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Accumulation of iron-binding compounds in root of *Pinus sylvestris* challenged by *Heterobasidion annosum* sensu lato

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Abstract: We examined the interaction between the roots of *Pinus sylvestris* and closely related species *Heterobasidion annousum* s.l. (*H. annosum* s.s., *H. parviporum*, *H. abietinum*) that differ in host plant preference. The aim of the current study was to determine in roots the accumulation pattern of low molecular mass compounds such as catecholate and hydroxamate derivates, oxalic acid as well as iron-reduction ability of that low molecular mass compounds, that play important roles in wood degradation and they are also involved in pathogenesis. The accumulation of catechol and hydroxamate derivates increased during the early (2–6 h) and late (24–48 h) stages of interaction and similar pattern of oxalic acid accumulation were observed. The level of catecholate derivates in *P. sylvestris* roots that were challenged with *H. parviporum* or *H. abietinum* correlated strongly with iron reducing ability. However, when host was exposed to *H. annosum* s. s. hydroxamates rather than catecholates regulated iron reducing ability. The extracellular Fe^{3+} reducing activity was greater for *H. annosum* s. s. isolates than for isolates of two other species, and reduction of ferric iron may promote oxidative burst in host cell and fungal colonization. Catecholate concentration in the presence of *H. annosum* s.s. contributing to host cell death, confirm iron involvement in infection success.

Additional key words: siderophores; organic acids; iron reduction, infection, Scots pine

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Introduction

Iron contributes to the plant-pathogen interactions, and there is well-documented evidence of its involvement in the pathogenesis evoked by bacteria and fungi (see review of Expert et al. 2012 and literature cited therein) but iron also participates in plant defence (Expert et al. 2012). Iron binding compounds have been also reported to actively participate in the decomposition of wood by several fungi (Goodell et al. 1997; Machuca et al. 1999; Milagres et al. 2002; Tanaka et al. 1993; Tanaka et al. 1996).

Heterobasidion annosum sensu lato (Fr.) Bref. is an intriguing model to study the involvement of the iron assimilation system in wood decay and pathogenesis, because unlike other wood decay fungi, *H. annosum* infects conifers at all developmental stages. As a consequence, *H. annosum* causes severe disease in Europe and North America (Korhonen and Stenlid 1998). In addition, *H. annosum* s. l. is a complex

that comprises *H. annosum* sensu stricto (Fr.) Bref., *H. parviporum* Niemelä & Korhonen, and *H. abietinum* Niemelä & Korhonen (Korhonen and Stenlid 1998). These species were previously known as the P, S, and F intersterility (IS) groups, which have host preferences for pine, spruce, and fir, respectively (Werner et al. 2005), although they also have the ability to invade other conifers. Such characteristics provide the opportunity to study the connection between iron assimilation and host preference. As described by Werner et al. (2005), fungal isolates from various *H. annosum* s.l. species show different mechanisms of root penetration, which has previously been related to differences in cortical cell wall structure in main host trees or pathogenic and saprotrophic activity of fungi. Different mechanisms of root penetration by various *H. annosum* s.l. species were also shown by Johansson et al. (2004) and confirmed by Mucha et al. (2012).

Regarding the pathogenic activity of *H. annosum* s. l., most attention has been paid to the enzymes that digest the host cell wall. Johansson et al. (1998) and Daniel et al. (1998) reported that *H. annosum* differs in the laccase production. Strains that belong to *H. annosum* s. s. are characterized by greater laccase than *H. parviporum*. However, it remains unknown whether differences in enzymes synthesis designate pathogens host preference. Laccase was, moreover, found to be able to oxidase iron in other organism (Zhu and Williamson 2004; Dwivedi et al. 2011) or iron uptake (McCaig et al. 2005). *Heterobasidion annosum* s. l. can also produce oxalic acid during infection, but there are many questions about its role in pathogenesis. Oxalate possesses strong metal-chelating activity, particularly for calcium and copper, and reduces the oxidative burst and concomitant defense response in plant tissue (Van Kan 2006). Asiegbu et al. (1998) speculated that, by chelating Ca^{2+}, oxalic acid could weaken the cell wall and acidify the environment, which stimulates the accumulation and activity of a spectrum of secreted enzymes.

Studies of Olson et al. (2012) confirmed the presence of ferric reductase – necessary to acquire iron from infected host – in pathogenic isolates of *H. irregulare* Garbel. & Otrosina in contrast to other non-pathogenic basidiomycetes. The regions essential in virulence of *H. annosum* s.s. coded proteins involved in iron metabolism (Dalman et al. 2013). Our previous studies showed that iron together with H_2O_2 is involved in cell death of *P. sylvestris* challenged by *H. annosum* s.s. (Mucha et al. 2012). The production of siderophore and oxalic acid and its regulation by iron availability has been confirmed in liquid culture of three *H. annosum* s.l. species, which may results different level of wood decay (Mucha et al. 2013). However, accumulation of iron-binding compounds with reducing ability has not been studies during interaction with actively growing roots of host challenged by pathogen. Therefore, the purpose of the current study was to establish the dynamics of accumulation of iron-binding compounds during the early stages of interaction between a host, *Pinus sylvestris*, and isolates belonging to the *H. annosum* s.l.

Materials and methods

Organisms and growth conditions

Nine isolates of differing pathogenicity were used: Borowiec P, 95107 P, and 02/139 P, which represented *H. annosum* s. s.; 96087 S, 02/58 S and 02/48 S, which belonged to *H. parviporum*; and 96066 F, 96067 F and 96071 F, which represented *H. abietinum*. The isolates were cultivated on malt extract agar (MEA) (Difco, Detroit, MI, USA) plates at 24°C in the dark and stored between trials at 4°C, but inoculation of the host was also applied. All isolates were obtained from the collection of the Laboratory of Root System Pathology, Institute of Dendrology, Kórnik, Poland. *Pinus sylvestris* L. seed came from Bolewice, Western Poland (52°28' N and 16°03' E).

Inoculation of *P. sylvestris* roots to *H. annosum* s. l.

Pine seeds were surface sterilized with 0.2% $HgCl_2$ (w/v; Polish Chemical Reagents, Gliwice, Poland) for 2 min, washed three times (5 min each) in sterile distilled water, and germinated on 0.6% water agar (w/v; Difco) at 24°C in the dark. Then, five germinated seeds were placed on the medium previously described by Ingestad (1979). Prior to placing seeds on the solidified Ingestad medium, half of it was removed and the remaining part was covered with sterile filter paper (dry autoclaved; Whatman no. 1, Springfield Mill, UK) to prevent roots from growing into medium. Five germinated seeds were placed on the filter paper and covered with cellophane foil to prevent desiccation. Plants were incubated for 2 weeks under fluorescent lighting (Osram L36/W77 Flora; 100 $\mu Em^{-2}\ s^{-1}$ for 16 h a day) at 60% relative humidity and a temperature regime of 24°C during the day and 20°C at night.

Fungal inoculum was prepared as follows: sterile filter paper (dry autoclaved) was placed on MEA and allowed to overgrow with isolates of *H. annosum* s. l. After 2 weeks, filter paper overgrown with a mycelial mat was placed on the roots of *P. sylvestris* seedlings under cellophane foil growing in the conditions as previously stated. Plant roots and fungal inocula were also separated by a Particle Track-etched Membrane (PTM; 10 μm thick, 0.2 μm size of the mesh; The Institute for Nuclear Chemistry and Technology,

Warsaw, Poland) to ensure migration of metabolites but prevent hyphae from growing into the host roots since the study undertake the role of extracellular metabolite. After 2, 4, 6, 12, 24, 48, and 72 h of incubation, plants were carefully removed from the dishes using tweezers and roots were subjected to further analysis. Control seedlings were treated in the same way as test seedlings and grown in the same conditions except that the filter paper used did not contain any of isolates. Three time-independent replications of each treatment, i.e. each combination of incubation time and fungal strains, were studied. Each replication contained roots from 5 different seedlings.

Collection of target compounds on or in roots

Roots were ground to a powder in liquid nitrogen, and then incubated in 2 ml of water for 1 h with shaking throughout (200 rpm on TTS 2, IKA Works, Wilmington, NC). Subsequently, the samples were centrifuged and the supernatant was filtered using an Amicon Ultra-15 Ultrafree-15 centrifugal filter device (Millipore, Billerica, USA) with a nominal molecular mass limit of 3 kDa. The low-molecular-mass fraction (<3 kDa) was tested for the presence of iron-binding compounds as outlined below.

Detection of catechol derivatives

The method described by Arnow (1937) was employed. Briefly, to 0.25 ml of filtrate (collected from root samples as described above), 0.25 ml of 0.5 M HCl was added, followed by 0.25 ml of ammonium molybdate reagent (10 g of $NaNO_2$ and 10 g of $Na_2MoO_4 \times 2\ H_2O$ were added to 100 ml of deionized water). In the final step, 0.25 ml of 1 M NaOH were added. After addition of each reagent the mixture was shaken and another was applied after 1 min. Reagents were prepared one day before experiments and stored at 4°C. The absorbance was measured after 20 min at 510 nm using UV-2501 PC spectrophotometer (Shimadzu, Kyoto, Japan). 2,3-Dihydroxybenzoic acid (2,3-DHBA) was used as a standard. The concentration of catecholate siderophores was expressed as μg 2,3-DHBA equivalent in 1 ml g^{-1} root dry weight.

Detection of hydroxamate derivatives

To detect hydroxamate derivates the method of Neilands and Nakamura (1991) was employed. Briefly, 1 ml of filtrate was added to 1 ml of assay solution d by combining 1.351 g of $FeCl_3 \times 6\ H_2O$ and 45 ml of 20% $HClO_4$ diluted in deionized water to a volume of 1 liter. Subsequently, absorbance was measured after 20 min at 495 nm using a spectrophotometer. Desferrioxamine B (DFO; Sigma, St Louis, USA) was used as a standard. The amount of hydroxamate derivatives was expressed as μg DFO equivalents in 1 ml $root^{-1}$ dry weight. To confirm the chemical nature of siderophores, tetrazolium test was applied (Snow 1954). After application of 8–10 mg of tetrazolium salt, then 1–2 drops of 2N NaOH and 0.75 ml of filtrate the appearance of deep red color indicated the hydroxamate siderophores.

Detection of oxalic acid

Oxalate content was analyzed with enzymatic assay kit No 591-D (Trinity Biotech, Co. Wicklow, Ireland) in accordance with the manufacturer's instructions. Firstly, pH of filtrate samples were adjusted, if necessary, to reach value between 5 to 7 using 1N sodium hydroxide and then 2 ml of samples were purified in tubes included by manufacturer. Briefly, 50 μl of filtrate was added to 1 ml of Oxalate Reagent A (containing 3-methyl-2-benzothiazolinone hydrazone (MBTH), 3-(dimethylamino)benzoic acid (DMAB) and buffer), and then 0.1 ml of Reagent B (containing oxalate oxidase and peroxidase) and the resulting solution was mixed immediately. After 5 min of incubation, the absorbance was measured at 590 nm using a spectrophotometer.

Detection of citric acid

Citric acid was estimated with an enzymatic assay kit (R-Biopharm AG, Pfungstadt, Germany). To 1 ml of a solution consisting of glycylglycine buffer, pH 7.8; L-malate dehydrogenase, L-lactate dehydrogenase and NADH, 0.2 ml of filtrate and 1.8 ml of redistilled water were added and then mixed. After 5 min absorbance (A1) was measured at 340 nm. Then 20 μl of solution 2 (citrate lyase) was added and after 20 min absorbance (A2) was measured again at 340 nm (UV-2501 PC spectrophotometer; Shimadzu, Kyoto, Japan). The absorbance differences (A1–A2) for both blanks (where sample solution was replaced by water) and samples were evaluated. The subtraction of the absorbance difference of the blank from the absorbance difference of the sample was used to calculated the citric acid contents in sample filtrates.

Extracellular Fe^{3+} reducing activity – (EC-FeRA)

The ability to reduce Fe^{3+} to Fe^{2+} was estimated with the Ferrozine assay (Stookey 1970). To 0.8 ml of acetate buffer (50 mM, pH 4.5) and 0.4 ml of ferrozine solution (1% w/v), 0.75-ml of filtrate was added. The reaction was started by the addition of 0.05 ml of

$FeCl_3$ (20 mM) and monitored for 15 min at 562 nm. The results were expressed in terms of μmol Fe^{2+} min^{-1} g^{-1} (E_{562nm} = 27900 M^{-1} cm^{-1}).

Cell death

Cell death was estimated by the Evans blue method (Baker and Mock 1994). After incubation with the isolates, *P. sylvestris* roots were flooded in 0.25% (w/v) Evans blue solution (Sigma, St Louis, MO, USA). After 20 min, roots were washed twice with distilled water and then homogenized with 1.2 ml of 1% (w/v) sodium dodecyl sulfate (SDS) and 50% (v/v) methanol solution and centrifuged at 14.000*g* for 15 min. The optical density was determined after 10 min at 600 nm. Measurements were expressed as a percentage, with 100% corresponding to an absorbance of completely dead roots (roots boiled for 30 min were treated as dead).

Statistical analysis

Each treatment was repeated three times and each experiment consisted of three replicates. Data are presented as means from the three independent experiments since there were no statistical differences between the replicates. All data were checked for normality using the Shapiro–Wilk test and for homogeneity of variance with the Brown–Forsythe test. Differences between fungal species and time were analyzed using a hierarchical analysis of variance, comparing the means using Tukey's honestly significant difference (HSD) test at $P < 0.05$. Student's t-test was applied to uncover differences between control and studied variants. Pearson's correlation coefficients were applied to verify the relation between low molecular mass compounds and Fe^{3+} reducing activity. To explain the influence of studied factors on cell death, multiple regression was used. All statistical tests were conducted using Statistica version 8.0 (StatSoft, USA).

Results

In the study, we compared low-molecular compounds accumulation during interaction *P. sylvetris* roots and different species of *H. annosum* s.l. As was confirmed by hierarchical analysis of variance, time turned out to be significant ($P < 0.001$) but species ($P = 0.131$) and isolates nested within species ($P = 0.716$) did not differentiate the formation of catecholates. Isolates of *H. parviporum* and *H. abietinum* caused decreases in catecholate accumulation by 27% at 2h and by 69% at 12h in comparison to control (Fig. 1A). For all studied species, catecholate concentrations at 24 and 48 h were higher than during earlier measurement (in 12h), catecholate concentrations at these times were actually lower than those in the controls (by about 38% and 50% at 48h for roots exposed to *H. annosum* s.s. and *H. abietinum*, respectively). At 72h catecholate concentrations decreased for all the studied species and reached levels 83% lower than the controls for *H. annosum* s.s., 75% lower for *H. abietinum* and 62% lower for *H. abietinum*.

The dynamics of hydroxamate siderophore accumulation was similar to those observed for catecholate compounds (Fig. 1B), but neither species nor isolates was a key determinant ($P = 0.563$ and 0.825, respectively). There was a significant difference in hydroxamate derivates appearance across time ($P < 0.001$). The greatest accumulation of hydroxamate in early time points (from 4h to 6h) was detected after exposure to *H. annosum* s.s. its level reached from 78% to 184% above control similarly to *H. parviporum* (discrepancy between 94% and 152%) and *H. abietinum* (discrepancy between 102% to 106% above control) measured at 4h and 6h. Similar pattern of hydroxamate siderophore accumulation was observed at 24h and 48h.

Oxalic acid concentrations in roots were time related ($P < 0.001$) but not species ($P = 0.941$) or isolates ($P = 0.448$). Significant increase of oxalic acid appearance above the level in control was only detected in early stage of interaction (4h) for root exposed to *H. parviporum* (Fig. 1C). The greatest decrease in oxalic acid level was noted for *H. annosum* s.s. (65%, 69% and 85%%), *H. parviporum* (85%, 72% and 92%) and *H. abietinum* (84%, 64% and 82%) at 12h, 48h and 72h, respectively.

As for the other parameters studied, iron reducing capacity fluctuated over time ($P < 0.001$). For all fungal species, the intensity of EC-FeRA increased at approximately 4 or 6 h and again at 48 h (Fig. 1D). The greatest reducing capacity reached 808% of control 6h after root exposure to *H. annosum* s.s but it was also high at 4h, 12h and 48h (413%, 220% and 321%, respectively). The presence of *H. parviporum* caused accumulation of EC-FeRA at 4h, 6h and 24h to be 606%, 481% and 552% greater than the control, respectively. Out of three studied species, *H. abietinum* caused the lowest increased in intensity of EC-FeRA level (about 400% higher than the controls at 4h and 6h and 120% higher at 48h). The intensity of EC-FeRA depended strongly on species ($P = 0.045$), and with the greatest intensity of EC-FeRA observed in *P. sylvestris* roots exposed to *H. annosum* s.s. and the lowest in those exposed to *H. abietinum*. Nevertheless, the intensity of EC-FeRA was not dependent on isolates nested within species ($P = 0.406$).

The EC-FeRA of the low-molecular-weight fraction correlated positively with levels of catecholates

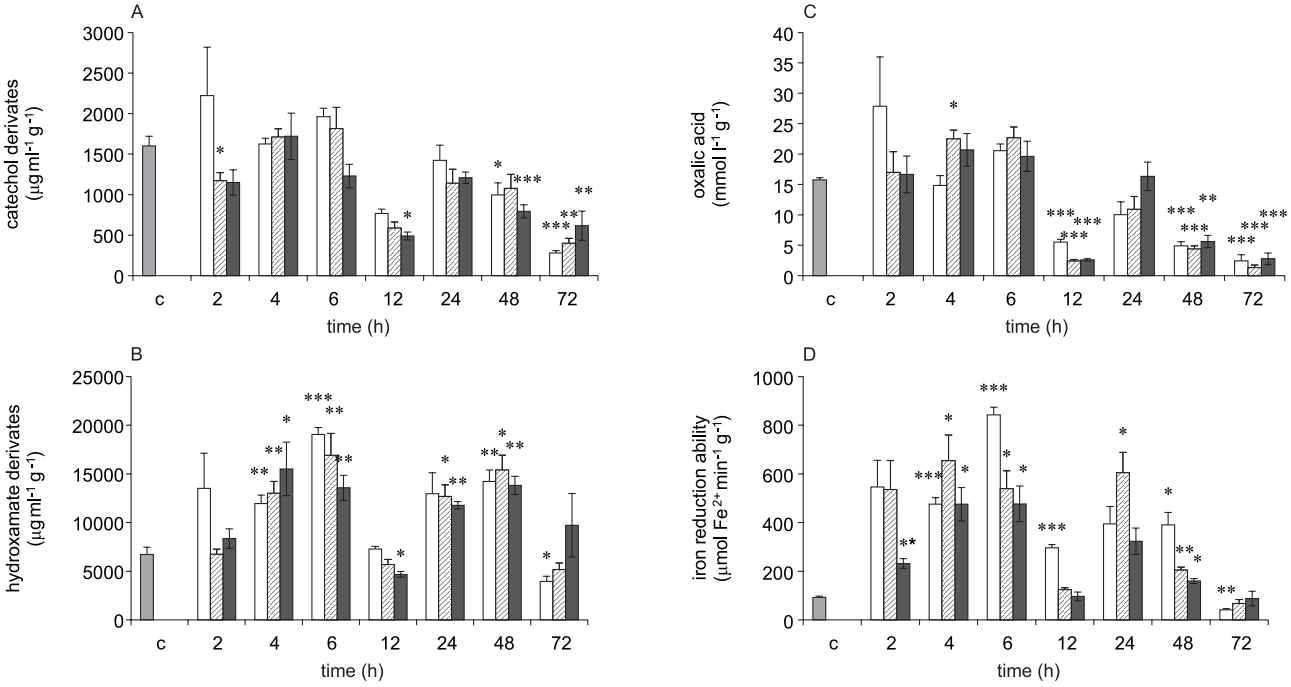

Fig. 1. Accumulation of catecholate compounds (A), hydroxamate derived compounds (B), oxalic acid (C) and changes in iron reduction ability by low molecular mass compounds (D) extracted from in *Pinus sylvestris* roots exposed to isolates representing three related species *H. annosum* s.s. (open bars), *H. parviporum* (hatched bars) and *H. abietinum* (closed bars) in different interval times and in control (grey bars) (average value ± SE). Values matched with asterisks differ statistically from control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (t-Student test)

($r = 0.792$, $P < 0.001$), hydroxamate derivates ($r = 0.676$, $P < 0.001$), and oxalic acid ($r = 0.761$, $P < 0.001$) when analyzing all species together. *H. annosum* s. s. showed a stronger correlation between EC-FeRA and hydroxamates ($r = 0.922$, $P < 0.001$) than between EC-FeRA and catecholate ($r = 0.862$, $P < 0.001$). In contrast, *H. parviporum* and *H. abietinum* showed the opposite tendency and the correlation between EC-FeRA and hydroxamates was lower ($r = 0.543$ and $P < 0.05$ for *H. parviporum*; $r = 0.596$ and $P < 0.01$ for *H. abietinum*) than that with catecholates ($r = 0.771$ and $P < 0.001$ for *H. parviporum*; $r = 0.740$ and $P < 0.001$ for *H. abietinum*).

To estimate the influence of the studied compounds dynamics on the percentage of cell death, multiple regression was used. When all of the studied isolates were analyzed together, two predictors (oxalic acid and hydroxamates) explained 31% of cell death variance ($R^2 = 0.31$, $P < 0.001$ for overall model) with following regression equation: y = 22.7565 – 0.4808*oxalic acid + 0.0005*hydroxamate. However, analysis of each species separately allowed to explained more percentage of cell death variance for *H. annosum* s. s. and *H. parviporum*. The best predictors for *P. sylvestris* root cell death exposed to *H. annosum* s. s. were oxalic acid and catecholates, which explained 42% of variance ($R^2 = 0.42$, $P = 0.036$, y = 20.1223 – 1.2668*oxalic acid + 0.0143*catecholates). In the presence of *H. parviporum* isolates, *P. sylvestris* cell death was negatively related to oxalic acid but positively to pH ($R^2 = 0.53$, $P = 0.011$, y = –34.9683 – 0.3638*oxalic acid + 11.7748*pH). Only one predictor explained 26% of host cell death ($R^2 = 0.27$, $P = 0.049$, y = 25.1816 – 0.2922*oxalic acid) when *P. sylvestris* was exposed to *H. abietinum*.

Discussion

In the study described herein, we analyzed the dynamics of iron binding low-molecular-mass compounds within *P. sylvestris* roots exposed to different species of *H. annosum* s. l. Our results highlight that the dynamics of catecholate, hydroxamate, and oxalic acid level in *P. sylvestris* have the same pattern during exposure to different species of *H. annosum* s. l., but *H. annosum* s. s. isolates elicited/induced greater extracellular Fe^{3+} reducing activity than in the other species.

Given that catechol and hydroxamate derivates can play different roles (promote or prevent) in the generation of reactive oxygen species (ROS) (Arantes et al. 2006; Boughammoura et al. 2007; Dellagi et al. 1998), the pattern of their accumulation is important to elucidate pathogenic activity. Two peaks of increased accumulation of catecholate and hydroxamate derivates (at 4 and 6 h and then at 24 and 48 h) observed in present studies could potentially influence the concentration of H_2O_2 in the host, especially because we detected similar fluctuation of

H_2O_2 accumulation in *P. sylvestris* roots exposed to different species of *H. annosum* s. l. (Mucha et al. 2012). Hydroxamate derivates have been reported to reduce the accumulation of H_2O_2 in *Arabidopsis thaliana* cells infected by *Erwinia chrysanthemi* (Boughammoura et al. 2007). However, hydroxamates do not likely account for the variable H_2O_2 levels in *P. sylvestris*, because the concentration of hydroxamate derivates in *P. sylvestris* roots was similar for all the fungal species, whereas earlier studies showed a greater number of cells contained H_2O_2 after infection with *H. annosum* s.s. as compared with the other two species (Mucha et al. 2012).

The greater bioavailability of compounds able to reduce iron is a key factor for pathogenic potential via ROS level modulation. The reduced form Fe^{2+} may react with oxidants such as H_2O_2 to form free radical species that are oxidizers of cellulosic and phenolic compounds (Goodell et al. 1997). Catecholates and hydroxamate differ in their ability to reduce iron; a previous study showed that catecholate chelators show a much stronger iron-reducing ability than hydroxamate chelators (Arantes and Milagres 2007). The accumulation of catecholate derivatives in *P. sylvestris* roots was strongly correlated with EC-FeRA, regardless of the *H. annosum* s.l. species. As was shown by Willimas et al. (2011), host defence reaction is suppressed by reducing environment in host cell e.g. oxidative burst and callose deposition. Callose was deposited in *P. sylvestris* roots challenged by *H. annosum* s.s. and *H. parviporum*, but not *H. abietinum* (Zadworny et al. 2012) at 6h after inoculation. Additionally, our earlier studies indicated higher accumulation of reactive oxygen species before 6h post inoculation necessary for evoking cell death making earlier time point studies essential in the outcome interactions (Mucha et al. 2012). We also observed a greater accumulation of iron in *P. sylvestris* roots in response to exposure to *H. annosum* s.s. than in response to the other two species (Mucha et al. 2012). *Heterobasidion parviporum* and *H. abietinum* are genetically related to each other more closely than to *H. annosum* s. s. (Karjalainen 1996; Kasuga et al. 1993), which was confirmed by evolutionary studies by Dalman et al. (2010). This can explain the similar response of the host to *H. parviporum* and *H. abietinum*.

Wood rot fungi can use the Fenton reaction to decompose components of the cell wall. However, this reaction leads to the accumulation of toxic levels of hydroxyl radicals, which raises the question of how such fungi modulate the level of generated ROS. The level of siderophores and their ability to modulate Fe^{2+} availability might strongly influence the concentration of hydroxyl radicals in the Fenton reaction, especially given that the roots of *P. sylvestris* contain more iron than, for example, those of *Picea abies* (Palviainen et al. 2004). Catecholate turned out to be one of the factors positively correlated with root cell death exposed to *H. annosum* s. s., which also supports our finding in previous study (Mucha et al. 2012) about involvement of iron in *P. sylvestris* cell death in the presence of *H. annosum* s. s. isolates. Studies of Olson et al. (2012) confirmed the presence of ferric reductase – necessary to acquire iron from infected host – in pathogenic isolates of *H. irregulare* Garbel. & Otrosina in contrast to nonpathogenic basidiomycetes. Besides, *H. annosum* s.s. genom studies showed that genes coded proteins responsible for virulence factor are also connected with iron metabolism (Dalman et al. 2013). Oxalic acid has also been reported to play a role in the pathogenicity of *Sclerotinia sclerotiorum* (Lib.) de Bary by manipulating host ROS (Kim et al. 2008). In the present study, the dynamics of oxalic acid was the same as for the other compounds studied, and similar to that for the concentration of H_2O_2 in *P. sylvestris* exposed to *H. annosum* species. Oxalic acid may act as a transfer agent and provide iron for reduction by other chelators, as has been confirmed by the increase in hydroxyl radicals in the presence of oxalate and catecholate derivate chelators (Goodell et al. 1997). However, after the increase in oxalic acid, we observed a decrease in its synthesis, as for catecholate and hydroxamate derivates. Acidic pH results in the reduced accumulation of siderophores (Bertrand et al. 2009). Moreover, Williams et al. (2011) have shown that reducing conditions in the cell generated by oxalate correlate with inhibition of the host oxidative burst and other defense responses. Conversely, our studies determined that oxalic acid correlated negatively with *P. sylvestris* root cell death regardless of the species challenged the plant host. In the line of agreement with our results, oxalic acid increased resistance of *P. abies* against necrotrophic fungus *Ceratocystis polonica* (Siemaszko) C. Moreau (Krokene et al. 2008) or it could be connected with activation of oxalate oxidase, the enzyme release H_2O_2 and CO_2 from oxalic acid (Zhang et al. 1995). Pectolytic enzymes proved to be involved in necrotrophic process of *H. annosum* s. l., but their activity was not correlated with oxalic acid synthesis (Johansson et al. 1998). Besides, oxalic acid is not accumulated in active growth phase of *H. annosum* s. l. (Dutton et al. 1993).

In conclusion, *H. annosum* s.s. caused the highest extracellular Fe^{3+} reducing activity in root and catecholates were positive regulators of host cell death evoked by *H. annosum* s. s. This is an assumption to confirm the substantial role of iron-binding compounds in the infection success. The following step will be identification of extracted siderophores and examining their dynamics in roots of *P. sylvestris* challenged by different species of *H. annosum* s.l.

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