiarize with the effect of genetic potential and diversity on one of the key enzymes involved in the stress response, which is also responsible for lignification process in cell wall, enzyme activity of peroxidases was monitored in the needles of Austrian pine (*Pinus nigra* Arn.) half-sib lines. Genetic diversity causes various expression and reactivity of the enzyme, as well as various isoenzyme forms. In addition, activity of this enzyme in dependence on medium acidity was monitored. By means of isoelectrofocusing, isoenzymes were determined which participate in the mentioned reactions and processes in eight selected Austrian pine lines incorporated in generative seed orchard.

Generative seed orchard of Austrian pine on Jelova Gora mountain has metapopulation structure and area of 3.10 ha. It was established with 40 half-sib lines of Austrian pine, with the total of 5700 plants. On the basis of relatively small orchard area, it can be assumed that environmental factors are more or less uniform, whereby almost the same conditions for the growth and development of the trees of all half-sib lines are provided. Therefore, the results obtained from the analyses which were carried out to the greatest extent reflect the influence of genetic diversity of the half sib lines.

In two-year-old needles, the activity of soluble peroxidases (POD) present in cytoplasm and apoplast was measured, as well as the concentration of total proteins in both fractions. In addition to enzyme activity, isoenzyme profile on polyacrylamide gel was also monitored.

Concentration of heavy metals was determined in two half-sib lines which showed the largest part of statistically significant differences in the activities of peroxidases in relation to other lines.

MATERIALS AND METHODS

Experiments were done on two-year-old needles of Austrian pine (*Pinus nigra*). Needles were taken from ten-year-old Austrian pine trees. Material was frozen and transported to laboratory where the analyses were performed. Samples for the extraction of enzymes were homogenized, 1 g each. Activities of peroxidase enzyme were analyzed. Isoenzyme profile of this enzyme was determined in soluble fraction. In addition, the concentration of total proteins was determined. Concentration of heavy metals in the needles was determined by atomic absorption spectrophotometry. Total of four repetitions were applied for each genetic line. Based on this, non-parameter statistics was performed, i.e. Mann-Whitney U test, with reliability of 0.95 %, in order to evaluate the significance of differences among the values for each line.

Extraction of enzymes was carried out by means of homogenization in 5 mL 0.1 M TRIS-HCl buffer pH 7.6 containing 1 mM DTT and 1 mM EDTA in freezing bath according to the procedure of MOCQUOT et al. (1996) and WECKX and Clijsters (1996). Plant material was homogenized for 1 min. Homogenizer type 18/10 (*Janke & Kunkel Ika-werk*) was used. The sample homogenized in that way are strained through eight layers of gauze and centrifuged at 12000 g for 10 min at 4°C (*Sorvall RC-5B*). Supernatant contains soluble (inter-cell and apoplast) fractions of peroxidase enzyme. Samples prepared in that manner are ready for analysis. Determination of peroxidase activity was performed by the use of spectrophotometry method with hydrogen-peroxide and guaiacol, universal substrate for the total POD (CHANCE & MAEHLY, 1955). Enzyme activity is calculated on the basis of tetraguaiacol extinction coefficient which amounts to ε_{470}=25.5 $mM^{-1}cm^{-1}$ (GEORGE, 1953).

$$AKT = Vk * \Delta A / Ve * 25.5,$$

Where the following applies: Vk – volume of the solution in cuvette (mL), ΔA - absorbance change in one minute, Ve – volume of the sample added to reaction mixture (mL), value of 25.5 relates to guaiacol extinction coefficient.

Concentration of proteins was determined with the use of Bradford (BRADFORD, 1976) method. This method is based on binding Coomassie Brilliant Blue G-250 to aromatic amino acid residue, whereby maximum color absorption is moved from 465 nm to 595 nm. The color is developed after 2 min and the increase of absorbance is measured at 595 nm.

For the determination of the concentration of metals in the needles of Austrian pine, samples were prepared according to the procedure for "dry burning" whereby flame atomic absorption spectrophotometry was used. The sample was read on the absorber *Varian AA-10*. Detection limit for Cd is 0.02 – 3 µg/mL, for Cu 0.03-10 µg/mL and for Ni 0.1-20 µg/mL.

RESULTS

Genetic diversity causes various expression and reactivity of the enzyme, as well as a variety of isoenzyme forms. In order to obtain an answer to the question how large the effect of genetic diversity is on one of the key enzymes involved in the stress response, which is also responsible for the process of lignification in cell wall, the enzyme activity of peroxidases was monitored. In addition, activity of this enzyme in dependence on medium acidity was monitored. By means of isoelectrofocusing, isoenzymes were determined which participate in physiology processes in the needles of the tested Austrian pine lines.

Specific enzyme activity of peroxidases was first tested with regard to different pH values of medium, i. e. buffer on which the reaction was measured. In that way, optimum pH value was precisely determined for the enzyme reaction of total peroxidases in the needles of Austrian pine. It was determined that optimum value of pH is 5.5 for peroxidase reaction (Figure 1). Reaction was performed in the mixture of isocitrate-phosphate buffers with various pH values (3 – 8). PH optimum of the reaction is the same as for peroxidases isolated from spruce needles.

pH correlation POD from pine

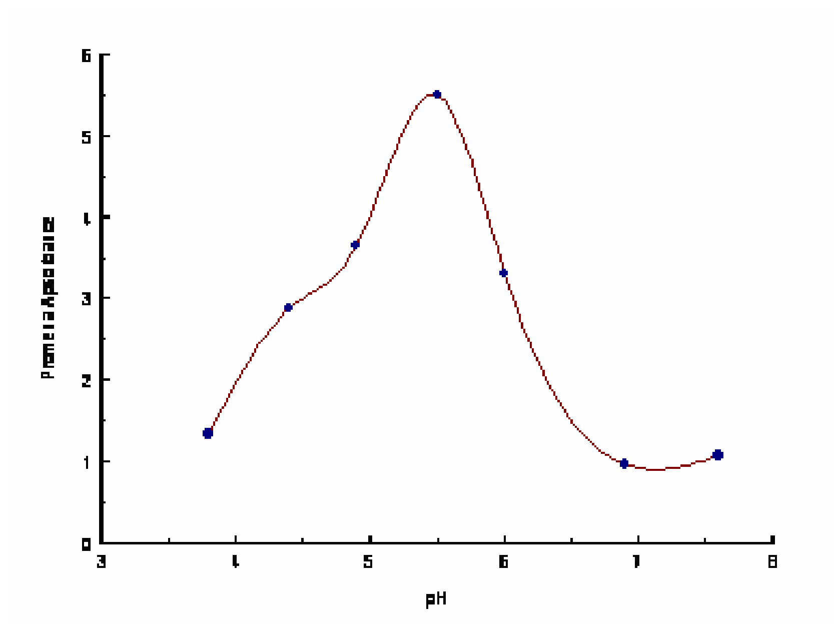

Figure 1 Optimum pH value for the enzyme reaction of total peroxidases from the needles of Austrian pine

Figure 2 shows the concentration of total proteins in the isolate of Austrian pine needles. Protein content in different lines ranges from 0.05 to 0.20mg/ml. The lowest protein content was measured in the needles of the line 23, and the highest in the line 32. In the figure, statistically significant differences among the lines are marked. Statistically, the lines 23 and 40 differ from the other lines to the greatest extent. Synthesis of total proteins depends on many factors (external and internal).

Concentration of proteins in the needles of various Austrian pine lines

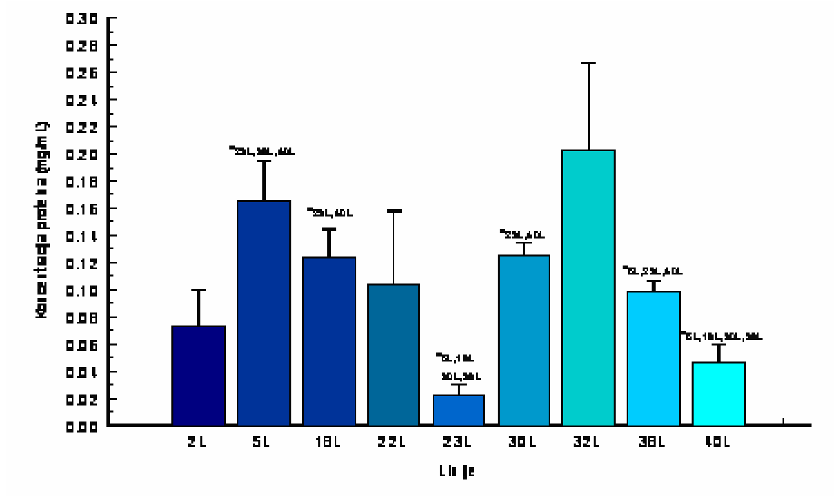

Figure 2 Concentration of proteins expressed in mg per mL in the needles of various Austrian pine lines.

- designates statistically significant difference (p≤0,05).

Specific activity in the needles of various Austrian pine lines

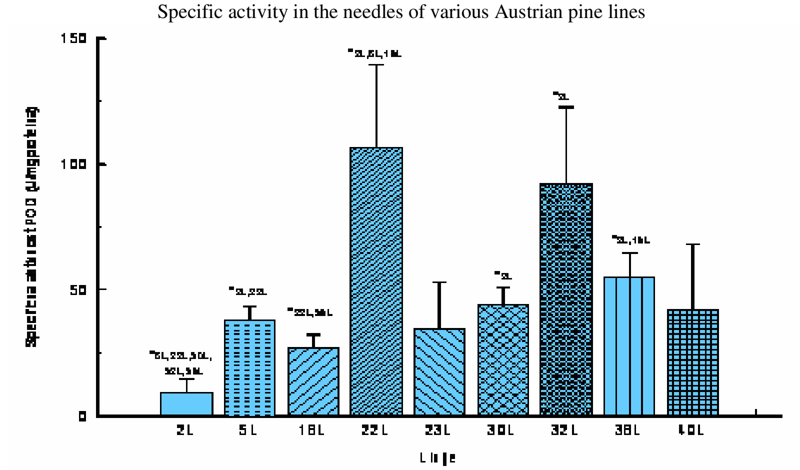

Figure 3 Enzyme activity of peroxidases expressed as specific activity (U/mg of proteins) in the needles of various Austrian pine lines.

* designates statistically significant difference (p≤0,05).

Figure 3 shows the results of specific enzyme activity of peroxidases in the needles of Austrian pine. The lowest value was measured in the line 2, and it amounted to 9.5U/mg of proteins, and the highest in the line 22, which amounted to 110U/mg of proteins. To the greatest extent, statistically significant difference was demonstrated between the lines 2 and 22.

Figure 4 shows the content of heavy metals in the lines where the highest and the lowest peroxidase activity were measured – i.e. the lines 2 and 22. The measured concentration of metals statistically differs in the needles of these two lines by the content of nickel and lead. Results of the analyses showed that the content of other metals in the needles did not significantly differ. The line 2 contained significantly higher concentration of lead than the one determined in the line 22, while the content of nickel was significantly higher in the needles of the line 22.

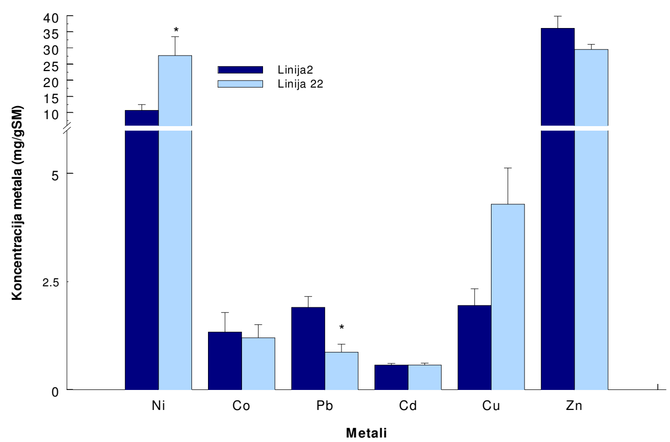

Figure 4 Concentration of heavy metals expressed in mg of metal per g of dry needle mass in the lines 2 and 22 of Austrian pine which showed the greatest difference in POD activity.

* designates statistically significant difference ($p \leq 0$)

Figure 5 shows the results of the analyses which illustrate interline variability in the content of copper, nickel and zinc in needle samples. It was determined that the content of these three heavy metals was the greatest in the needles of line No. 2.

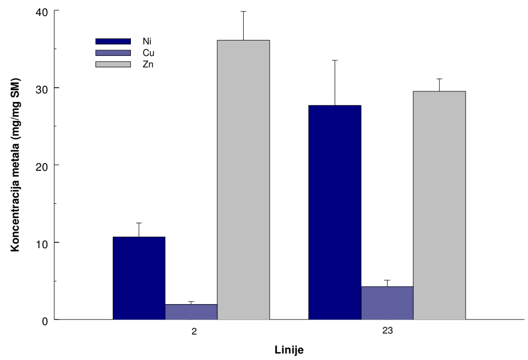

Figure 5 Concentration of Ni, Cu and Zn in Austrian pine lines 2 and 22.

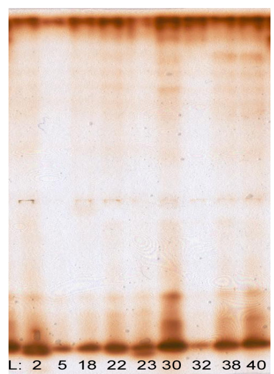

Figure 6 Isoelectric focusing of needle isolate of various Austrian pine lines.

The results of studies showed that the change in the concentration of total proteins and activities of peroxidase enzyme in the needles of Austrian pine lines, wherefrom the seed orchard of this species was established on Jelova Gora mountain near Užice, significantly differed (Figures 2 and 3). Proteins have various expression level in the individual Austrian pine lines, while at the same time peroxidase enzyme shows different activity which can be considered to be under direct genetic control, having in mind that the influence of external factor was minimized due to relatively small seed orchard area. Various activities of enzymes and proteins which have been recorded show that genetic variation of analyzed Austrian pine half-sib lines is highly significant for the further directed use of the genetic potential of this species.

Contents of heavy metals in the lines 2 and 22, which mostly differ from the other lines, shows statistically significant difference in concentration of the metals Pb and Ni, Figures 4 and 5. Based on this, it can be concluded that the accumulation and transport of metals differ in different lines, and more likely, that microhabitat characteristics differ in some parts of the orchard. The results of the researches of some authors, BLINDA et al., 1997, showed that exposition to heavy metals such as Ni and Cd caused *de novo* syntheses of proteins, especially in apoplast. This shows that this part of cell, together with cell wall, plays very important role in the response to stress caused by heavy metals.

The effect of these metals on the activity of enzymes is pronounced, so that the complexity of factors leading to various activities of peroxidases and total proteins should be taken into account in further analyses.

Other authors, e.g. Leinemann, demonstrated that the composition of isoenzymes of other enzymes, such as glucose-6-phosphate dehydrogenase (G-6-PDH) or isocitrate dehydrogenase (IDH) had special importance during the separation of various subspecies of *Prunus spinosa* (LEINEMANN, 2000). This method is currently successfully applied for reliable determination of genetic base in species and subspecies variability of trees. On the other hand, we may conclude that isoenzyme monitoring of peroxidases, as a method for determination of genetic differences among Austrian pine half-sib lines, is not sufficiently reliable, although statistically significant difference was determined during the spectrophotometry monitoring of enzyme activity. Results of the analyses performed with spruce needles, RADOTIĆ et al. 2000, showed that the occurrence of new isoenzymes of peroxidases in spruce needles was an indicator of stress caused by heavy metals (Cd, Ni, Cu). This poses new questions for future researches in the sense – are the other enzymes reliable indicators of divergences inside species and could some enzymes of antioxidant defense be reliably used in order to monitor environmental pollution?

CONCLUSIONS

Results of the performed analyses show the presence of pronounced variability in the concentration of total proteins, content of heavy metals and activities of peroxidase enzyme in two-year-old needles of analyzed Austrian pine half-sib lines. Concentration of total proteins and activities of peroxidase enzyme are significantly different in various Austrian pine half-sib lines (Figures 2 and 3) while the proteins have various level of expression in individual lines.

In addition, peroxidase enzyme shows different activity which depends on genetic constitution of the trees from analyzed half-sib lines, because based on the seed orchard size and the planting pattern, it can be assumed that the effect of external factors is reduced to minimum. Peroxidases present as soluble in apoplast, or covalently bound to cell wall, play an important role in lignification, suberization, healing injuries, defense against pathogens and airborne pollutants.

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VARIJIABILNOST AKTIVNOSTI PEROKSIDAZA I SADRZAJA TEŠKIH METALA U ČETINAMA LINIJA POLUSRODNIKA CRNOG BORA (Pinus nigra Arn.)

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Izvod

Ispitivana je aktivnost enzima peroksidaza ukupnih proteina i sadrzaja teških metala u četinama osam različitih linija polusrodnika crnog bora. Uzroci su uzeti iz četrnajstogodišnje generativne semenske plantaže na Jelovoj Gori (kod Užica), osnovane po modelu metapopulacione strukture. Ekstrakcija enzima je radjena najpre homogenizacijom u 5 mL 0.1 M TRIS-HCl puferu pH 7.6 koji sadrži 1 mM DTT i 1 mM EDTA na ledenom kupatilu po proceduri . Koncentracija proteina je uradjena po metodi (Bradford, 1976). Za odredjivanje koncentracije metala u četinama crnog bora, uzorci su pripremani prema proceduri za "suvo spaljivanje" Uzorak je očitavan na apsorberu *Varian AA-10*. Detekcioni limit za Cd je od 0.02 do 3 µg/mL, za Cu je 0.03-10 µg/mL i za Ni 0.1-20 µg/mL. U cilju utvrđivanja koliki je uticaj genetičke raznolikosti linija polusrodnika na jedan od ključnih enzima uključenih u odgovor na stres, i zaduženog za proces lignifikacije u ćelijskom zidu, praćena je aktivnost enzima peroksidaza. Takodje je praćena aktivnost ovog enzima u zavisnosti od kiselosti medijuma. Izoelektrofokusiranjem utvrdjeni su izoenzimi koji su prisutni u četinama analiziranih osam linija crnog bora.

Rezultati oviog rada pokazuju prisustvo izraženog varijabiliteta u koncentraciji ukupnih proteina, sadržaju teških metala i aktivnosti enzima peroksidaza kod analiziranih linija polusrodnika crnog bora. Ovo govori da proteini imaju različit nivo ekspresije u pojedinim linijama crnog bora. Takodje enzim peroksidaza pokazuje različitu aktivnost koja zavisi od genetske konstitucije stabala iz analiziranih linija polusrodnika, jer se, na osnovu veličine plantaže i šeme sadnje biljaka, može smatrati da je uticaj spoljašnjih faktora sveden na minimum. Peroksidaze prisutne kao solubilne u apoplastu ili kovalentno vezane za ćelijski zid, imaju važnu ulogu u lignifikaciji, suberizaciji, zalačivanju povreda, odbrani od patogena i zagađivača iz vazduha.

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