

The origin of reactive oxygen during interaction of *Pinus sylvestris* root and *Heterobasidion annosum* s.l. – the linkage with the iron

Joanna Mucha · Anna Budka · Dariusz Kayzer ·
Marzenna Guzicka · Piotr Łakomy ·
Marcin Zadworny

Accepted: 11 May 2015 / Published online: 21 May 2015
© The Author(s) 2015. This article is published with open access at Springerlink.com

Abstract Reactive oxygen species (ROS) can be promoted differently, although iron is regarded as a *condicio sine qua* of their generation. We assessed how enzymes involved in ROS production and iron accumulation impact the interactions between *Pinus sylvestris* root and pathogens with different host preferences (*Heterobasidion annosum sensu stricto*, *H. parviporum* and *H. abietinum*). Studies with inhibitors of ROS generation showed that diphenylene iodonium (DPI) and potassium cyanide (KCN) decreased the mortality of *P. sylvestris* root cells when exposed to *H. parviporum* in the elongation zone. In the meristematic zone, inhibited production of NADPH oxidase and oxalic acid decreased mortality of host root cells challenged with *H. parviporum* or *H. abietinum*. In the meristematic zone, iron immobilization with desferoxamine (DFO), inhibited percentage of dead host cells exposed to

H. annosum s.s. Scots pine inoculation with high compatible *H. annosum* s.s., irrespectively in the root zone, led to an accumulation of superoxides (O_2^-) and a colocalisation of H_2O_2 with ferric or ferrous ions involved in production of the most harmful ROS – hydroxyl radicals at 4 h. After 48 h of mutual interaction between *P. sylvestris* and *H. annosum* s.s., a similar pattern as at 4 h was found in the root meristematic zone. We also showed that O_2^- accumulation in the *P. sylvestris* – *H. annosum* s.s. pathosystem was not NADPH oxidase dependent. During the interaction of *P. sylvestris* and *H. annosum* s.s., colocalisation of H_2O_2 with Fe^{2+} and Fe^{3+} decreased by disturbing ROS production; this pattern was less apparent when *H. parviporum* or *H. abietinum* were identified as the invader. Our work implies that different mechanisms related to plant cell death can operate in both the root elongation and meristematic zone of *P. sylvestris* colonized with *H. annosum* s.s. pathosystem.

Electronic supplementary material The online version of this article (doi:10.1007/s10658-015-0679-7) contains supplementary material, which is available to authorized users.

J. Mucha (✉) · M. Guzicka · M. Zadworny
Institute of Dendrology, Polish Academy of Sciences,
Parkowa 5, 62-035 Kórnik, Poland
e-mail: jmucha@man.poznan.pl

A. Budka · D. Kayzer
Department of Mathematical and Statistical Methods, Poznan
University of Life Sciences, Wojska Polskiego 28,
60-637 Poznan, Poland

P. Łakomy
Department of Forest Pathology, Faculty of Forestry, Poznan
University of Life Sciences, Wojska Polskiego 28,
60-637 Poznan, Poland

Keywords Iron · NADPH oxidases · Pathogen · Peroxidases · Scots pine

Introduction

Reactive oxygen species (ROS), mostly O_2^- and H_2O_2 , are produced during plant development, but abiotic and biotic stresses can also result in the rapid formation of reactive oxygen (Lamb and Dixon 1997; Dat et al. 2000). ROS induced by pathogens can act in different manners: (i) as a signal for polymerizing and cross-

linking reactions of various cell wall components, at sites surrounding attempted invasion to retard further penetration (Asiegbu et al. 1994); or (ii) as a direct defence reaction against invading microorganism in the apoplast, since O_2^- and H_2O_2 or their products (e.g., OH $^-$) show harmful activity towards microorganisms (Torres 2010). Additionally, the function of ROS is strongly related to the infection mode of a pathogen. While necrotrophic fungi kill host cells to access targeted nutrients, biotrophic fungi profit from living in host cells. Thus, plants, which induce ROS bursts should limit the spread of biotrophic, but not necrotrophic fungi (Shetty et al. 2008). However, this assumption is an over-generalization, because even genotypes of *Botrytis cinerea* Pers., known as a necrotrophic pathogen, can generate reactive oxygen bursts (Govrin and Levine 2000) might also be restricted by ROS (Unger et al. 2005).

The use of various inhibitors allows determining the source of ROS formation (Bolwell et al. 1998; Torres et al. 2002; Taheri et al. 2014). The inactivation of cell wall peroxidases through azide and cyanide and the inactivation of plasma membrane NADPH oxidases through diphenylene iodonium (DPI) revealed that different factors might be involved in various pathosystems, increasing complexity in a system (Lamb and Dixon 1997; de Gara et al. 2003; Wang et al. 2010). There is also evidence of a lack of correlation between NADPH oxidase and the production of ROS (Glazener et al. 1996; Torres et al. 2006), suggesting that we still do not fully understand the process or processes that establish the character of plant-pathogen interactions and the role of ROS in hypersensitive response (HR) (Shetty et al. 2007). Hüchelhoven and Kogel (2003) showed that H_2O_2 production by *Hordeum vulgare* in response to *Blumeria graminis* (DC.) Speer is not sensitive to DPI - the NADPH oxidase inhibitor. In a study by Liu et al. (2007), the oxidative burst in *Triticum monococcum* exposed to *B. graminis* f. sp. *tritici* was mediated by iron. In such a pathosystem, increased peroxidase production was localized in the leaf epidermis (Liu et al. 2005), probably responsible for H_2O_2 formation. Blocking the H_2O_2 production by desferrioxamine (DFO - impede Fe loading into peroxidase) in a *T. monococcum* – *B. graminis* f. sp. *tritici*. pathosystem, indicating that iron is essential for the oxidative burst (Liu et al. 2007). One explanation is that the availability and release of “catalytic” Fe^{2+} from storage or transport proteins or iron-containing enzymes

may be important in regulating H_2O_2 toxicity to plant cells. Another factor contributing to the formation of oxidative bursts is a decrease in intracellular pH releasing Fe^{2+} during HR (Baker and Orlandi 1995) and therefore may enhance the transformation of H_2O_2 into active hydroxyl radicals (Bestwick et al. 1997). The involvement of iron in this process has rarely been analyzed during interaction of closely related species with the same host in different root zones. Thus, we made the effort to trace operated mechanisms in such a pathosystem.

The formation of ROS could have different origins. One origin could be a particular plant-pathogen interaction (Torres 2010) or second, the activation of enzymes (peroxidase and NADPH oxidase among others), which are responsible for the formation of ROS in a response to different pathogen species (Torres et al. 2002). *Heterobasidion annosum* s.l. can be divided into *H. annosum* s.s. (Fr.) Bref., *H. parviporum* Niemelä & Korhonen, and *H. abietinum* Niemelä & Korhonen. These species were also known as the P, S, and F intersterility groups, showing strong preferences for a main host tree, e.g., pine, spruce, or fir, respectively (Niemelä and Korhonen 1998). All the species can cause severe damage in coniferous forest, endangering the timber production in Europe (Asiegbu et al. 2005). Johansson et al. (2004) studied *H. parviporum* infection and showed that peroxidase activity was higher in *Picea abies* than in *Pinus sylvestris*. Similarly, an increase in peroxidase transcription was observed in roots of *P. sylvestris*, infected by *H. annosum* s.s. (Li and Asiegbu 2004). We also showed patterns of H_2O_2 and O_2^- accumulation in *P. sylvestris* exposed to *H. annosum* s.s., the accumulation differing when induced by *H. parviporum* or *H. abietinum* (Mucha et al. 2012). In addition, we also found two peaks in H_2O_2 accumulation. Kärkönen et al. (2009) studied the mechanism of H_2O_2 production in spruce cell culture, elicited by *H. parviporum*, and found that azide, an inhibitor of haem-containing enzymes, strongly delayed the elicitor-induced H_2O_2 production. Diphenylene iodonium, an inhibitor of flavin-containing enzymes, efficiently inhibited both constitutive and elicited H_2O_2 production (Kärkönen et al. 2009). The authors showed that peroxidase gene and a respiratory burst oxidase homolog (rboh) may contribute to H_2O_2 generation.

The anatomy and physiology of cells in different root zones, such as elongating and meristematic regions, is unique and each root zone may indicate different

mechanisms of host responses to pathogen. Asiegbu et al. (1999) and Jaber et al. (2013) showed strong reaction of root tips in response to *H. annosum* presence. Similar responses were reported by Czymmek et al. (2007), here the colonization of *Arabidopsis* root by the necrotrophic pathogen *Fusarium oxysporum* E.F. Sm. & Swingle was found in the meristematic zone of tap root tips and emerging lateral roots. In general, roots can be in various stages of development e.g., meristematic and elongation (Evert 2006). In a comparison between the meristematic and elongation zones of tap roots, Mucha et al. (2014) showed that accumulation of ROS significantly differed in both zones when challenged by trophically diverse fungi, including a pathogen *F. oxysporum* and saprotrophic and ectomycorrhizal fungi. The authors also concluded that the mechanisms that promote establishment of pathogenic fungi operate in a different way in these different root zones.

In conclusion, plasma membrane localized NADPH/NADP oxidase and/or a cell wall peroxidase-dependent oxidative burst were found in the presence of pathogens (Apel and Hirt 2004). However, it is important to determine whether such enzymes are responsible for oxidative burst in response to different species of *H. annosum* sensu lato, in roots of *P. sylvestris*, for example. While more than one mechanism can result in the formation of H_2O_2 , these mechanisms may vary between different fungal species and physiological contexts (Bolwell and Wojtaszek 1997). We assessed whether mechanisms that determine ROS formation in pathosystem of *H. annosum* s.s. with high preferences to *P. sylvestris* differ from that operating in *H. parviporum* and *H. abietinum*, in different root zones.

Material and methods

Organisms and growth conditions

Pinus sylvestris L. seedlings came from the provenance of Bolewice (52° 28' N and 16 ° 03' E). We studied isolates of three fungal pathogens: Borówiec P, 95107 P, 02/139 P representing *H. annosum* s.s.; 96087 S, 02/58 S and 02/48 S belonging to *H. parviporum*; 96066 F, 96067 F and 96071 F of *H. abietinum*. Isolates of the pathogen used in the studies were maintained in cultures on malt extract agar (Difco) plates grown at 24 and stored at 4 °C.

After sterilization (3 min in 0.2 % $HgCl_2$ followed by washing three times for 5 min in sterile distilled water), seeds were placed on 0.6 % water agar (*w/v*; Difco, Detroit, MI, USA) in the dark. In 14 cm (diameter) Petri dishes, 100 ml of Ingestad medium (1979) was poured and after solidification, half of the nutrient medium was removed to allow space for the developing upper part of seedlings. The medium was then covered with a filter paper. Germinated seeds were aseptically transferred to the Ingestad medium, covered with foil to prevent desiccation and incubated for 1 week in a growing room under lighting emitted by fluorescent tubes (Osram L36/W77 Flora; $100 \mu Em^{-2} s^{-1}$). Lights were on 16 h per day, relative humidity was maintained at 60 % RH, and we used a 24:20 °C day to night temperature ratio. One week after transplanting the seedlings, filter paper overgrown with *H. annosum* was put on the seedling roots. Control plants were seedlings of the same age treated in the same way but without inoculums of the pathogen. In our earlier studies we observed accumulation of two ROS (O_2^- and H_2O_2) and iron ions in these pathosystems (Mucha et al. 2012) and for further experiments the six seedling roots were sampled after 4 and 48 h.

Chemical treatment

To unravel the source of ROS, roots were treated with one of the following inhibitor compounds in 10 mM MES buffer pH 7.2: 3 mM potassium cyanide (KCN—a cell wall peroxidase inhibitor), 1 mM sodium azide (NaN_3 —a cell wall peroxidase inhibitor), 10 μM diphenylene iodonium (DPI—a NADPH oxidase inhibitor), 20 μM 3-amino-1,2,4-triazole (ATZ – a catalase inhibitor) or 3 mM itaconic acid (IA—a oxalic acid inhibitor). These compounds inhibit enzymes involved in ROS generation in plants (Bestwick et al. 1997; Tiwari et al. 2002). Membrane bound NADPH oxidase generates superoxide, which can convert to hydrogen peroxide (Doke 1983). Cell wall peroxidases respond to the presence of superoxide and generate hydrogen peroxide (Bolwell et al. 1998). Catalases decompose hydrogen peroxide (Torres et al. 2006). Oxalic acid suppresses ROS production (Kim et al. 2008) and can scavenge iron (Shimada et al. 1997). The minimum concentrations required to inhibit ROS production was estimated by a preliminary study in which we gradually increased the concentration of inhibitors from 0.03 to 30 mM. Since both KCN and NaN_3 may inhibit also

other enzymes apart from peroxidase, we used both of the widely applied inhibitors. The KCN, NaN_3 and ATZ were taken from stock solution prepared in deionized water whereas DPI was dissolved in pure DMSO and IA in pure methyl alcohol. Inhibitors were applied for 1 h and then briefly washed with deionized water before inoculation with pathogens. Inoculated roots were then processed for ROS and iron detections as described below. Control seedlings were incubated in 10 mM MES buffer (pH 7.2) supplemented with the same inhibitors used in stock solution in respective concentration.

To reveal the importance of iron on the generation of ROS, roots were treated with 1 mM desferoxamine (DFO—a compound binding iron) for 6 h. Afterwards, roots were washed, inoculated with pathogen inoculum and subsequently processed as described below.

Detection of ROS and iron

O_2^- was visualized by immersing *P. sylvestris* roots for 1 h in a solution of 0.1 mg mL^{-1} nitroblue tetrazolium (NTB; Sigma, St Louis, MO, USA) in pH 7.0, 20 mM phosphate buffer at 22°C in the dark (Dunand et al. 2007). To localise iron ions, roots were fixed in metacam (60:30:10 methanol:chloroform:acetic acid) at 4°C for 24 h. Afterwards, the material was rehydrated in ethanol series and incubated for 24 h in 7 % (w/v) potassium ferrocyanide (for Fe^{3+} detection) or for 48 h in 7 % (w/v) potassium ferricyanide (for Fe^{2+} detection) in aqueous 3 % (v/v) hydrochloric acid at room temperature (Smith et al. 1997; Liu et al. 2007). For double staining of iron and H_2O_2 , iron staining was carried out followed by the DAB reaction. H_2O_2 was estimated by submerging root fragment in 1 mg mL^{-1} 3,3'-diaminobenzidine (Sigma), pH 3.8, for 75 min (Thordal-Christensen et al. 1997).

Longitudinal sections were made by hand from the apical zone and the next 0.5 cm from the root apex. The longitudinal sections were mounted in 1:1 glycerin/water solution before examination. Changes in iron and ROS were estimated using an Axioskop 20 microscope (Carl Zeiss) with the $\times 40$ lens and $\times 100/1.4$ oil-immersion lens.

Cell death

Cell death, as indicated by disruption of the plasma membrane integrity, was verified according to the Evans

blue method (Baker and Mock 1994). Roots of *P. sylvestris* firstly were treated with chemical compounds as described above, secondly were challenged with isolates of *H. annosum* s.l. for 4 and 48 h, and then were placed in 0.25 % (w/v) Evans blue solution (Sigma) for 20 min. Then, roots were washed twice in distilled water prior to examination under an Axioskop 20 microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

The percentage of cells demonstrating specific colour reactions were calculated according to the following formula: the number of stained cortical cells taken from one optical layer / the number of all cells from the same optical layer $\times 100$. The data for individual staining were obtained from seven root pieces from different seedlings, each containing 30 cells taken from one optical layer. The percentages of cells with O_2^- , iron ions and double reaction of H_2O_2 iron ions and dead cells were transformed before analysis according to the C. I. Bliss equation to normalize the data (Snedecor and Cochran 1967): $x = \arcsin \sqrt{(p/100)} \times 180/\pi$, where p is the percent value. Analysis of variance (ANOVA) followed by Tukey's HSD test was applied to unravel the influence of inhibitors on cell death for the isolates and the impact of studied inhibitors on the percentages of cells with O_2^- , iron ions and double reaction of H_2O_2 with iron ions for the studied species. Student test was used to compare the percentage of dead cells in both studied zones. Statistical analysis were carried out using the statistical analysis software, Statistica (version 8.0, StatSoft Tulsa, USA). The data was also analysed using canonical variate analysis (CVA) (Lejeune and Caliński 2000; Kayzer et al. 2009; Budka et al. 2011), a form of multivariate analysis, to estimate the similarity between the nine studied isolates belonging to three different species using Bliss transformed data of the percentages of cells with O_2^- , iron ions and double reaction of H_2O_2 with iron ions through all used inhibitors. The method of CVA, similar to principal component analysis, was chosen because considering data sets coming from the multidimensional experimental structure (besides replicates for isolates the other source of variation are taken into consideration, such as time after inoculation, inhibitors and interactions). CVA analyses were undertaken using the original procedure proposed by the author of this study in Excel.

Results

O₂⁻ staining

Four hours after exposure to pathogen inocula, an increase in O₂⁻ above the level observed in control roots was noted for *H. annosum* s.s. after application of ATZ and for *H. parviporum* and *H. abietinum* after treatment of *P. sylvestris* with DFO in elongation zone (Table 1, S1, Fig. 1a and b). Moreover, the analysis of O₂⁻ occurrence revealed its accumulation in the presence of *H. annosum* s.s. 48 h after treatment with DPI (Fig. 1c). Dissimilar to the results observed for the root elongation zone, only the presence of *H. annosum* s.s. accelerated accumulation of O₂⁻ in the meristematic zone; more O₂⁻ was observed within 4 h after treatment with KCN, NaN₃ or IA and within 48 h after treatment with ATZ (Table 2, S1).

Fe³⁺ staining and co-localisation with H₂O₂

In the elongation zone, all of the applied compounds limited Fe³⁺ accumulation in host cells after 4 h of

contact with *H. annosum* s.s., but only KCN, DPI and DFO limited Fe³⁺ accumulation when roots were in contact with *H. parviporum* in contrast to control (Table 1, S2). At 48 h after inoculation, higher accumulation of Fe³⁺ after application of DPI or IA in the presence of *H. annosum* s.s. was observed but a decrease in accumulation after DPI or DFO treatment in contact with *H. parviporum* was observed. In the meristematic zone, the lowest Fe³⁺ accumulation was found after application KCN, NaN₃, DPI or itaconic acid for *H. annosum* s.s. at 48 h and KCN, NaN₃, DPI or DFO for *H. parviporum* at 4 h but none at 48 h. However, all applied compounds limited Fe³⁺ accumulation in the meristematic zone at 4 and 48 h (except for NaN₃) in contact with *H. abietinum*.

All of the inhibitor compounds limited double staining of H₂O₂ with Fe³⁺ in *P. sylvestris* cells in contact with *H. annosum* s.s. in the elongation zone at 4 h (Table 2, S3). Treatment of host roots with KCN, NaN₃, ATZ, IA or DFO before exposure to *H. abietinum* caused the highest decrease in H₂O₂ with Fe³⁺ at 48 h. In the meristematic zone of *P. sylvestris*

Table 1 The scheme of changes in reactive oxygen species and iron ions in the elongation zone of *P. sylvestris* roots previously treated with inhibitors and then exposed to *H. annosum* s.s., *H. parviporum* or *H. abietinum* isolates for 4 or 48 h

Species		4 h						48 h					
		KCN	NaN ₃	DPI	ATZ	IA	DFO	KCN	NaN ₃	DPI	ATZ	IA	DFO
<i>H. annosum</i> s.s.	O ₂ ⁻				↑					↑			
	Fe ³⁺	↓	↓	↓	↓	↓	↓		↑	↑	↑		
	Fe ³⁺ & H ₂ O ₂	↓	↓	↓	↓	↓	↓						
	Fe ²⁺	↓		↓	↓		↓						
	Fe ²⁺ & H ₂ O ₂						↓	↓	↓	↓	↓	↓	↓
<i>H. parviporum</i>	O ₂ ⁻						↑						
	Fe ³⁺	↓		↓			↓		↓			↓	
	Fe ³⁺ & H ₂ O ₂												↓
	Fe ²⁺	↑									↓		
	Fe ²⁺ & H ₂ O ₂							↓	↓	↓	↓	↓	↓
<i>H. abietinum</i>	O ₂ ⁻						↑						
	Fe ³⁺												
	Fe ³⁺ & H ₂ O ₂							↓	↓		↓	↓	↓
	Fe ²⁺	↓	↓		↓		↓	↑	↑				
	Fe ²⁺ & H ₂ O ₂	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

The inhibitors studied include: potassium cyanide (KCN), sodium azide (NaN₃), diphenylene iodonium (DPI⁻), 3-amino-1,2,4-triazole (ATZ), IA (itaconic acid), and desferoxamine (DFO). Detailed information with statistical analysis is provided in Supplementary Data Tables S1–S5

roots, the double localization of H_2O_2 with Fe^{3+} was restricted after application of all studied compounds in the presence of *H. annosum* s.s. and *H. abietinum* at 4 and 48 h (except for sodium azide, ATZ or DFO at 4 h for the latter) (Fig. 1 d, e, and f).

Fe^{2+} staining and colocalisation with H_2O_2 staining

In the root elongation zone, the inhibitors influenced different pathways of iron-dependent ROS production over time (Table 1, S4). In the meristematic zone, all of

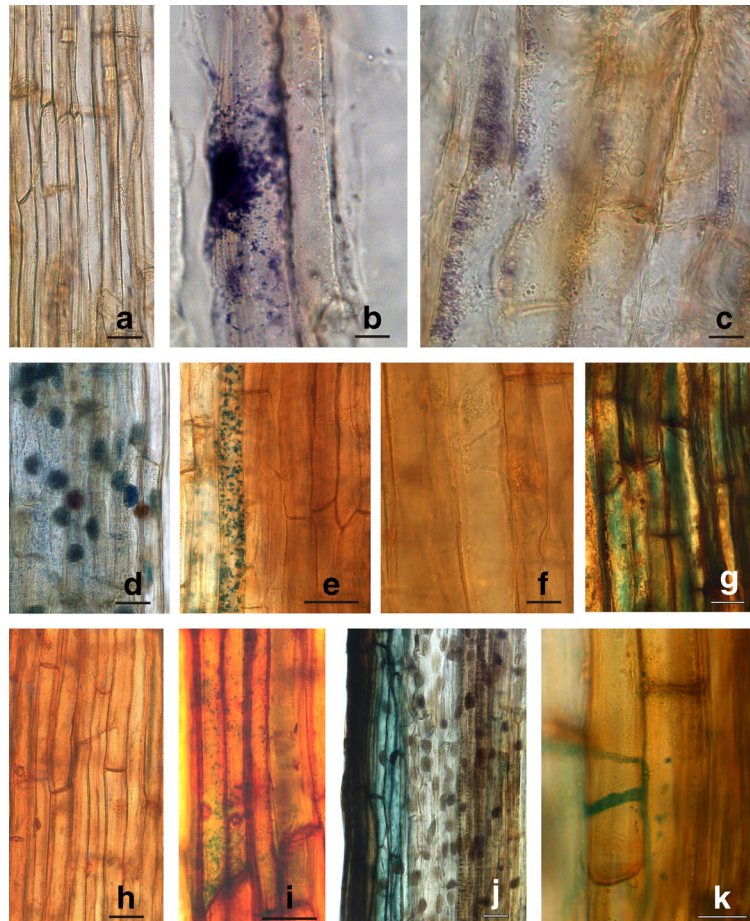


Fig. 1 Detection of O_2^- (violet-blue colour), H_2O_2 (redish-brown colour) and ferric or ferrous iron (blue colour) in main tap root of *P. sylvestris* tree seedling, treated with ROS inhibitors and then infected by different species of *H. annosum* s.l. **a** Lack of O_2^- localization in *P. sylvestris* cells 48 h after exposure to isolates 02/139P of *H. annosum* s.s. in the elongation zone (bar=20 μ m) **b** O_2^- presence 4 h after contact with 95107P of *H. annosum* s.s. *P. sylvestris* when first treated with ATZ in the elongation zone (bar=10 μ m). **c** O_2^- presence 48 h after DPI application to tap root of *P. sylvestris* and exposure to 95107P of *H. annosum* s.s. in the elongation zone (bar=10 μ m). **d** ferric iron staining in root after application of KCN and then exposed 4 h to 95107P isolates of *H. annosum* s.s., note the lack of H_2O_2 presence in the meristematic zone (bar=20 μ m). **e** Co-presence of ferric iron and H_2O_2 when root were treated with DPI and contact with isolates 96087S of *H. parviporum* for 4 h in the meristematic zone (bar=20 μ m). **f** Lack of co-presence of ferric iron and H_2O_2 in roots treated with DPI and exposure to 96066 F of *H. abietinum* for 48 h in the

meristematic zone (bar=10 μ m). **g** Co-presence of ferrous iron and H_2O_2 in *P. sylvestris* roots after KCN application to tap root, followed by exposure to 96087S isolates of *H. parviporum* at 4 h in the elongation zone (bar=10 μ m). **h** Lack of ferrous iron localization in double staining for ferrous and H_2O_2 in tap roots exposed for 4 h to 96066 F of *H. abietinum*., tap root of *P. sylvestris* was first treated with inhibitors DPI at 4 h in the elongation zone (bar=20 μ m). **i** Double staining for ferrous iron and H_2O_2 in *P. sylvestris* roots exposed to 95107P of *H. annosum* s.s., note that tap root was not treated with any inhibitors at 4 h in the meristematic zone (bar=20 μ m). **j** Lack of H_2O_2 localization in double staining for ferrous and H_2O_2 in roots contact for 4 h to 02/139P of *H. annosum*., tap root of *P. sylvestris* was first treated with inhibitor AZ in the meristematic zone (bar=20 μ m). **k** Double staining of ferrous iron and H_2O_2 in *P. sylvestris* roots after KCN application to root, followed by exposure to Borowiec P isolates of *H. annosum* s.s. at 48 h in the meristematic zone (bar=10 μ m)

Table 2 The scheme of changes in reactive oxygen species and iron ions in the meristematic zone of *P. sylvestris* roots previously treated with inhibitors and then exposed to *H. annosum* s.s., *H. parviporum* or *H. abietinum* isolates for 4 or 48 h

Species		4 h						48 h					
		KCN	NaN ₃	DPI	ATZ	IA	DFO	KCN	NaN ₃	DPI	ATZ	IA	DFO
<i>H. annosum</i> s.s.	O ₂ ⁻	↑	↑			↑					↑		
	Fe ³⁺							↑	↑	↑	↑	↑	
	Fe ³⁺ & H ₂ O ₂	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
	Fe ²⁺							↓	↓		↓	↓	↓
	Fe ²⁺ & H ₂ O ₂	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
<i>H. parviporum</i>	O ₂ ⁻												
	Fe ³⁺	↓	↓	↓			↓						
	Fe ³⁺ & H ₂ O ₂												
	Fe ²⁺	↓	↓	↓		↓	↓	↑					
	Fe ²⁺ & H ₂ O ₂												
<i>H. abietinum</i>	O ₂ ⁻												
	Fe ³⁺	↓	↓	↓	↓	↓	↓			↓	↓	↓	↓
	Fe ³⁺ & H ₂ O ₂							↓	↓	↓	↓	↓	↓
	Fe ²⁺												↑
	Fe ²⁺ & H ₂ O ₂		↓	↓		↓							

The inhibitors studied include: potassium cyanide (KCN), sodium azide (NaN₃), diphenylene iodonium (DPI), 3-amino-1,2,4-triazole (ATZ), IA (itaconic acid), and desferoxamine (DFO). Detailed information with statistical analysis is provided in Supplementary Data Tables S1–S5

the applied compounds, except for ATZ, negatively influenced Fe²⁺ appearance in *P. sylvestris* root cells in the presence of *H. parviporum* at 4 h. Similarly, almost all compounds (except for DPI) restricted Fe²⁺ accumulation in host cell 48 h after exposure to *H. annosum* s.s. Only DPI or IA significantly restricted double staining H₂O₂ with Fe²⁺ in roots exposed to *H. parviporum*, while almost all compounds (apart from DFO) decreased H₂O₂ and Fe²⁺ co-localization in contact with *H. abietinum* in the elongation zone at 4 h (Table 2, S5, Fig. 1g and h). The occurrence of H₂O₂ with Fe²⁺ decreased 48 h after exposure of *P. sylvestris* roots in the elongation zone to *H. annosum* s.s., *H. parviporum* and *H. abietinum*. In the meristematic zone, double staining H₂O₂ with Fe²⁺ was limited by application of all of the studied inhibitors 4 and 48 h after exposure of *P. sylvestris* roots to *H. annosum* s.s. (Fig. 1 i, j, and k).

Canonical variate analysis

Multivariate analysis of isolates by their effects on ROS and iron showed separation of different *H. annosum* s.l. isolates, though the results varied depending on the time

since inoculation and the root zone (Fig. 2). First axis explained most of the observed variability of ROS and iron accumulation in the meristematic zone (86 % for 4 h and 82 % for 48 h) and separated isolates of *H. annosum* s.s. from isolates of two other species (*H. parviporum* and *H. abietinum*). As compared to isolates of *H. parviporum* and *H. abietinum*, 4 h after inoculation, the isolates of *H. annosum* s.s. showed greater ROS and iron accumulation and the different *H. annosum* s.s. isolates were more similar to each other. This pattern was associated with higher values of cell percentage stained with O₂⁻, Fe³⁺ as well as observed co-localisation of Fe²⁺ with H₂O₂. However, co-localisation of Fe³⁺ with H₂O₂ and Fe²⁺ had no impact in this structure of dataset for differentiation of the studied isolates. At 48 h, the separation of *H. annosum* s.s. isolates was combined with ferric iron and O₂⁻ with lower meaning of the percentage of cells showing co-localization of Fe³⁺ with H₂O₂ that was lower. Variation among isolates of *H. annosum* s.s. based on the studied factors (O₂⁻, co-localization of Fe³⁺ and Fe²⁺ with H₂O₂, Fe³⁺ and Fe²⁺) was higher 48 than 4 h after inoculation in the meristematic zone (Fig. 2c and d).

The isolates of *H. annosum* s.s. 95107P had lower value than the two other – Borówiec P and 02/139 P after 48 h post inoculation (Fig. 2d). However, there was a great variation among isolates of *H. annosum* s.s. in both time points in the elongation zone. In the elongation zone after 4 h of exposure to the pathogens, the first CVA axis explained 73.4 % of the variability among isolates and separated *H. annosum* s.s. from the rest of studied isolates. In addition, the two isolates of *H. annosum* s.s. (95107P and 02/139P) were associated with a

decreased co-localisation of Fe^{2+} or Fe^{3+} with H_2O_2 than Fe^{2+} , but only Borówiec P – ferric ion and superoxide. The isolates of the rest two species *H. parviporum* and *H. abietinum* was characterized with similar value of the studied factors after 4 h post inoculation in the elongation zone (Fig. 2a). There was no such pattern observed in the elongation zone at 48 h. As referred to studied factors, the isolates 96087 S was similar to Borówiec P and 02/139 P. But the third isolates of *H. annosum* s.s. – 95107 P grouped with the

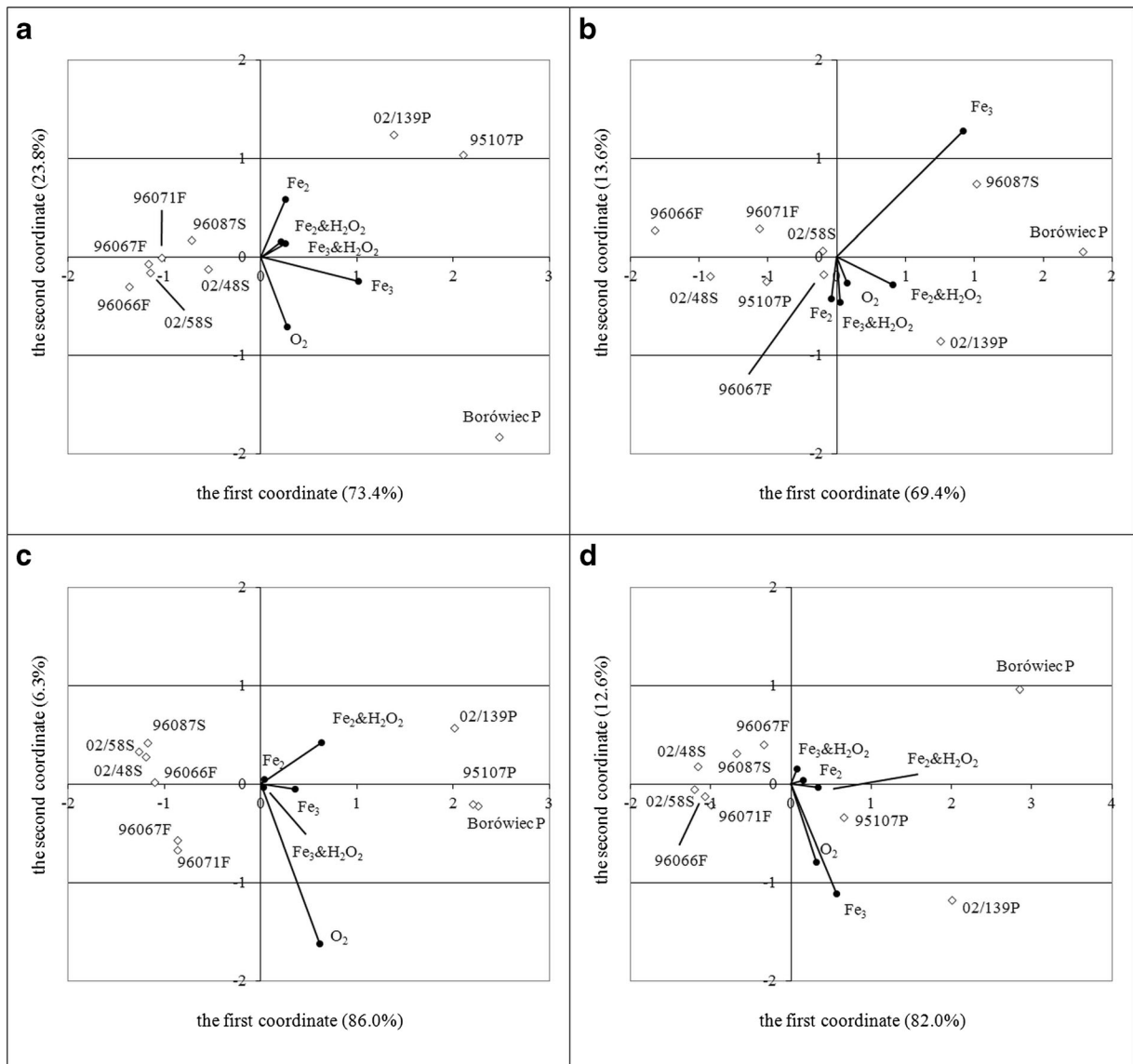


Fig. 2 Positions of the various pathogen isolates on the first two canonical variate axes, based on accumulation of reactive oxygen species and iron ions in the elongation zone at 4 h (a) or 48 h (b), and the meristematic zone at 4 h (c) or 48 h (d) after exposure to

the pathogens. Each fungal species was represented by three different isolates: *H. annosum* s.s. (Borówiec P, 95107P, 02/139 P), *H. parviporum* (96087 S, 02/58 S, 02/48 S) and *H. abietinum* (96066 F, 96067 F, 96071 F)

Table 3 Percentage of dead cells in the elongation zone of *P. sylvestris* roots treated with different inhibitors and then challenged with various isolates of *H. annosum* s.l

Species	Isolates	Inhibitors						<i>P</i>
		KCN	NaN ₃	DPI	ATZ	IA	DFO	
<i>H. annosum</i> s.s.	Borówiec	2.62±0.32 a	0.84±0.52 ab	2.08±0.28 ab	0.20±0.13 b	3.93±1.84 a	3.62±1.42 a	< 0.001
	95107 P	2.18±1.16	1.67±0.97	2.99±0.82	0.68±0.33	3.13±1.24	3.18±0.88	=0.300
	02/139 P	3.19±0.82	4.03±1.89	2.02±1.96	1.95±0.69	1.91±0.64	2.83±1.47	=0.485
<i>H. parviporum</i>	96087 S	0.71±0.68 b	8.69±2.13 a	0.06±0.06 b	2.54±1.02 ab	11.10±4.02 a	1.13±1.05 b	< 0.001
	02/58 S	0.63±0.42 b	6.38±1.42 a	0.31±0.20 b	2.42±0.90 ab	6.12±2.32 a	2.84±1.16 ab	< 0.001
	02/48 S	0.92±0.46 b	7.73±1.98 a	0.31±0.25 b	0.82±0.60 b	0.42±0.28 b	2.65±1.06 ab	< 0.001
<i>H. abietinum</i>	96066 F	0.12±0.12 b	7.84±2.10 a	2.10±1.29 ab	3.32±2.24 ab	8.06±2.42 a	3.80±1.46 ab	=0.018
	96067 F	0.02±0.02	0.33±0.21	1.29±0.98	3.32±2.24	0.75±0.38	4.37±1.69	=0.703
	96071 F	0.12±0.12	0.68±0.39	2.05±0.86	0.97±0.77	1.74±0.68	0.87±0.58	=0.201

The inhibitors studied include: potassium cyanide (KCN), sodium azide (NaN₃), diphenylene iodonium (DPI), 3-amino-1,2,4-triazole (ATZ), IA (itaconic acid), desferoxamine (DFO). Values represent means of the cell death caused by the inhibitors±SE. Means followed by the same letter in the row do not differ statistically (Tukey test, *P*<0.05)

rest of *H. parviporum* and *H. abietinum* isolates (except for 96087S) (Fig. 2b).

Influence of inhibitors on cell death evoked by pathogens

The catalase inhibitor elicited cell death in the lowest degree within root elongation zones challenged by Borówiec P (isolates of *H. annosum* s.s.) and 02/48S (isolates of *H. parviporum*), while within other isolates of *H. annosum* s.l. we did not find any statistically

significant reduction in cell death due to the catalase inhibitor (Table 3). In contrast, inhibition of cell wall peroxidase and NADPH oxidase most effectively limited cell death after roots were inoculated with *H. parviporum* isolates. Similarly, decrease in percentage of dead cells in root challenged by *H. parviporum* isolates was observed after application of KCN, but not NaN₃. The results also confirm KCN involvement in root cell death when challenged by 96066 F isolates *H. abietinum*. Surprisingly, oxalic acid was not involved in cell death in the *P. sylvestris* root elongation zone in any cases except

Table 4 Percentage of dead cells in the meristematic zone of *P. sylvestris* roots treated with different inhibitors and then challenged with various isolates of *H. annosum* s.l

Species	Isolates	Inhibitors						<i>P</i>
		KCN	NaN ₃	DPI	ATZ	Itaconic acid	DFO	
<i>H. annosum</i> s.s.	Borówiec	8.17±1.31 a	4.11±0.87 ab	4.44±1.16 ab	5.62±0.78 ab	3.48±1.16 ab	1.65±0.50 b	=0.004
	95107 P	3.37±0.57 b	3.57±0.47 ab	6.38±1.32 ab	8.19±1.55 a	6.00±0.74 ab	3.36±0.79 b	= 0.014
	02/139 P	22.52±7.18 a	6.40±1.18 ab	5.37±1.59 ab	0.16±0.11 b	4.61±1.38 ab	3.01±1.48 b	= 0.004
<i>H. parviporum</i>	96087 S	6.25±1.40 a	2.52±1.14 ab	0.31±0.31 b	3.25±0.75 ab	2.12±0.79 ab	0.44±0.15 b	= 0.002
	02/58 S	12.79±4.87 a	3.81±1.14 ab	0.72±0.47 b	11.72±2.46 a	1.27±0.61 b	3.73±1.14 ab	<0.001
	02/48 S	2.11±0.81 bc	6.92±2.03 ab	0.18±0.12 c	10.76±2.00 a	0.79±0.30 c	1.01±0.35 c	<0.001
<i>H. abietinum</i>	96066 F	10.11±5.61 ab	8.01±2.24 ab	1.66±1.09 ab	12.79±0.59 a	0.45±0.33 b	2.97±1.54 ab	=0.014
	96067 F	19.81±5.32 a	2.89±1.07 bc	1.42±1.06 c	11.44±3.39 ab	0.25±0.25 c	4.67±1.86 bc	<0.001
	96071 F	13.29±3.05 a	3.74±1.29 ab	2.46±1.49 b	13.28±2.92 a	5.09±0.99 ab	3.37±1.23 ab	=0.010

The inhibitors studied include: potassium cyanide (KCN), sodium azide (NaN₃), diphenylene iodonium (DPI), 3-amino-1,2,4-triazole (ATZ), IA (itaconic acid), desferoxamine (DFO). Values represent means of the cell death caused by the inhibitors±SE. Means followed by the same letter in the row do not differ statistically (Tukey test, *P*<0.05)

one isolate of *H. parviporum* (02/48S). Iron limitation, by binding with DFO, decreased the percentage of cell death but this decrease was only statistically significant for isolate 96087S of *H. parviporum*.

In the meristematic zone, a slightly different pattern was observed. Reduction of available iron resources by DFO restricted mortality of host cells exposed to *H. annosum* s.s., but KCN and ATZ decreased *P. sylvestris* cell death when exposed to isolates 95107P and 02/139P, respectively. NADPH oxidase inhibition significantly reduced the percentage of cell death evoked by all of the isolates of *H. parviporum* and two isolates (96067 and 96071 F) of *H. abietinum* (Table 4). The outcome of Evans blue reaction was significantly dependent on the presence of the oxalic acid inhibitor within root exposed to isolates of *H. parviporum* (02/58S and 02/48S) and isolates of *H. abietinum* (96066 and 96067 F). The lowest percentage of cell death after iron binding was found after contact with all isolates of *H. annosum* s.s., two isolates of *H. parviporum* (96087S and 02/48S), and isolates 96067 F of *H. abietinum*.

Comparison of mortality caused by pathogens in both zones showed that higher mortality was evoked in meristematic than elongation zone by isolates of *H. annosum* s.s. (Borówiec P and 95107P, $P < 0.001$; 02/139P, $P = 0.045$) and *H. abietinum* (96067 and 96071 F, $P < 0.001$). Such variation was not observed in *P. sylvestris* root infected by isolates of *H. parviporum*.

Discussion

Most plants generate harmful ROS as a response to pathogens. Our previous studies revealed the patterns of H_2O_2 , O_2^- , or iron ion accumulation in root cells may reflect aggressiveness of different pathogen species during the *P. sylvestris* defense response to *H. annosum* s.l. infection (Mucha et al. 2012). In the study described herein, we analyzed whether the outcome of the interaction between *H. annosum* s.l. and *P. sylvestris* may be dependent on different mechanisms activated during the host response by enzymes responsible for H_2O_2 or O_2^- formation and iron scavenging.

The source of ROS and their relation to evoking hypersensitive response (HR) and subsequent cell death are still discussed (Bolwell and Wojtaszek 1997; Torres et al. 2002). The first oxidative burst was reported to be a crucial step in mutual regulation of H_2O_2 and salicylic

acid (SA) and exert the effect on the gene dependent second oxidative burst and the HR (Shirasu et al. 1997; Mur et al. 2000). In our previous studies, H_2O_2 accumulation dynamics observed in seedling roots inoculated with *H. parviporum* and *H. abietinum* strains were similar to those for *H. annosum* s.s. strains, although the reaction in H_2O_2 in *H. parviporum* and *H. abietinum* strains was weaker after 4 h than that after 48 h (Mucha et al. 2012). Biphasic burst during the HR in soybean and potato was suggested to be the result of a membrane bound neutrophil-like NADPH oxidase generating O_2^- with the subsequent dismutation to H_2O_2 (Doke 1983). However, our studies revealed that production of O_2^- was increased by inhibition of haem-containing peroxidase by potassium cyanide and sodium azide at 4 h in the meristematic zone exposed to *H. annosum* s.s.. Additionally, an inhibitor of NADP oxidase, DPI, did not influence O_2^- accumulation, apart from increased O_2^- at 48 h in the elongation zone challenged by *H. annosum* s.s.; this suggests a lack of NADPH oxidase involvement in O_2^- accumulation. Similarly, oxidative burst in a barley - *Blumeria graminis* pathosystem was not sensitive to NADPH (Hückelhoven and Kogel 2003; Liu et al. 2007). The authors showed that oxidative burst in that pathosystem was dependent on iron alteration. Our studies reported herein also showed that disturbing ROS production negatively influenced co-localisation of H_2O_2 and Fe^{2+} in the meristematic root zone after 4 h of common incubation of *P. sylvestris* and *H. annosum* s.s. and in both root zones after 48 h. Co-localisation of H_2O_2 with Fe^{2+} was found to be important in the *P. sylvestris* – *H. annosum* s.s. pathosystem as described by Mucha et al. (2012). ROS, especially H_2O_2 , may evoke intracellular iron depletion, which can have caused disruption of Fe-S cluster proteins (enzymes involved in ROS detoxification) and lead to oxidative damage (Walter et al. 2002; Choi et al. 2004). Fewer cells with cellular iron was observed after exposure of *P. sylvestris* roots to *H. annosum* s.s. in comparison to *H. parviporum* (unpublished data). Reduced levels of co-localized H_2O_2 with Fe^{2+} was also found in the elongation zone irrespective to the duration of root exposure to *H. abietinum*, but were only observed at 48 h in the elongation zone for *H. parviporum*; this discrepancy might be explained by different mechanisms involved in the first and second oxidative bursts. As stated by Thordal-Christensen et al. (1997), different aspects of defence may be regulated by H_2O_2 responses at different time-points. H_2O_2 toxicity to plant cells may be regulated by the availability of

“catalytic” Fe^{2+} (Baker and Orlandi 1995; Bestwick et al. 1997). Independent regulation of oxidative burst and iron availability may allow for more intense disease spread of fungal pathogens, as shown by Olson et al. (2012). The authors found that necrotrophic fungi participate in production of ROS to facilitate infection, but ferric reductase was an additional factor involved in pathogenicity of the necrotroph *H. irregulare* Garbel. & Orosina. Although it seems that the present studies revealed similar mechanisms of *P. sylvestris* response to *H. annosum* s.s. and *H. abietinum* in some root zones, canonical variate analysis showed that factors such as: iron accumulation, and co-localisation of hydrogen peroxide with iron and superoxide was bound with isolates of *H. annosum* s.s., in both studied time in meristematic zone. It was also found in elongation zone 4 h after challenged for the same isolates.

Iron-containing peroxidases with ability to generate H_2O_2 are also very important factors in plants challenged with pathogens and oxidative burst may be blocked by iron scavenging compounds such as DFO (Liu et al. 2007). This compound was found to be one of the most inhibiting factors in co-localisation of H_2O_2 with Fe^{2+} and Fe^{3+} in the *P. sylvestris* – *H. annosum* s.s. pathosystem at 4 h. Studies on elicitation of *Picea abies* cells with *H. parviporum* showed that azide, an inhibitor of haem-containing enzymes including peroxidase, significantly delay H_2O_2 formation (Kärkönen et al. 2009). The role of peroxidase in the oxidative burst is so important since it was found to enhance cell death of *Arabidopsis thaliana* in contrast to NADPH oxidases (Torres et al. 2005) and since exogenous H_2O_2 may stimulate salicylic acid concentration and cell death (Shirasu et al. 1997). Unlike peroxidase, plasmalemma-bound NADPH oxidases produce O_2^- responsible for oxidative burst and prevent the spread of salicylic acid-dependent *Arabidopsis thaliana* cell death (Torres et al. 2005). Likar and Regvar (2008) found that *H. annosum* was able to induce salicylic acid.

Here we provide evidence that *P. sylvestris* activated different mechanisms in response to *H. parviporum* and *H. abietinum* than *H. annosum* s.s. in generating oxidative burst and utilizing iron to evoke cell death. We showed that isolates of *H. annosum* s.s. and *H. abietinum* caused higher mortality in meristematic root zones as compared to elongating zones, with no difference observed for *H. parviporum*. Generally, necrotrophic fungi preferably infect meristematic region to invade plant host root (Rodriguez-Galvez and Mendgen 1995; Mucha et al. 2014), where cells are similar to

parenchymatic cells, and are characterized by thin walls and little amount of storage compounds (Evert 2006). Thus, the higher mortality of *P. sylvestris* meristematic root cells could be associated with a preference for meristematic region as a point of pathogen entry (Asiegbu et al. 1994). Since *H. annosum* s.l. is a necrotrophic pathogen able to cause necrosis in any region of *P. sylvestris* roots (Asiegbu et al. 1994), it was uncertain if there were differences in the mechanisms operating during infection in meristematic and elongation zones between these closely related species. In the present studies, NADPH oxidase turned out to participate in the cell death after root inoculation by most of the *H. parviporum* and *H. abietinum* isolates in both zone. Oxalic acid was involved in cell death of *P. sylvestris* exposed to *H. parviporum* and *H. abietinum* isolates only in meristematic one. Involvement of oxalic acid in host cell death was confirmed by Kim et al. (2008) through manipulation of host ROS. The authors underlined its role in host death signaling since the combination of oxalic acid and NADPH oxidase inhibitors decreased cell death. In our previous studies, the level of oxalic acid was dropping along with exposure time of *P. sylvestris* to the same isolates of *H. annosum* s.l. used in the current study. In addition, levels of oxalic acid were correlated negatively with cell death (Mucha et al. 2015). This may suggest that oxalic acid is used as a source of H_2O_2 , since oxalic acid is a substrate for oxalate oxidase, which releases H_2O_2 and CO_2 from oxalic acid (Zhang et al. 1995). This enzyme was shown to accumulate in the presence of pathogens in angiosperm (Zhang et al. 1995) and gymnosperm trees (Jöhnk et al. 2005). Oxalate oxidase activity was also found in *P. sylvestris* among other tree species (Dunwell et al. 2000) and this enzyme may confer resistance against oxalic acid producing pathogen, as was confirmed for *Sclerotia sclerotiorum* (Hu et al. 2003). Thus, we provide clear confirmation of role for oxalic acid as a source of hydrogen peroxide during infection of *P. sylvestris* by closely related *H. parviporum* and *H. abietinum* at least some isolates, which prefer other host species (spruce and fir, respectively).

The studies of Kim et al. (2008) also showed that heat-stable non-proteinaceous compounds are involved in programmed cell death. Apart from oxalic acid, such compounds may be represented by siderophores (Neilands 1984). Our studies showed that the decrease of available pool of iron by DFO negatively influenced host mortality in the presence of *H. annosum* s.s. in the meristematic zone. However, the elongation zone did

not show such a pattern. The differentiation between zones may reflect the dynamics of changes in enzyme activation. Dissimilarity in the role of iron in the infection process was found in our earliest study (Mucha et al. 2012), where *H. annosum* s.s. in contrast to *H. parviporum* required iron for successful infection. Moreover, the role of iron in pathogenicity of *H. irregulare* was also confirmed by Olson et al. (2012).

In conclusion, the use of inhibitors in this study confirmed the role of iron in H₂O₂ accumulation (Mucha et al. 2012) due to peroxidases to evoke HR and eventually cell death in the *P. sylvestris* – *H. annosum* s.s. pathosystem in meristematic, more frequently infected zone than elongation one, while strains with less preference for *P. sylvestris* as a host, including *H. parviporum* and *H. abietinum*, employ other mechanisms to invade the host cell.

Acknowledgments The research received financial support from the National Science Centre (project no NN 309 136935) and the Institute of Dendrology of the Polish Academy of Sciences. We would like to thank Ludmila Bladocha for excellent technical support. We thank Dr. Marc Goebel, the Department of Natural Resources at Cornell University for valuable comments on the manuscript.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

- Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, 55, 373–399.
- Asiegbu, F. O., Daniel, G., & Johansson, M. (1994). Defence related reactions of seedling roots of Norway spruce to infection by *Heterobasidion annosum* (Fr.) Bref. *Physiological and Molecular Plant Pathology*, 45, 1–19.
- Asiegbu, F. O., Johansson, M., & Stenlid, J. (1999). Reactions of *Pinus sylvestris* (Scots pine) root tissues to the presence of mutualistic, saprotrophic and necrotrophic microorganisms. *Journal of Phytopathology*, 147, 257–264.
- Asiegbu, F. O., Adomas, A., & Stenlid, J. (2005). Conifer root and butt rot caused by *Heterobasidion annosum* (Fr.) Bref. sl. *Molecular Plant Pathology*, 6(4), 395–409.
- Baker, C. J., & Mock, N. M. (1994). An improved method for monitoring cell death in a cell suspension and leaf disk assays using Evans blue. *Plant Cell Tissue and Organ Culture*, 39, 7–12.
- Baker, C. J., & Orlandi, E. W. (1995). Active oxygen in plant pathogenesis. *Annual Review of Phytopathology*, 33, 299–321.
- Bestwick, C. S., Brown, I. R., Bennett, M. H. R., & Mansfield, J. W. (1997). Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola*. *Plant Cell*, 9, 209–221.
- Bolwell, G. P., & Wojtaszek, P. (1997). Mechanism for generation of reactive oxygen species in plant defence—a broad perspective. *Physiological and Molecular Plant Pathology*, 51, 347–366.
- Bolwell, G. P., Davies, D. R., Gerrish, C., Auh, C. K., & Murphy, T. M. (1998). Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiology*, 116, 1379–1385.
- Budka, A., Borowiak, K., Zbierska, J., Kayzer, D., & Krzesiński, W. (2011). Application of a multidimensional linear model to compare degrees of tobacco leaf injury caused by tropospheric ozone at rural and urban exposure sites. *Fresenius Environmental Bulletin*, 4, 969–975.
- Choi, E. Y., Kim, E. C., Oh, H. M., Kim, S., Lee, H. J., Cho, E. Y., Yoon, K. H., Kim, E. A., Han, W. C., Choi, S. C., et al. (2004). Iron chelator triggers inflammatory signals in human intestinal epithelial cells: involvement of p38 and extracellular signal regulated kinase signaling pathways. *The Journal of Immunology*, 172, 7069–7077.
- Czymmek, K. J., Fogg, M., Powell, D. H., Sweigard, J., Park, S. Y., & Kang, S. (2007). In vivo time-lapse documentation using confocal and multi-photon microscopy reveals the mechanisms of invasion into the *Arabidopsis* root vascular system by *Fusarium oxysporum*. *Fungal Genetics and Biology*, 44, 1011–1023.
- Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inze, D., & Van Breusegem, F. (2000). Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences*, 57, 779–795.
- De Gara, L., de Pinto, M. C., & Tommasi, F. (2003). The antioxidant system vis-à-vis reactive oxygen species during plant-pathogen interaction. *Plant Physiology and Biochemistry*, 41, 863–870.
- Doke, N. (1983). Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiological Plant Pathology*, 23, 345–358.
- Dunand, C., Crèvecoeur, M., & Penel, C. (2007). Distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: possible interaction with peroxidases. *New Phytologist*, 174, 332–341.
- Dunwell, J. M., Khuri, S., & Gane, P. J. (2000). Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiology and Molecular Biology Reviews*, 64(1), 153–179.
- Evert, R. F. (2006). *Esau's plant anatomy: Meristems, cells, and tissues of the plant body: their structure, function, and development*. Wiley.

- Glazener, J. A., Orlandi, E. W., & Baker, C. J. (1996). The active oxygen response of cell suspensions to incompatible bacteria is not sufficient to cause hypersensitive cell death. *Plant Physiology*, *110*, 759–763.
- Govrin, E. M., & Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology*, *10*, 751–757.
- Hu, X., Bidney, D. L., Yalpani, N., Duvick, J. P., Crasta, O., Folkerts, O., & Lu, G. (2003). Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiology*, *133*(1), 170–181.
- Hückelhoven, R., & Kogel, K. H. (2003). Reactive oxygen intermediates in plant-microbe interactions: who is who in powdery mildew resistance? *Planta*, *216*, 891–902.
- Ingestad, T. (1979). Mineral nutrient requirements of *Pinus sylvestris* and *Picea abies* seedlings. *Physiology Plantarum*, *45*, 373–380.
- Jaber, E., Xiao, C., & Asiegbu, F. O. (2013). Comparative pathobiology of *Heterobasidion annosum* during challenge on *Pinus sylvestris* and *Arabidopsis* roots: an analysis of defensin gene expression in two pathosystems. *Planta*, 1–17.
- Johansson, S. M., Lundgren, L. N., & Asiegbu, F. O. (2004). Initial reactions in sapwood of Norway spruce and Scots pine after wounding and infection by *Heterobasidion parviporum* and *H. annosum*. *Forest Pathology*, *34*, 197–210.
- Jöhnk, N., Hietala, A. M., Fossdal, C. G., Collinge, D. B., & Newman, M. A. (2005). Defense-related genes expressed in Norway spruce roots after infection with the root rot pathogen *Ceratobasidium bicorne* (anamorph: *Rhizoctonia* sp.). *Tree Physiology*, *25*(12), 1533–1543.
- Kärkönen, A., Warinowski, T., Teeri, T. H., Simola, L. K., & Fry, S. C. (2009). On the mechanism of apoplastic H₂O₂ production during lignin formation and elicitation in cultured spruce cells; peroxidases after elicitation. *Planta*, *230*, 553–567.
- Kayzer, D., Borowiak, K., Budka, A., & Zbierska, J. (2009). Study of interaction in bioindication research on tobacco plant injuries caused by ground level ozone. *Environmetrics*, *20*, 666–675.
- Kim, K. S., Min, J. Y., & Dickman, M. B. (2008). Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Molecular Plant-Microbe Interactions*, *21*, 605–612.
- Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*, 251–275.
- Lejeune, M., & Caliński, T. (2000). Canonical analysis applied to multivariate analysis of variance. *Journal of Multivariate Analysis*, *72*, 100–119.
- Li, G., & Asiegbu, F. O. (2004). Use of scots pine seedling roots as an experimental model to investigate gene expression during interaction with the conifer pathogen *Heterobasidion annosum* (P-type). *Journal of Plant Research*, *117*, 155–162.
- Likar, M., & Regvar, M. (2008). Early defence reactions in Norway spruce seedlings inoculated with the mycorrhizal fungus *Pisolithus tinctorius* (Persoon) Coker & Couch and the pathogen *Heterobasidion annosum* (Fr.) Bref. *Trees*, *22*, 861–868.
- Liu, G., Sheng, X., Greenshields, D. L., Ogieglo, A., Kaminskyj, S., Selvaraj, G., & Wei, Y. (2005). Profiling of wheat class III peroxidase genes derived from powdery mildew-attacked epidermis reveals distinct sequence-associated expression patterns. *Molecular Plant-Microbe Interactions*, *18*, 730–741.
- Liu, G., Greenshields, D. L., Sammynaiken, R., Hirji, R. N., Selvaraj, G., & Wei, Y. (2007). Targeted alterations in iron homeostasis underlie plant defence responses. *Journal of Cell Science*, *120*, 596–605.
- Mucha, J., Guzicka, M., Łakomy, P., & Zadworny, M. (2012). Iron and reactive oxygen responses in *Pinus sylvestris* root cortical cells infected with different species of *Heterobasidion annosum* sensu lato. *Planta*, *236*, 975–988.
- Mucha, J., Guzicka, M., Ratajczak, E., & Zadworny, M. (2014). Strategies utilized by trophically diverse fungal species for *Pinus sylvestris* root colonization. *Tree Physiology*, *34*, 73–86.
- Mucha, J., Guzicka, M., Łakomy, P., & Zadworny, M. (2015). Accumulation of iron-binding compounds in root of *Pinus sylvestris* challenged by *Heterobasidion annosum* sensu lato. *Dendrobiology*, *73*, 103–110.
- Mur, L. A., Brown, I. R., Darby, R. M., Bestwick, C. S., Bi, Y. M., Mansfield, J. W., & Draper, J. (2000). A loss of resistance to avirulent bacterial pathogens in tobacco is associated with the attenuation of a salicylic acid-potentiated oxidative burst. *Plant Journal*, *23*, 609–621.
- Neilands, J. B. (1984). Siderophores of bacteria and fungi. *Microbiological Sciences*, *1*(1), 9–14.
- Niemelä, T., & Korhonen, K. (1998). Taxonomy of the genus *Heterobasidion annosum*. In S. Woodward, J. Stenlid, R. Karjalainen, & A. Hüttermann (Eds.), *Heterobasidion annosum: Biology, ecology, impact and control* (pp. 346–360). Wallingford: CAB International.
- Olson, Å., Aerts, A., Asiegbu, F., Belbahri, L., Bouzid, O., Broberg, A., Canbäck, B., Countinho, P. M., Cullen, D., Dalman, K., Deflorio, G., van Diepen, L. T. A., Dunand, C., Duplessis, S., Durling, M., Gonthier, P., Grimwood, J., Fossdal, C. G., Hansson, D., Henrissat, B., Hietala, A., Himmelstrand, K., Hoffmeister, D., Högberg, N., James, T. Y., Karlsson, M., Kohler, A., Kües, U., Lee, Y. H., Lin, Y. C., Lind, M., Lindquist, E., Lombard, V., Lucas, S., Lundén, K., Morin, E., Murat, C., Park, J., Raffaello, T., Rouzé, P., Salamov, A., Schmutz, J., Solheim, H., Ståhlberg, J., Véléz, H., de Vries, R. P., Wiebenga, A., Woodward, S., Yakovlev, I., Garbelotto, M., Martin, F., Grigoriev, I. V., & Stenlid, J. (2012). Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. *New Phytologist*, *194*, 1001–1012.
- Rodriguez-Galvez, E., & Mendgen, K. (1995). The infection process of *Fusarium oxysporum* in cotton root tips. *Protoplasma*, *189*(1–2), 61–72.
- Shetty, N. P., Mehrabi, R., Lütken, H., Haldrup, A., Kema, G. H. J., Collinge, D. B., et al. (2007). Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. *New Phytologist*, *174*, 637–647.
- Shetty, N. P., Lyngs Jørgensen, H. J., Jensen, J. D., Collinge, D. B., & Shekar Shetty, H. (2008). Roles of reactive oxygen species in interactions between plants and pathogens. *European Journal of Plant Pathology*, *121*(3), 267–280.
- Shimada, M., Akamtsu, Y., Tokimatsu, T., Mii, K., & Hattori, T. (1997). Possible biochemical roles of oxalic acid as a low

- molecular weight compound involved in brown-rot and white-rot wood decays. *Journal of Biotechnology*, 53(2), 103–113.
- Shirasu, K., Nakajima, H., Rajasekhar, V. K., Dixon, R. A., & Lamb, C. (1997). Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell*, 9, 261–270.
- Smith, M. A., Harris, P. L. R., Sayre, L. M., & Perry, G. (1997). Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 9866–9868.
- Snedecor, W., & Cochran, W. G. (1967). *Statistical methods* (6th ed., pp. 327–329). Ames: The Iowa State University Press.
- Taheri, P., Irannejad, A., Goldani, M., & Tarighi, S. (2014). Oxidative burst and enzymatic antioxidant systems in rice plants during interaction with *Alternaria alternata*. *European Journal of Plant Pathology*, 1–11.
- Thordal-Christensen, H., Zhang, Z., Wei, Y., & Collinge, D. B. (1997). Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. *Plant Journal*, 11, 1187–1194.
- Tiwari, B. S., Belenghi, B., & Levine, A. (2002). Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant Physiology*, 128, 1271–1281.
- Torres, M. A. (2010). ROS in biotic interactions. *Physiologia Plantarum*, 138, 414–429.
- Torres, M. A., Dangl, J. L., & Jones, J. D. G. (2002). Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 517–522.
- Torres, M. A., Jones, J. D. G., & Dangl, J. L. (2005). Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. *Nature Genetics*, 17, 1130–1135.
- Torres, M. A., Jones, J. D. G., & Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiology*, 141, 373–378.
- Unger, C., Kleta, S., Jandl, G., & von Tiedemann, A. V. (2005). Suppression of the defence-related oxidative burst in bean leaf tissue and bean suspension cells by the necrotrophic pathogen *Botrytis cinerea*. *Journal of Phytopathology*, 153, 15–26.
- Walter, P. B., Knutson, M. D., Paler-Martinez, A., Lee, S., Xu, Y., Viteri, F. E., & Ames, B. N. (2002). Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 2264–2269.
- Wang, C. F., Huang, L. L., Zhang, H. C., Han, Q. M., Buchenauer, H., & Kang, Z. S. (2010). Cytochemical localization of reactive oxygen species (O₂⁻ and H₂O₂) and peroxidase in the incompatible and compatible interaction of wheat – *Puccinia striiformis* f. sp. *tritici*. *Physiological and Molecular Plant Pathology*, 74, 221–229.
- Zhang, Z., Collinge, D. B., & Thordal-Christensen, H. (1995). Germin-like oxalate oxidase, a H₂O₂-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. *Plant Journal*, 8, 139–145.