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Redistribution of iron and hydrogen peroxide in *Pinus sylvestris* roots in response to trophically diverse fungi

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Abstract The simultaneous over-accumulation of Fe, hydrogen peroxide and superoxide can result in the generation of hydroxyl radicals, which play a significant role in the interactions between plant hosts and invading fungi. The physiological response to infection by pathogenic or mycorrhizal fungi may, however, be determined by the root zone. The objective of the present study was to use light microscopy to determine whether iron and hydrogen peroxide are differentially distributed in *P. sylvestris* roots in response to colonization by necrotrophic pathogens (*Fusarium oxysporum* and *Rhizoctonia solani*) vs. mycorrhizal fungi (*Hebeloma crustuliniforme* and *Laccaria bicolor*) in in vitro culture. It was also of interest to determine how hydrogen peroxide, superoxide, and iron added to substrate in vitro impacted the ability of trophically different fungi to grow. Infection by necrotrophic fungi, especially by *F. oxysporum,* were associated with a significantly higher accumulation of both forms of iron (Fe^{2+} and Fe^{3+}) in the meristematic root zone, relative to the accumulation of these forms of iron in response to ectomycorrhizal fungi. Iron supplementation of solid culture medium reduced the growth of pathogenic fungi to a greater extent than the growth of mycorrhizal fungi. In contrast to the distribution of iron, cells in the elongation zone of *P. sylvestris* roots generally exhibited the highest accumulation of hydrogen peroxide. The growth of mycorrhizal fungi was more inhibited by the presence of hydrogen peroxide in the solid culture medium than by any other supplemented compounds. This research indicate that the distribution of iron and hydrogen peroxide in roots of *P. sylvestris* is related to the trophic strategy of the colonizing fungi.

Introduction

Plants are constantly challenged by microorganisms and their interactions range from parasitism to mutualism. Mechanisms associated with plant defense responses to trophically different fungi have been extensively studied. It has been generally recognized that a considerable variation exists in the extent of the formation of reactive oxygen species (ROS) at the initial stage of the interaction and that this initial response can determine the nature of the subsequent association (Asiegbu et al. 1999; Adomas et al. 2008; Zhang and Tang 2012). ROS accumulation can lead to a hypersensitive response and cell death in plant hosts, which functions as a defense mechanism against biotrophic pathogens, but fosters plant colonization by necrotrophic pathogens (Govrin and Levine 2000). ROS accumulation in host

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tissues resulting from the colonization of fungi establishing a mutualistic association (e.g. mycorrhizal fungi), however, does not appear to result in the massive death of plant cells (Baptista et al. 2007; Mucha et al. 2014). Thus, specific redox levels associated with reactive oxygen species, as well as levels of iron, during plant-fungus interactions may either foster or prevent fungal establishment and disease. Iron levels also play a role in formation of hydroxyl radicals (Pierre and Fontecave 1999; Baker and Orlandi 1995) but whether the relationship between iron and hydrogen peroxide accumulation in plant roots differs between trophically different fungi (necrotrophic vs. mycorrhizal fungi) is relatively unknown.

Mechanisms regulating iron homeostasis in pathogenic and symbiotic fungi are not well understood. Acquisition of iron is necessary for the growth and development of fungal pathogens, and iron plays an essential role in activating the transcription of fungal pectinase genes that are engaged in the infection process (Expert 1999; Franza et al. 2002, 2005). Thus, fungal pathogens that can acquire iron from their hosts when the available iron in the environment is limiting are at a distinct advantage. Fungi utilize two processes to regulate iron accumulation; specifically, a reduction in iron assimilation and siderophore-assisted iron uptake. The mechanism utilized to acquire iron from plant tissues depends on the nature of the plant-fungus interaction (Eichhorn et al. 2006; Greenshields et al. 2007; Oide et al. 2006; Schrettl et al. 2004; Ramanan and Wang 2000), suggesting that separate strategies may exist for biotrophic vs. necrotrophic pathogens (Greenshields et al. 2007). Indeed, fungal siderophores can act as virulence determinants in necrotrophs (Oide et al. 2006; Schrettl et al. 2004), but also play a role in maintaining beneficial plant-fungal interactions (Johnson et al. 2007). Although ectomycorrhizal fungi are capable of producing siderophores (Haselwandter 1995; Renshaw et al. 2002), how iron accumulation in plant cells reflects the relationship between a plant and fungi with different trophic strategies at the initial stage is not known.

ROS catalysis by a small "transit" pool of cellular iron is possible (Johnson 2008) where Fe^{2+} is oxidized by hydrogen peroxide to Fe^{3+}, resulting in the formation of a harmful hydroxyl radicals (Pierre and Fontecave 1999). The distribution of iron in host cells and tissues may act as a defense signal in plant cells and induce the activation of many host defense systems against an invading pathogen (Dellagi et al. 2009); including those involved in oxidative metabolism (Johnson 2008). For example, targeted alterations in iron homeostasis associated with the production of H_2O_2 induce the expression of pathogenesis-related genes as a part of the defense response in leaves of *Blumeria graminis* (DC). Speer f. sp. *tritici* Marchal (Liu et al. 2007). The processes associated with plant defense responses can either assist the plant in resisting pathogen invasion, or may actually benefit the establishment and spread of a fungal pathogen.

In roots, meristematic and elongation zones differ in their anatomy and functional physiological roles (Scheres et al. 2002). These differences make the meristematic zone of root tips more conducive to the colonization of necrotrophs (Rodriguez-Galvez and Mendgen 1995) than ectomycorrhizal fungi (Felle et al. 2009). Our previous results demonstrated that a hypersensitive response related to Fe and H_2O_2 can regulate the initial stage of *P. sylvestris* root infection by the pathogen, *Heterobasidion annosum* s.s. (Mucha et al. 2012). It was subsequently demonstrated that an iron-dependent mechanism primarily operates in the meristematic zone of *P. sylvestris* roots invaded by *H. annosum* s.s., and to a much lesser extent, in roots invaded by pathogens (*H. parviporum* and *H. abietinum*) that are more atypical for *P. sylvestris* (Mucha et al. 2015). It is unclear, however, whether iron levels, and its distribution in different root zones, is another factor that impacts the redox state in trophically diverse fungus – plant root interactions.

The initial stages of the interaction between plants and trophically different fungi may be determined by the dynamics of iron accumulation. A model that accounts for ROS and Fe dynamics between a host and an invading fungus may more accurately reflect the strategy of a necrotrophic fungus vs. a mutualistic symbiont. The objective of the present study was to determine whether the accumulation and distribution of iron and hydrogen peroxide in plant host tissues is affected by trophically diverse fungal species (necrotrophic pathogens vs. ectomycorrhizal fungi). We also hypothesize that ectomycorrhizal fungi will be more sensitive to hydrogen peroxide than to superoxide or iron, as compared to necrotrophic fungi that will be more restricted by hydrogen peroxide and iron than ectomycorrhizal fungi.

Material and methods

Plant material and experimental design

The seeds of Scots pine (*Pinus sylvestris* L.) used in the current study originated from the provenance of Bolewice, Poland (52° 28′ N and 16 ° 03′ E). *Laccaria bicolor* (Maire) P.D. Orton and *Hebeloma crustuliniforme* (Bull.) Quél. and two isolates of the fungal pathogens, *Fusarium oxysporum* E.F. Sm. & Swingle and *Rhizoctonia solani* J.G. Kühn were used to examine iron redistribution in response to trophically diverse fungal species. The strains of *F. oxysporum* and *R. solani* were isolated from a forest nursery in the Myslibórz Forest District (N 52°51′04″, E 14°51′44″). The ectomycorrhizal fungi were isolated from fruiting bodies growing in a forest near Sulęczyno (N 54°13′30″, E 17°46′4″). All of the fungal isolates were maintained on malt extract agar (MEA) (malt agar extract, Difco, Detroit, MI, USA) in the dark. Plants were inoculated with the different fungi every 2 years and then re-isolated in order to avoid a loss of infective properties due to long-term storage on artificial MEA media.

Scots pine seeds were sterilized with 0.1% $HgCl_2$ (w/v) (Polish Chemical Reagents, Gliwice, Poland) for 3 min, washed three times (5 min each) in sterile distilled water, and then germinated on 0.6% water agar (w/v) (Difco, Detroit, MI, USA) in the dark. Germinated seeds were subsequently transferred under sterile conditions to Ingestad medium (Ingestad 1979) in 14 cm (diameter) Petri dishes. Roots of one-week-old seedlings were covered with a piece of filter paper containing actively growing fungi and were then covered by cellophane foil to prevent desiccation. All manipulations were performed under sterile conditions. Seedlings were grown under fluorescent lights (Osram L36/W77 Flora; 100 $\mu Em^{-2} s^{-1}$) using a 16 h a day, 60% RH and a 24:20 °C day: night temperature regime. Roots of control plants were covered with sterile filter paper and cellophane foil. *Pinus sylvestris* roots were harvested at 2 h, 6 h, 24 h, 48 h, 96 h, and 10 d.

Light microscopy visualization of iron and hydrogen peroxide

The protocol described by Thordal-Christensen et al. (1997) was used to visualize ROS, mainly H_2O_2, in Scots pine roots. Prior to visualization of ROS with light microscopy, roots were submerged for 75 min in 1 mg ml^{-1} 3,3′-diaminobenzidine, pH 3.8.

Iron accumulation in Scots pine roots was visualized using the protocol described by Smith et al. (1997) and Liu et al. (2007). Pieces of roots were placed in a fixative solution of methacarn (methanol:chloroform:acetic acid, 60:30:10) at 4 °C for 15 h. Roots were then dehydrated in a graded series (70–100%) of ethanol solutions and subsequently incubated in 7% potassium ferrocyanide for 24 h to detect Fe^{3+}. In order to detect Fe^{2+}, roots were incubated for 48 h in 7% potassium ferricyanide in aqueous 3% hydrochloric acid. Root samples were sectioned by hand, placed on glass slides, and observed under an Axioscope 20 microscope (Carl Zeiss, Jena, Germany) at a magnification of 40x.

Stress tolerance assay

Isolates of the fungal pathogens, *F. oxysporum* and *R. solani,* and the ectomycorrhizal fungi, *L. bicolor* and *H. crustuliniforme,* were grown on MEA (Difco, Detroit, MI, USA) supplemented with increasing concentrations (1, 5, 10 or 15 mM) of H_2O_2 or KO_2 (superoxide generator), or the varying concentrations (1, 5, 10 or 15 mM) of $FeCl_3$. Colony diameter was measured daily for all of the isolates growing in each of the concentrations of stress compounds to evaluate the sensitivity of each of the fungal species to the stress reagent. Results were expressed as a percentage of fungal colony growth relative to a control growing on non-amended medium.

Statistical analyses

All experiments utilized five biological replicates for each of the experimental units and each experiment was repeated three times. The number of cells exhibiting iron or hydrogen peroxide staining was used to calculate a percentage of cells with staining relative to the total number of cells observed in a field of view using the Bliss equation (Snedecor and Cochran 1967). A hierarchical analysis of variance was used to determine the significance of the effect of root zone (meristematic and elongation zone), fungal trophic strategy, fungal species nested in fungal trophic strategy, and length of time of the interaction. A one-way ANOVA, followed by a Tukey's HSD test, was used to determine significant differences between the time of sampling within an experimental unit (fungal strain) and among different root parts within the same time point for the different fungal strains. Pearson's correlation coefficients were

used to determine the strength of the interdependence of the studied parameters. All statistical analyses were conducted using JMP 8 software (SAS Institute, Cary, NC, USA) and were considered significant at $P \leq 0.05$.

Results

Hydrogen peroxide (H_2O_2)

P. sylvestris infected with trophically diverse fungi exhibited an increase in the percentage of cells stained with H_2O_2, relative to the control (Table 1). The percentage of cells with H_2O_2 in *P. sylvestris* roots was not dependent on fungal trophic strategy but a strong influence was found for species nested within fungal trophic strategy ($P = 0.004$). The presence of mycorrhizal fungi caused a gradual increase in the percentage of cells with hydrogen peroxide. In contrast to the response of roots to ectomycorrhizal fungi, a high percentage of cells with hydrogen peroxide were present in roots of *P. sylvestris* seedlings in response to *R. solani*. Percentage of cells exhibiting H_2O_2 staining were also more frequently ($P < 0.010$) observed in the elongation zone than in the meristematic zone of roots. This zonation was largely evident in roots invaded by ectomycorrhizal fungi. The pathogenic fungus, *F. oxysporum,* induced a lower level of H_2O_2 accumulation in the elongation zone than in the meristematic zone. Percentage of stained cells of both zones was similar in roots infected with *R. solani*. Differences in H_2O_2 accumulation in *P. sylvestris* root cells in response to the presence of the studied fungal species was mainly observed at the later stages of the interaction time (48 h and later).

Ferric iron (Fe^{3+})

The pattern of accumulation of Fe^{3+} observed in roots was in contrast to those observed for hydrogen peroxide. *P. sylvestris* roots infected with fungal pathogens exhibited a greater percentage of cells with Fe^{3+} staining than roots colonized by mycorrhizal fungi. The lowest percentage of cells stained with ferric ion was observed in roots colonized by the ectomycorrhizal fungus, *H. crustuliniforme,* and the highest ($P < 0.001$) accumulation of cell with ferric ions was observed in roots infected with the fungal pathogen, *F. oxysporum*. Similar to the H_2O_2 response, a higher percentage of host cells exhibited Fe^{3+} staining in the elongation than in the

meristematic zone ($P = 0.023$). Additionally, the number of stained cells increased with time ($P < 0.001$) (Table 2). Differential accumulation of Fe^{3+} in root cells of *P. sylvestris* in response to the trophically different fungi was observed at 2 h and at the latter sampled time points (48 h, 96 h and 10 days).

Ferrous iron (Fe^{2+})

Percentage of cell with Fe^{2+} in root of *P. sylvestris* was strongly dependent on fungal trophic strategy ($P < 0.001$), though percentage of cells with Fe^{2+} accumulation also varied in response to species nested within trophic strategy ($P < 0.001$). Pathogenic fungi induced the greatest accumulation of cell stained with Fe^{2+}, and *F. oxysporum* induced the highest percentage of cell with Fe^{2+} between the two species of pathogenic fungi (Table 3). Percentage of cell with Fe^{2+} was higher in the meristematic zone than in the elongation zone of roots ($P = 0.038$) (Table 3). This pattern, however, was only true for pathogenic fungi. No differences in zonal accumulation of percentage of cells with Fe^{2+} was observed in roots colonized by mycorrhizal fungi. In contrast to the accumulation of hydrogen peroxide and Fe^{3+}, the percentage of root cells with Fe^{2+} staining significantly increased at the initial stage of the interaction (2 h), followed by a decrease (6 h), and reached a maximum percentage at 10-day ($P < 0.001$) in roots infected with necrotrophic fungi.

Correlation coefficients for all of the studied fungi indicated that the percentage of cells exhibiting H_2O_2 staining in the elongation zone was positively correlated ($r = 0.40$, $P = 0.044$) with the percentage of cells exhibiting Fe^{3+} staining and negatively correlated ($r = -0.41$, $P = 0.038$) with Fe^{2+} staining. A positive correlation ($r = 0.54$, $P = 0.005$) was also observed between the percentage of cells exhibiting H_2O_2 and Fe^{3+} staining in the meristematic zone of roots.

Stress tolerance to iron and ROS

All of the stress-inducing compounds utilized in the current study inhibited the growth of both ectomycorrhizal and pathogenic fungi (Fig. 1). Growth inhibition was highly dependent on the concentration of the tested compound ($P < 0.001$) (Fig. 1). The highest level of inhibition of the fungal pathogen species, *F. oxysporum* and *R. solani*, was observed on MEA medium containing either hydrogen peroxide or iron.

Table 1 Percentage of cells exhibiting H$_2$O$_2$ fluorescence staining in roots infected by fungal pathogens (*Rhizoctonia solani* and *Fusarium oxysporum*), ectomycorrhizal fungi– (*Hebeloma crustuliniforme* and *Laccaria bicolor*), and non-infected controls. Data are presented as the mean and SE (in brackets). Values with the same uppercase letter within each horizontal row or the same lowercase letter within each vertical column do not differ statistically from one another (Tukey's test, $P < 0.05$). ***$P < 0.001$, **$P \leq 0.01$, * $P \leq 0.05$, n. s. – not significant

variants	Elongation zone				Meristematic zone				P for the row
	H. crustuliniforme	*L. bicolor*	*F. oxysporum*	*R. solani*	*H. crustuliniforme*	*L. bicolor*	*F. oxysporum*	*R. solani*	
Control	5.0 (2.4) c	5.0 (2.4) d	5.0 (2.4) b	5.0 (2.4) b	6.1 (3.1) b	6.1 (3.1) b	6.1 (3.1) d	6.1 (3.1) b	n.s.
2 h	14.4 (3.1) bc	15.0 (3.9) cd	18.3 (3.2) ab	22.6 (3.1) a	13.9 (4.0) ab	11.1 (3.4) ab	9.4 (3.1) cd	18.9 (2.3) a	n.s.
6 h	13.3 (3.1) bc	21.7 (3.8) abc	10.6 (2.9) b	21.7 (3.5) a	12.2 (3.2) ab	11.1 (2.5) ab	15.0 (3.1) abcd	18.9 (4.8) ab	n.s.
24 h	22.2 (5.3) ab, AB	24.2 (3.9) abc, AB	10.0 (2.4) b, B	21.7 (4.3) a, AB	17.2 (5.1) ab, AB	13.9 (1.8) ab, AB	30.0 (5.2) ab, A	20.6 (3.8) a, AB	n.s.
48 h	31.1 (6.5) ab, A	19.2 (4.7) bcd, AB	6.1 (2.0) b, C	20.0 (2.8) a, AB	18.3 (2.9) ab, AB	19.2 (5.0) ab, AB	11.1 (2.9) bcd, B	22.8 (4.8) a, AB	P = 0.003
96 h	26.1 (4.7) ab, AB	39.2 (7.1) ab, A	11.7 (3.1) b, B	26.7 (3.9) a, AB	18.9 (6.2) ab, AB	18.9 (3.1) a, AB	20.0 (4.2) abc, AB	21.7 (4.9) a, AB	P = 0.019
10 d	37.8 (6.7) a, AB	46.4 (6.8) ab, A	35.0 (4.9) a, AB	18.9 (3.7) a, B	22.2 (4.2) a, AB	26.4 (4.28) a, AB	31.7 (6.1) a, AB	28.9 (6.4) a, AB	P = 0.022
P for the column	P < 0.001	P < 0.001	P < 0.001	P < 0.001	n.s.	P = 0.002	P < 0.001	P < 0.003	

Independent variable	F-value
2003Trophic strategy (TS)	1.10 n.s.
Fungal species (trophic stategy)	5.46**
Root zone (RZ)	6.37**
Harvest time (HT)	11.89***
TS × RZ	15.70***
TS × HT	2.37*
RZ × HT	0.75 n.s.
TS × RZ × HT	1.49 n.s.

Table 2 Percentage of cells exhibiting Fe^{3+} staining in roots infected by fungal pathogens (*Rhizoctonia solani* and *Fusarium oxysporum*), ectomycorrhizal fungi (*Hebeloma crustuliniforme* and *Laccaria bicolor*), and non-infected controls. Data are presented as the mean and SE (in brackets). Values with the same uppercase letter within each horizontal row or the same lowercase letter within each vertical column do not differ statistically from one another (Tukey's test, $P < 0.05$). ***$P < 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, n. s. – not significant

Variants	Elongation zone				Meristematic zone				P for the row
	H. crustuliniforme	L. bicolor	F. oxysporum	R. solani	H. crustuliniforme	L. bicolor	F. oxysporum	R. solani	
Control	5.0 (1.3)	5.0 (1.3) bc	5.0 (1.3) cd	5.0 (1.3) c	4.4 (1.8)	4.4 (1.8)	4.4 (1.8) c	4.4 (1.8) c	
2 h	9.4 (2.1) A	1.3 (0.7) c, B	4.1 (1.8) d, AB	5.0 (1.7) bc, AB	2.7 (1.1) AB	7.2 (2.1) AB	2.4 (1.2) c, AB	2.4 (0.8) c, AB	P = 0.026
6 h	8.3 (2.0)	11.4 (3.0) abc	6.4 (2.0) bcd	5.3 (1.2) bc	4.8 (1.8)	5.6 (1.8)	7.3 (2.6) bc	5.6 (1.8) bc	n.s.
24 h	8.6 (3.5)	9.4 (2.1) abc	8.0 (2.4) bcd	9.4 (1.6) abc	7.2 (2.7)	7.8 (2.5)	2.8 (1.2) c	9.4 (2.1) abc	n.s.
48 h	7.8 (2.2)	6.7 (2.4) bc	16.7 (4.4) abc	14.4 (2.7) ab	5.9 (2.2)	13.9 (2.9)	15.6 (2.9) b	12.8 (2.2) ab	P = 0.015
96 h	4.7 (1.9) CD	26.7 (6.4) a, A	19.4 (3.7) ab, AB	13.9 (1.8) ab, ABC	4.4 (2.6) D	13.3 (2.6) ABCD	8.9 (2.7) bc, BCD	13.9 (2.9) ab, ABCD	P < 0.001
10 d	13.0 (3.6) BCD	12.2 (2.7) ab, BCD	26.7 (4.9) a, AB	20.6 (6.4) a, BC	4.2 (1.2) D	16.7 (3.1) BCD	42.8 (5.5) a, A	15.0 (2.0) a, BCD	P < 0.001
P for the column	n.s.	P < 0.001	P < 0.001	P < 0.001	n.s.	P = 0.021	P < 0.001	P < 0.001	

Independent variable	F-value
Trophic strategy (TS)	13.55***
Fungal species (trophic stategy)	6.46**
Root zone (RZ)	5.18*
Harvest time (HT)	20.63***
TS × RZ	0.13 n.s.
TS × HT	6.13***
RZ × HT	1.43 n.s.
TS × RZ × HT	0.90 n.s.

Table 3 Percentage of cells exhibiting Fe^{2+} staining in roots infected by fungal pathogens (*Rhizoctonia solani* and *Fusarium oxysporum*), ectomycorrhizal fungi (*Hebeloma crustuliniforme* and *Laccaria bicolor*), and non-infected controls. Data are presented as the mean and SE (in brackets). Values with the same uppercase letter within each horizontal row or the same lowercase letter within each vertical column do not differ statistically from one another (Tukey's test, $P < 0.05$). ***$P < 0.001$, **$P \leq 0.01$, * $P \leq 0.05$, n. s. – not significant

Variants	Elongation zone				Meristematic zone				P for the row
	H. crustuliniforme	L. bicolor	F. oxysporum	R. solani	H. crustuliniforme	L. bicolor	F. oxysporum	R. solani	
Control	2.1 (1.2)	2.1 (1.2)	2.1 (1.2) b	2.1 (1.2) d	1.0 (0.6)	1.0 (0.6)	1.0 (0.6) d	1.0 (0.6) c	
2 h	3.2 (1.1) C	4.2 (1.9) C	39.4 (6.2) a, AB	56.1 (4.5) a, A	0.8 (0.6) C	3.9 (1.8) C	34.4 (5.1) bc, B	55.6 (4.6) a, AB	P < 0.001
6 h	3.2 (1.3) CD	3.8 (1.5) BCD	25.6 (4.5) a, A	13.7 (3.2) c, AB	3.0 (1.9) D	1.9 (1.2) D	20.0 (4.1) c, A	11.1 (1.9) b, ABC	P < 0.001
24 h	3.6 (1.8) C	2.0 (1.0) C	27.2 (2.7) a, B	13.7 (1.5) c, B	3.2 (1.9) C	3.1 (1.3) C	53.9 (3.6) ab, A	15.0 (3.7) b, B	P < 0.001
48 h	6.3 (2.1) C	2.1 (1.1) C	38.4 (4.0) a, A	22.9 (3.7) bc, AB	3.0 (1.3) C	3.0 (1.3) C	38.1 (3.4) bc, A	16.7 (2.2) b, B	P < 0.001
96 h	6.9 (2.9) C	1.7 (1.1) C	21.7 (2.6) a, A	22.3 (4.2) bc, AB	7.8 (2.7) BC	5.3 (2.2) C	36.2 (6.0) bc, A	42.0 (5.9) a, A	P < 0.001
10 d	2.6 (1.1) C	2.6 (1.4) C	38.3 (3.3) a, B	38.9 (4.8) ab, B	4.0 (1.2) C	2.2 (1.2) C	64.4 (6.3) a, A	42.6 (7.3) a, B	P < 0.001
P for the column	n.s.	n.s.	P < 0.001	P < 0.001	n.s.	n.s.	P < 0.001	P < 0.001	

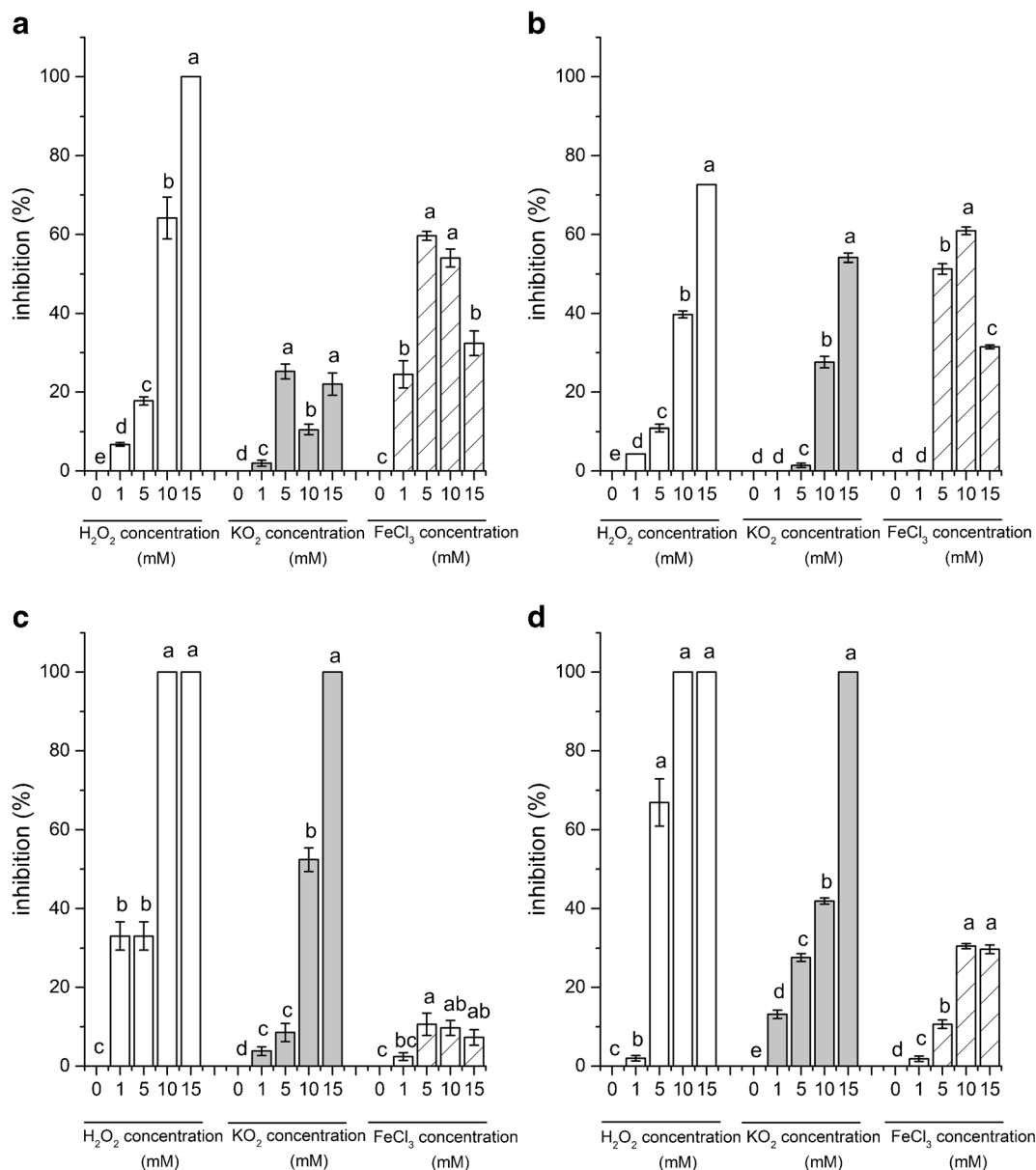| Independent variables	F-value
Trophic strategy (TS)	890.21***
Fungal species(trophic stategy)	9.84***
Root zone (RZ)	3.73 m.s.
Harvest time (HT)	11.99***
TS × RZ	5.20*
TS × HT	12.07***
RZ × HT	4.09**
TS × RZ × HT	0.94 n.s.

Fig. 1 Inhibition of *F. oxysporum* (**a**), *R. solani* (**b**), *H. crustuliniforme* (**c**), and *L. bicolor* (**d**) growth in vitro by hydrogen peroxide (H_2O_2), a ROS generating compound (KO_2), and iron ($FeCl_3$). Data represent the average percentage ± SE of growth inhibition relative to the control (growth in the absence of the tested compound) in the presence of increasing concentrations of hydrogen peroxide (white bars), superoxide anion radical (grey bars), and iron (hatched bars). Values marked with the same letter within one compound do not differ statistically (Tukey test, $P = 0.05$)

In contrast, growth of the ectomycorrhizal fungi, *H. crustuliniforme* and *L. bicolor*, was only weakly affected by increasing concentrations of iron, while increasing concentrations of hydrogen peroxide had an increasingly negative effect on growth. The negative effect of increasing concentrations of the stress-inducing compounds on growth was most readily observed in mycorrhizal fungi where lower concentrations of the inhibiting agent (1 mM or 5 mM) were required to obtain similar levels of inhibition observed with pathogenic fungi. *F. oxysporum* and *R. solani* were most strongly inhibited at 10 mM and 15 mM concentrations of hydrogen peroxide ($P < 0.001$).

The growth of *L. bicolor* and *H. crustuliniforme* was strongly inhibited at 5 mM hydrogen peroxide

($P < 0.001$). The growth of ectomycorrhizal fungi was completely inhibited at 10 mM hydrogen peroxide, while the same concentration inhibited the growth of the pathogenic fungi *F. oxysporum* by 64% and *R. solani* by 40%.

The reaction of the examined fungi to the presence of superoxide anion radicals was dependent on the species examined, and independent of the trophic strategy. The greatest level of growth inhibition among the necrotrophic fungi was observed in *R. solani*, while both species of ectomycorrhizal fungi were significantly inhibited.

Discussion

ROS are involved at the initial stage of a plant's response to necrotrophic pathogens and mycorrhizal fungus colonization. There is growing evidence that iron may also be one of the essential factors regulating plant-fungal interactions (Expert and O'Brian 2012; Johnson et al. 2007; Mucha et al. 2012, 2015). Whether the role of iron is linked to the role of ROS generation and whether a linkage of iron and ROS to host response is dependent on the trophic strategy of the invading fungus are still not well understood. In the present study, the pattern of iron accumulation in root cells of Scots pine (*Pinus sylvestris*) was strongly dependent on the trophic strategy of the invading fungus. The results also indicated that hydrogen peroxide was a stronger inhibitor of the growth of ectomycorrhizal growth in vitro than iron. In contrast, the in vitro growth of necrotrophic fungi was inhibited by both hydrogen peroxide and iron, indicating that the growth of trophically diverse fungi are differentially affected by stress-inducing compounds.

In the present study, fungi with contrasting trophic strategies all induced hydrogen peroxide accumulation in *P. sylvestris* roots. The pattern of accumulation of hydrogen peroxide, however, was dependent on species. More specifically, Scots pine roots infected with necrotrophic pathogens exhibit a different pattern of hydrogen peroxide accumulation than roots colonized by mycorrhizal fungi. ROS, including hydrogen peroxide, are induced in host tissues by invading fungi with different trophic strategies (Pellinen et al. 2002), but the function of the generated ROS could vary depending on the presence and concentration of other compounds. Notably, in the present study, iron was a significant factor that differentiated the response of necrotrophic vs. ectomycorrhizal strategy fungi. Given the redox potential of iron, hosts can utilize it to induce the production of hydroxyl radicals at the site of invasion that are very toxic to cells (Zwilling et al. 1999; Schaible and

Kaufmann 2004; Ong et al. 2006). Although previous studies have indicated that reactive oxygen species, including hydroxyl radicals, foster disease development by *R. solani* (Singh et al. 2011), the potential role of iron in the hydroxyl radical induction of disease development was not considered. Our present results revealed that an increase in the percentage of cells stained with H_2O_2 in root tissues infected with a necrotrophic fungus was also accompanied by an increase in the percentage of cells exhibiting Fe^{3+} staining. Fe^{3+} and Fe^{2+}, in the presence of hydrogen peroxide, can catalyze the formation of hydroxyl radicals through the Fenton/Haber-Weiss reaction (Pierre and Fontecave 1999) and induce cell death. Cell death is essential for necrotrophic fungi as they can only acquire nutrients from dead tissues (Govrin and Levine 2000). Cell death has been reported to facilitate fungal establishment in *P. sylvestris* roots during the initial stage of invasion (Adomas et al. 2008; Mucha et al. 2014). In contrast to necrotrophic pathogens, the observed correlation between lower levels of hydrogen peroxide and iron accumulation may facilitate the colonization of roots by ectomycorrhizal fungi, as they rely on living tissues to acquire carbon (Nehls et al. 2010). The establishment of ectomycorrhizal fungi, however, does not always require or induce cell death (Baptista et al. 2007; Ragnelli et al. 2013; Mucha et al. 2014); even though an accumulation of H_2O_2 occurs. In this scenario, hydrogen peroxide restricts fungal growth rather than promoting fungal proliferation by killing plant tissues. Notably, hydrogen peroxide limited the growth of mycorrhizal fungi in vitro more than iron.

The current study provides evidence that the role of ROS accumulation in root tissues in response to colonization by mycorrhizal fungi may be less dependent on the presence of iron and its role in increasing the vulnerability of plant cells to H_2O_2. The higher correlation between ROS accumulation in Scots pine root cells and colonization by mycorrhizal fungi than with necrotrophic pathogens suggests that the activation of other processes is involved in the specific response to H_2O; including the induction of specific genes that actively restrict fungal growth. ROS have been reported to be induced at the initial stage of the interaction between plant and ectomycorrhizal fungi (Passardi et al. 2005) and to be a necessary factor in the establishment and maintenance of plant endophytes (Tanaka et al. 2006). Since ROS are known to induce defense-related genes, the sequence of events associated with defense reactions may inhibit the invasion of ectomycorrhizal fungi into deeper tissue layers (Peterson et al. 2004). The premise

that hydrogen peroxide in host cells may prevent the further spread of ectomycorrhizal hyphae is supported by the fact that ectomycorrhizal fungi exhibit a greater sensitivity to hydrogen peroxide in vitro than necrotrophic fungi. Maintaining ROS levels may be an important parameter in establishing an equilibrium in mycorrhizal symbiosis (Rodriguez and Redman 2005). Therefore, the potential role of redox reactions and iron in the oxidative burst associated with plant root-ectomycorrhizal fungi interactions warrants further investigation.

Iron levels have been found to play an essential role in both fungal virulence and plant defense response (Dong et al. 2016; Expert and O'Brian 2012). The effect of iron and ROS production on fungal colonization has indicated that the role of iron is more important for the establishment of necrotrophic pathogens than ectomycorrhizal fungal species. Our present work provides evidence that iron dynamics play an important role in the interaction between root tissues of *P. sylvestris* and necrotrophic fungi. Iron accumulation was also shown to play an important role in determining the response of *P. sylvestris* roots to the pathogenic fungus, *F. oxysporum* (Ascomycota). The presence of available iron greatly enhances the virulence of *Ascomycetes*, possibly by enhancing their tolerance to H_2O_2 (Oide et al. 2006). Indeed, iron and other nutrients (e.g. Zn^{2+}, Mn^{2+}, Cu^{2+}) known as reductants in redox reactions have a deleterious effect on the establishment of *Fusarium* species in plants (Ruiz et al. 2015; Dong et al. 2016), while mycorrhizal fungi provide iron to plants (Adeleke et al. 2012). Our current results indicate that iron availability is highly associated with the vulnerability of plant roots to necrotrophic pathogens.

Mucha et al. (2014) previously reported that the response of Scots pine to trophically diverse fungi is dependent on the root zone that is being invaded and that necrotrophic fungi preferentially colonize the meristematic zone. The preferential colonization of the meristematic zone of roots by the necrotrophic fungus, *Fusarium oxysporum*, is also supported by studies reported by Rodriguez-Galvez and Mendgen (1995) and Asiegbu et al. (1999). This pattern of colonization agrees with our findings in the current study that greater increases in Fe^{3+} and Fe^{2+} occurred in the meristematic zone of *P. sylvestris* roots in the presence of necrotrophic fungi. The observation that iron, when combined with ROS, form hydroxyl radicals that lead to cell death; this supports the premise that necrotrophic pathogen obtain

nutrients after killing host tissues (Asiegbu et al. 1994; Mayer et al. 2001). Therefore, it is possible that disruption of cells in the meristematic zone of roots may be a mechanism that supports the invasion of host roots. The data obtained from the present study indicate that iron availability is highly correlated with the vulnerability of plant roots to necrotrophic pathogens.

Summary

The current study revealed that a significant increase in hydrogen peroxide in *P. sylvestris* root cortical cells infected with a pathogenic fungus is correlated with the accumulation of iron, Similarly, the ionic status of iron (Fe^{3+}, Fe^{2+}) is trophic specific and potentially determines the ability of fungi to establish an infection. Moreover, the pattern of accumulation of different forms of iron in different root zones may alter how the oxidative burst is induced, as well as its functional role. Increases in hydrogen peroxide in the elongation zone would limit the ability of a mycorrhizal fungus to spread into deeper root tissues (stele). In contrast to ectomycorrhizal fungi, necrotrophic fungi preferentially colonize tissues in the meristematic zone of roots (Rodriguez-Galvez and Mendgen 1995; Mucha et al. 2014; Asiegbu et al. 1999; Mayer et al. 2001) to kill roots and make nutrients readily available. The current study demonstrated the accumulation of ferrous iron in the meristematic zone of root cells, especially in the presence of *F. oxysporum*. Changes in the distribution of different forms of iron and hydrogen peroxide are likely to impact the initial response of host tissues to necrotrophs vs. mycorrhizal fungi. Thus, strategies have evolved for maintaining or altering the balance between iron demand for metabolic processes, and its role in exacerbating ROS-induced cell death; and potentially determine the interactions of plant tissues with necrotrophic and mycorrhizal fungi.

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Compliance with ethical standards

Conflict of interest The authors state that there are no conflicts of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

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