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Changes in antioxidant enzyme activities in *Pinus sylvestris* and *Larix decidua* seedlings after *Melolontha melolontha* attack

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Abstract. Plants are constantly exposed to the influence of biotic and abiotic stress factors that significantly affect the induction of resistance responses. Biotic factors include bacteria, fungi and insects such as the common cockchafer (*Melolontha melolontha*) that harm many tree species, including pine and larch. The adult beetles damage needles, while the larvae (grubs) feed on roots. The aim of the experiment was to determine how plants cope with the damage caused by these insects. The activity of antioxidant enzymes such as peroxidase (POD) and superoxide dismutase (SOD) were determined three and six weeks after the insect-induced damage and the results show that the peroxidase activity, as measured with guaiacol (GPOD), was higher in the damaged roots of larch. The POD activity measured with syringaldazine (SPOD) was slightly higher in damaged pine needles as well as pine roots. SOD activity was higher in the damaged roots of both species as compared to the needles. It is believed that changes in the activity of POD and SOD as well as the presence of another form of SOD in the roots of larch, indicate direct involvement of these enzymes in the plants' response to damage caused by *M. melolontha*.

Keywords: coniferous species, defense, cockchafer, superoxide dismutase, peroxidases

1. Introduction

A wide variety of insects living in forest habitats cause significant damage to trees and shrubs, acting as a serious threat to them. Depending on the type of plant injury, insects are divided into five groups (McGauley and Kirby 1991). There are defoliators (feeding on leaves), borers (pests of bark and wood), sucking insects (distortion and discoloration of attacked tissues), gall-makers (abnormal growths of tissue) and root-feeders (pests of plant roots). Defoliators are insects which feed on the foliage of live plants and can destroy them completely (Glavendekić and Medarević 2010). Adults of *Melolontha melolontha* (L.) belong to a group of defoliators, while grubs of this beetle feed the roots of many tree species, both broadleaved and coniferous. *M. melolontha* attacks mainly roots of the seedlings of woody species, namely pine, oak or linden (Švestka 2006). *M. melolontha* grubs usually cause greater damage than adult beetles. They are increasingly harmful pests in forestry, affecting nurseries and young plantations and may cause delay or inhibition of growth in very young trees. (Sukovata et al. 2015).

The knowledge about basic plant mechanisms developed against insects is limited, in comparison with well-proven mechanisms taking part in the fight of host plants against pathogens. The responses of plants damaged by herbivores are frequently altered by insect-specific elicitors, giving plants the potential to optimize their defences. Insects that differ in this respect trigger different, but overlapping, patterns of gene expression. The specific plant response may depend on differences in the mechanical damage to the tissue. This is related to the type of mouthparts, method of feeding, compounds that enter a plant, insect saliva and secretion of digestive tract (Howe and Jander 2008). The pests with same construction of their mouthparts induce expression of same genes.

Resistance to biotic stress of plants depends on constitutive and induced mechanisms. Stimuli including damage to plant tissues, secretions of salivary glands and gastrointestinal secretions induce signals triggering plant defense. Moreover, the type of damage and of elicitor activate specific signalling pathways and biosynthesis of secondary metabolites. Plants possess the ability to recognize compounds which are oral secretions of insects. These compounds elicit more intense volatile responses than only mechanical damage (Felton and Tumlinson 2008; Fürstenberg-Hägg et al. 2013). Frost et al.

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(2008) demonstrated that organic volatile compounds can prepare a plant tissue attacked by one pest for an attack of another one. Interaction cross-talk allows the victim plant to choose the most effective way of defense. Cross-talk minimizes costs which the plant has spent on defense. Unfortunately, pests and pathogens modulate the signal pathways so that they can take advantage of the induced immune suppression (Pieterse and Dicke 2007). Maffei et al. (2007) showed that inceptine (proteolytic fragments of subunits γ chloroplastic ATP synthase) functioned as strong, indirect signal triggering specific plant reactions in response to insect attack, they comprised of induction of volatile substances, phenylpropanoids or protease inhibitors.

The role of oral secretions in the defense response in roots is still unresolved (Fürstenberg-Hägg et al. 2013). Roots are exposed to more insect damage caused by grub of *M. melolontha* in comparison to needles. The beetle's grubs live in soil for 4 years; therefore, they cause great damage to the root system of plants.

In a tissue damaged by insects, there are a lot of biochemical processes disturbing the normal functioning of plant cells. Rapid increase in generation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) is an early response of plant cells to tissue damage. The increase in the activity of enzymatic antioxidants directly involved in metabolism of plants is the consequence of intensive and additional generation of ROS. Superoxide dismutase (SOD) is an enzyme that catalyses dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2, while peroxidase (POD) uses H_2O_2 for the oxidation of phenolic compounds.

The SOD gene family codes enzymes differing in the metal ion cofactor (iron superoxide dismutase (Fe-SOD), manganese superoxide dismutase (Mn-SOD), copper and zinc superoxide dismutase (Cu/Zn-SOD)) and in cell localization. The data on SOD gene regulation in coniferous plants are important to explain the molecular mechanisms of gene expression regulation and to understand the evolution of structure and function of SOD genes in higher plants (Katyshev et al. 2006).

PODs are an important group of enzymes that are involved in immediating the plants' response to post-insect damage. There are many processes related with plant defense that are regulated by PODs: lignification, suberization, somatic embryogenesis, auxin metabolism and wound healing. Plant defense is related with the production of phenoxy and other oxidative radicals by PODs and with the oxidation of phenols that can deter insects from feeding. PODs also produce toxins which affect insect growth and development (War et al. 2012).

Conifers are dominant plants in Polish forests. Unfortunately, they are victims of attacks by a wide range of organisms. It is interesting how coniferous trees cope with feeding of insects. In our work, we investigated the resistance of two different conifer species to *M. melolontha* attack because one of the important physical factors for plant resistance is the toughness of leaf. This feature affects biting, stinging or sucking mouthparts of insects and making it difficult for feeding (Schoonhoven et al. 2005; Howe and Schaller 2008). The selected trees' species differ in toughness of needles, which in our opinion may affect their defense abilities.

The aim of the study was to determine changes of POD and SOD activities after damage caused by *M. melolontha* grub. The expression of isoenzyme forms of POD and SOD was analysed by native electrophoresis on a polyacrylamide gel (PAGE).

2. Materials and methods

Plant material

One year old seedlings of Scots pine (*Pinus sylvestris* L.) and European larch (*Larix decidua* Mill.), obtained from the forest plantation in 2011 (Forest District Spała, 51°37'18.5''N, 20°07'37.3''E; Poland) damaged by grubs of *M. melolontha* were used. Control seedlings were plants not attacked by the grubs. The material was harvested during two periods (March and April in 2011). The samples were collected 3 and 6 weeks after visible symptoms of pest attack. The symptoms of *M. melolontha* grub feeding were monitored by collecting seedlings from the ground and determining the damage of root system. The experiment was arranged in a completely randomized design (ten seedlings were collected for each variant). The samples were immediately frozen in liquid nitrogen, stored at − 80°C and then used for analysis.

The plant tissue (1 g of roots and needles) was homogenized in 10 mL of 100 mM potassium phosphate buffer at pH 6.0 with 0.8 M NaCl. Additionally, in the assay of SOD, the 1 mM EDTA-Na and 1% PVPP was added. The resulting homogenate was centrifuged at 10000 rpm. After centrifugation, the supernatant was used as the material for the implementation of enzyme assays.

Peroxidase (EC 1.11.1.7) activity

Peroxidase activity with guaiacol (GPOD) was determined by an increase in the absorbance at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) (Maechly and Chance 1954). The reaction mixture contained 25 mM acetate buffer pH 5.6, 5 mM guaiacol, 15 mM H_2O_2 and 20 µL of enzyme extracts. Addition of H_2O_2 started the reaction. Peroxidase activity with syringaldazine (SPOD) was determined spectrophotometrically by an increase in the absorbance at 530 nm (ε = 27 mM^{-1} cm^{-1}) (Imberty et al. 1985). The reaction mixture contained 25 mM phosphate buffer pH 6.0, 41.6 µM syringaldazine 50 µL solution (3.1 mg in 4 ml methanol), 0.11 mM H_2O_2 and 20 µL of enzyme extracts. Addition of syringaldazine started the reaction. Enzyme activities were referred to fresh weight of the samples.

Superoxide dismutase (EC 1.15.1.1) activity

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit photochemical reduction of NBT using the method of Beauchamp and Fridovich (1971).

The reaction mixture of 3 mL contained 50 mM phosphate buffer at pH 7.8, 13 mM methionine (Sigma), 75 µM NBT, 2 µM riboflavin (Sigma), 0.1 mM EDTA (Sigma) and 20 µL of enzyme extract. Riboflavin was added last and the tubes were placed 30 cm below two 15 W fluorescent lamps. After 10 minutes, the SOD activity was determined spectrophotometrically by an increase in the absorbance at 560 nm. The amount of enzymes inhibiting 50% of the reaction was treated as the enzyme unit.

Enzyme activities were referred to fresh weight of the samples.

Assay of protein content

Protein was determined by the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard.

Native PAGE

Native protein electrophoresis was performed at 100 V, on 7% polyacrylamide gel for POD and 11% polyacrylamide gel for SOD, prepared using modified buffer system of Laemmli (1970). Samples contained 0.1–0.2 mg of proteins exhibiting POD or SOD activities. POD activity was visualized with 100 x 10^{-3} M diamonbenzidine and 20 mM H_2O_2. SOD was visualized with 2.45 x 10^{-3} M blue nitrotetrazolium (NBT), 0.028 M versenic acid (EDTA) and 0.28 x 10^{-5} M riboflavin with the use of the modified Lee et al. (2007) method.

Statistical analysis

Statistical analysis was performed with Statistica, version 10. Sample variability for n = 4 was given as standard deviation of mean. The statistical analyses were performed using a non-parametric Mann-Whitney rank sum test. Statistically significant differences were accepted at $P \leq 0.05$.

3. Results

We observed that insect feeding significantly increased GPOD activities in the roots of larch, and slightly in pine needles, after 3 and 6 weeks of pest attack (Fig. 1). In pine roots, the GPOD activity was higher in the control samples after 3 weeks, while the opposite pattern was observed after 6 weeks (Fig. 1). In pine, respective GPOD activities in the control were very similar after 3 and 6 weeks of pest attack (Fig. 1). We observed differences in GPOD activity between roots and needles of the investigated plants. The GPOD activity was higher in roots than needles for pine and larch.

In the damaged larch needles, 3 weeks after pest attack, the sample SPOD activity was only slightly higher compared to the control; while in larch roots, 3 weeks after the pest attack it was lower, and in larch needles after 6 weeks and larch roots after 6 weeks, it was greatly increased. The SPOD activities of control samples were similar in larch roots 3 and 6 weeks after pest attack. SPOD activities in pine needles and roots after 3 weeks

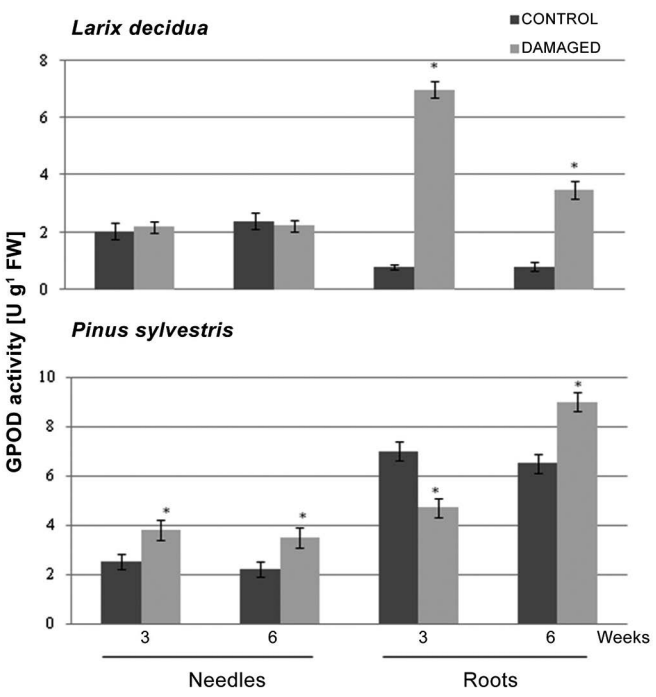

Figure 1. Guaiacol peroxidase (GPOD) activity in *L. decidua* and *P. sylvestris* needles and roots after insect-induced damaged by *M. melolontha* grubs. Samples were collected from a forest plantation twice – 3 and 6 weeks after visible symptoms of insect-induced damage. Values are means with SD, * indicates significant differences between the control at $P < 0.05$

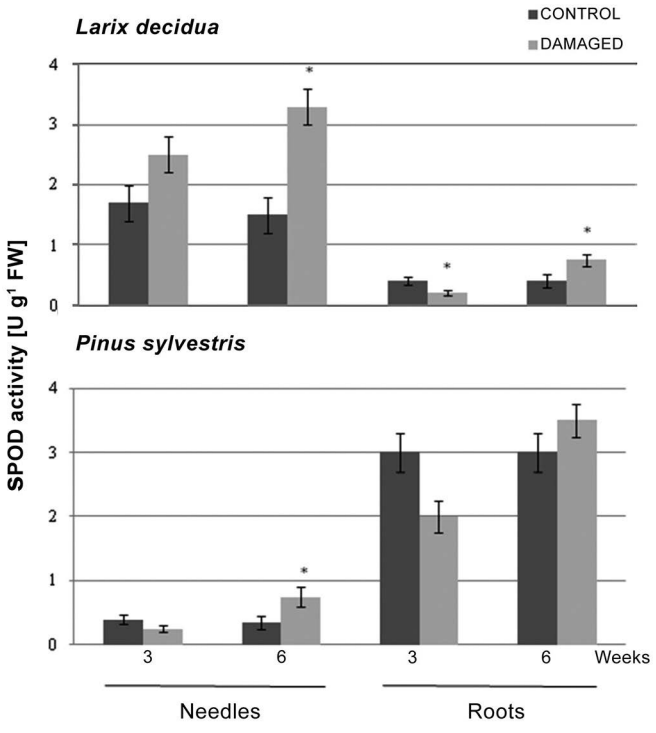

Figure 2. Syringaldazine peroxidase (SPOD) activity in *L. decidua* and *P. sylvestris* needles and roots after insect-induced damaged by *M. melolontha* grubs. Samples were collected from a forest plantation twice – 3 and 6 weeks after visible symptoms of insect-induced damaged. Values are means with SD, * indicates significant differences between the control at $P < 0.05$

were lower compared to the control (Fig. 2). SPOD activities in pine needles and roots 6 weeks after pest attack were slightly higher compared to the control (Fig. 2). We observed differences in SPOD activity between roots and needles after pest attack. SPOD activity in damaged larch needles was higher than in roots but SPOD activity in pine needles was lower than in pine roots.

The SOD activity in damaged larch needles 3 and 6 weeks after pest attack was lower; while in larch roots, 6 weeks after pest attack, it was higher in comparison to the control samples (Fig. 3). SOD activity in pine needles 3 and 6 weeks after pest attack was significantly lower compared to the control (Fig. 3). SOD activity in pine roots 3 weeks after pest attack was similar compared to the control; while in pine roots, 6 weeks after pest attack, it was slightly higher (Fig. 3). We did not observe differences in SOD activity between roots and needles of the same plant after pest attack.

The protein concentrations in needles and roots of both tree species were higher after 3 and 6 weeks of *M. melolontha*-induced damaged compared to the control samples (Fig. 4).

The analysis of POD isoenzymes on native PAGE showed no differences between damaged and control samples for larch needles (Fig. 5). POD patterns showed one extra band (POD 1) in the pine needles damaged by insects and lack of some forms present in the control needles (Fig. 5). The analysis of SOD isoenzymes on native PAGE showed no differences in pine roots between the control and damaged samples (Fig. 5). SOD patterns for larch roots showed one extra band (SOD 1) in the roots damaged by insects (Fig. 5).

4. Discussion

Plants are often exposed to attack by insects that feed on roots and aboveground parts of plants, such as leaves, flowers and stems (Kafle et al. 2014). Roots are an integral part of the plant system and their function is providing water and nutrients to leaves. They also play a role in plant defense and evidence suggests that plant defense induction occurs in spatially separated plant parts (Bezemer et al. 2003). Our work presents the results concerning the attack of *M. melolontha* grubs on both roots and leaves.

Barbehenn et al. (2010) studied the influence of super expression of horseradish POD in transgenic poplar seedlings on biochemistry of *Lymantria dispar* (L.) caterpillar intestine. The scientists demonstrated that the transgenic plants were more resistant to herbivore attack than wild ones. They found out that chlorogenic acid level rose only in the leaves injured by insect feeding. Injured leaves produced increased hydrogen peroxide levels, while horseradish POD was used for free radical production in the middle part of pest intestine. The research by Barbehenn et al. (2010) suggested that phenols were exerted to be oxidized by POD in herbivore insect intestine. It revealed that genetically modified *Liquidambar sp., Nicotiana sp., Solanum sp.* plants were more resistant to Lepidoptera, Coleoptera and Homoptera insects (Dowd and Lagrimini 1998). Roitto et al. (2003) studied defoliation-induced responses concerning peroxidases in Scots pine needles. They demonstrated increased peroxidase activity in pine needles after the attack of

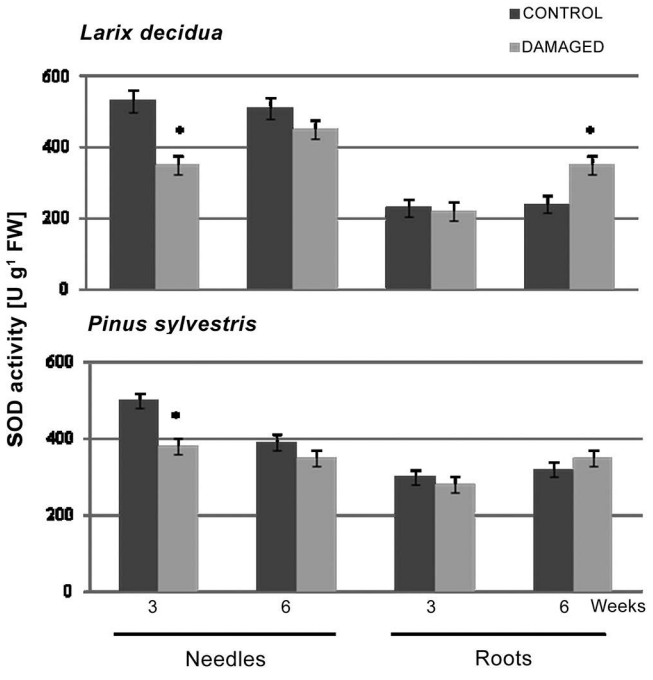

Figure 3. Superoxide dismutase (SOD) activity in *L. decidua* and *P. sylvestris* needles and roots after insect-induced damaged by *M. melolontha* grubs. Samples were collected from a forest plantation twice – 3 and 6 weeks after visible symptoms of insect-induced damaged. Values are means with SD, * indicates significant differences from the control at $P < 0.05$

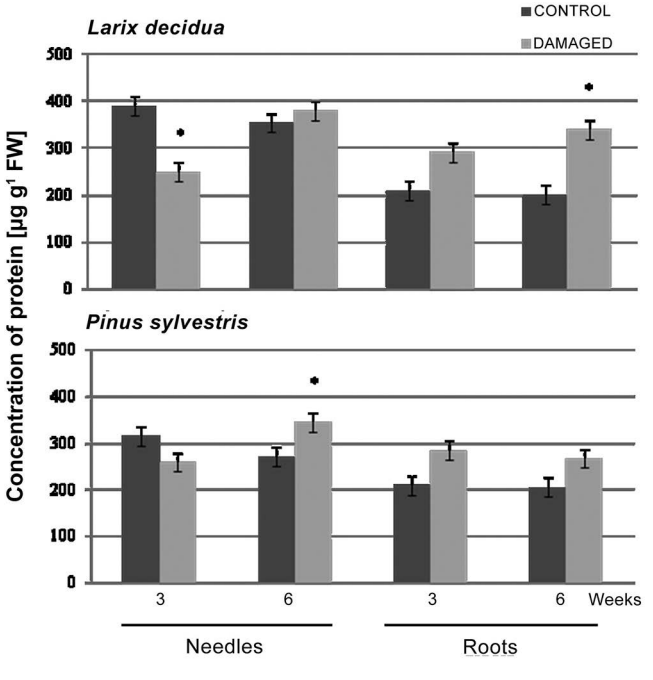

Figure 4. Concentration of protein in *L. decidua* and *P. sylvestris* needles and roots after insect-induced damaged by *M. melolontha grubs*. Samples were collected from a forest plantation twice – 3 and 6 weeks after visible symptoms of insect-induced damaged. Values are means with SD, * indicates significant differences from the control at $P < 0.05$

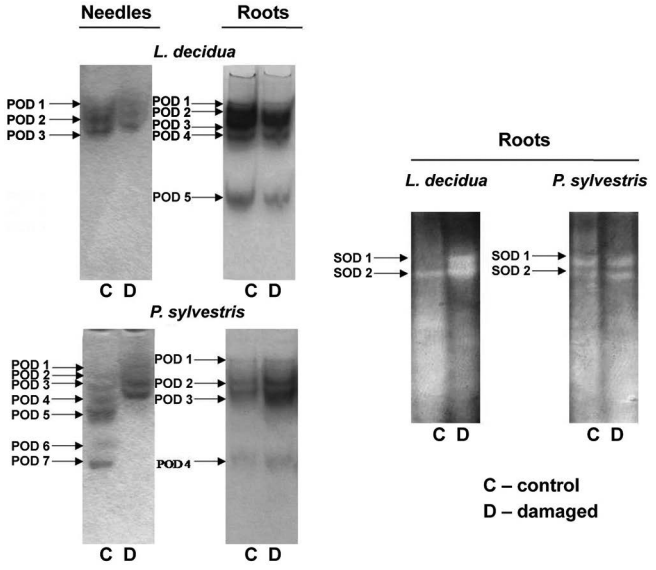

Figure 5. Identification of POD and SOD isoforms from *P. sylvestris* and *L. decidua* needles and roots damaged by *M. melolontha* grubs on native PAGE (POD1 and SOD1 – new bands). Electrophoretic patterns for larch needles and roots, pine needles and roots damaged by *M. melolontha* grubs compared to the control. Single variants were obtained 6 weeks after visible symptoms of insect-induced damaged.

the European pine sawfly *Neodiprion sertifer* (Geoffr.). In our work, POD activity was studied with two substrates: guaiacol and syringaldazine. We observed that in larch roots and in pine needles, the peroxidase activity with guaiacol increased after *M. melolontha*-induced damage. GPOD activity was high in damaged pine roots, but only in the second harvest material. Increased POD activity measured with syringaldazine was observed in the needles and roots of larch and pine in the second harvest of material after pest attack. Our research showed that high peroxidase activity remained elevated 6 weeks after the insect attack. Therefore, the function of this enzyme is not limited only to immediate reactions after insect damage (Roitto et al. 2003).

The research of Dowd et al. (2010) on transgenic plants (that contained tobacco anionic POD) demonstrated that they were more resistant to insect pests. Tissues containing high levels of this enzyme browned fast upon damage. The maize roots studied by Dowd et al. (2010) browned and contained anionic POD. The scientists paid attention to specificity of cDNA from corn that encodes anionic POD. After 48 hours of mechanical damage, the roots of maize seedlings showed browning near the injury regions and increased induction of POD isoenzyme activities. Increased level of expression of POD codine cDNA was the reason of callus browning and it increased plant resistance against insects in comparison with transgenic plants overexpressing glucuronidase.

In the present work, fractions of peroxidase received through gel acrylamide electrophoresis from larch and pine were analysed. Considerable differences between isoenzymes from the control and damaged plants were observed. The research of Nowogórska and Patykowski (2015) on common bean demonstrated the occurrence of an extra band of peroxidase after the *Pseudomonas syringae* pv. *phaseolicola* inoculation, which confirmed the induction of enzymatic response.

Defense against pest involves the activation of specific reactions comprising recognition and proper response to herbivorous insect attack. Within a few minutes, it triggers gene activation (signalling pathways) (Maffei et al. 2007). The present research on plant-insect reactions focuses on the use of genomic and proteomic methods, they help in better understanding of subsequent changes caused by a biotic stress. The events which occur during the first seconds or minutes following the damage, during which recognition of a pest insect and induction of signal transduction take place, are still poorly known.

Katyshev et al. (2006) analysed the nucleotide cDNA sequence of genes coding Mn-SOD and Cu/Zn-SOD in Dahurian larch *Larix gmelinii* (Ruprecht) Kuzeneva. They showed at least two different genes for Cu/Zn-SOD. Mn-SOD is coded by a few genes, for example, in maize, four. They believe that polyadenylations of pre-mRNA and following in vivo generation of transcripts differing with 3'-terminal sequences is the alternative for Mn-SOD genes as it was observed in research on callus.

In our work, we assayed SOD activity in larch and pine seedlings damaged by insects. Distinct changes were observed in the damaged roots of larch and pine, where SOD activity was considerably higher in the second harvest material. It seems possible that growth of SOD activity was related to the extended time of damage caused by grub of *M. melolontha*. The investigation revealed the greatest changes in SOD isozyme forms in the roots of larch damaged by the grubs of *M. melolontha*. We showed one extra form of SOD in the damaged larch roots using the gel poliacrylamide electrophoresis. The research of Garcìa-Limones et al. (2002) on *Cicer arietinum* L. after *Fusarium oxysporum* f. sp. *ciceris* inoculation showed the intensity of SOD electrophoretic bands in samples from infected plants as compared with the non-infected controls. The appearance of extra band of SOD as a result of the attack the pest or pathogen confirms the activity of SOD in defense reaction.

5. Conclusion

In summary, plants exposed to root herbivory showed differences in enzymatic antioxidant activities. Our results demonstrate that equal changes of POD and SOD activities, increased concentration of proteins and especially the extra form of SOD observed in the roots of larch are connected with direct plant reaction to *M. melolontha*.

Conflict of interest

The author declares no conflicts of interest.

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Author's contribution

M.S. – was responsible for methods, measurements preparation of the results and manuscript writing, J.P. – was responsible for the conception of manuscript and research, A.W. – was responsible for methods and measurements.