Strategies utilized by trophically diverse fungal species for *Pinus sylvestris* root colonization

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Physiological changes in host plants in response to the broad spectrum of fungal modes of infection are still not well understood. The current study was conducted to better understand the infection of in vitro cultures of *Pinus sylvestris* L. seedlings by three trophically diverse fungal species, *Fusarium oxysporum* E. F. Sm. & Swingle, *Trichoderma harzianum* Rifai and *Hebeloma crustuliniforme* (Bull.) Quél. Biochemical methods and microscopy were utilized to determine (i) which factors (apoplastic and cellular pH, reactive oxygen species, glutathione and cell death) play a role in the establishment of pathogenic, saprotrophic and mycorrhizal fungi, and (ii) whether cell death is a common response of conifer seedling tissues when they are exposed to trophically diverse fungi. Establishment of the pathogen, *F. oxysporum*, was observed more frequently in the meristematic region of root tips than in the elongation zone, which was in contrast to *T. harzianum* and *H. crustuliniforme*. Ectomycorrhizal (ECM) hyphae, however, were occasionally observed in the studied root zone and caused small changes in the studied factors. Colonization of the meristematic zone occurred due to host cell death. Independently of the zone, changes in cellular pH resulting in an acidic cytoplasm conditioned the establishment of *F. oxysporum*. Additionally, cell death was negatively correlated with hydrogen peroxide (H₂O₂) in roots challenged by a pathogenic fungus. Cell death was the only factor uniquely associated with the colonization of host roots by a saprotrophic fungus. The mechanism may differ, however, between the zones since apoplastic pH was negatively correlated with cell death in the elongation zone, whereas in the meristematic zone, none of the studied factors explained cell death. Colonization by the ECM fungus, *H. crustuliniforme*, was associated with a decreasing number of cells with acidic apoplast and by production of H₂O₂ in the elongation zone resulting in cell death. Saprotrophic and ECM fungi had a greater effect on cell acidification in the meristematic zone than the pathogenic fungus.

**Keywords**: colonization, conifer, fungal trophic strategy, host cell death, pH, reactive oxygen species.

Introduction

Conifer trees are crucial components of boreal and temperate forests and have great impact on ecosystem function (Bonello et al. 2006). Similar to other plants, conifers are constantly challenged by various microorganisms (Pearce 1996) and, in turn, these interactions impact plant ecology and evolution (Bardgett et al. 2005). Parasitic and symbiotic associations between plants and microorganisms are two ultimate outcomes of a continuum of interactions affecting plant productivity. Saprotrophs are relevant in litter decomposition and nutrient cycling in forest ecosystems and are not thought to directly interact with living tissues of forest trees (Boddy 1999). Nevertheless, research has been published demonstrating that fungi characterized by these widely different trophic strategies may functionally overlap (Arnold 2007, Vasaitis et al. 2007). Thus, the interactions of microorganisms that exhibit different trophic relationships with their plant hosts imply a highly dynamic plant response. Accordingly, the distinction between pathogenic and non-pathogenic microorganisms is crucial for understanding plant response (Asiegbu et al. 1999, Hahlbrock et al. 2003).
Cellular pH exerts a significant effect on biological processes such as nutrient uptake, cell growth and plant–microbe interactions (Feijó et al. 1999, Felle 2001, Michard et al. 2008). The plant cell apoplast undergoes alkalization in response to the presence of both pathogens and symbiotic organisms. The response of the cytosol, however, varies, becoming alkalinized in the presence of a symbiotic organism but acidic in response to pathogens (Felle 1996, 1998, 2001). Pathogens can also alter the local pH at the infection site to support the increased expression of pathogenicity factors and enzymes associated with pathogenicity (Denison 2000, Yakoby et al. 2000, Prusky et al. 2001, Eshel et al. 2002, Prusky and Yakoby 2003, Felle et al. 2004). Significant differences in apoplastic H\(^+\) concentration force cross-membrane transport of nutrients into pathogenic fungi (Hahn and Mendgen 2001). Modulation in pH may also activate the production of reactive oxygen species (ROS) in apoplast (Bolwell et al. 2002) or result in an oxidative burst, which is typical for defense reactions (Sakano 2001).

An oxidative burst is considered an integral aspect of the defense response (Wojtaszek 1997) and has been shown to occur in response to the presence of an avirulent isolate of a biotrophic microorganism (Lu and Higgins 1999), as well as a necrotroph (Govrin and Levine 2000). There are still few data, however, on the involvement of ROS accumulation during mycorrhizal establishment in ectomycorrhizal (ECM) associations. Baptista et al. (2007) reported three peaks of hydrogen peroxide (H\(_2\)O\(_2\)) production during the early stages of the symbiotic interaction between Castanea sativa Mill. and Pisolithus tinctorius (Mont.) E. Fisch. The pattern of ROS production was similar to that occurring during pathogen infection, but mycorrhizal colonization did not evoke cell death (Baptista et al. 2007). In particular, H\(_2\)O\(_2\) has been suggested to trigger cell death in some systems (Tenhaken et al. 1995). Some data, however, do not seem to support the role of ROS in cell death. For example, ROS production is not important in the death of cultured parsley cells invaded by Phytophthora infestans (Mont.) de Bary (Naton et al. 1996). Cell death is effective against biotrophic pathogens, because it results in nutrient deprivation (Greenberg and Yao 2004); however, it is not effective against necrotrophs (Asieigbu et al. 1994, Mayer et al. 2001). There is growing evidence that cell death may not be tightly linked to disease resistance (Heath 1998). Relatively small increases in ROS production may be removed by cell antioxidants, thus reestablishing redox homeostasis. This delicate redox balance is easily disturbed under growth-limiting environmental conditions, potentially leading to significant ROS accumulation (Van Breusegem and Dat 2006).

Several studies have concentrated on plant responses to mutualistic (Johansson et al. 2004, Manthey et al. 2004) or pathogenic (Bar-Or et al. 2005) colonization, but few have compared responses in the same plant (Mohr et al. 1998, Guimil et al. 2005, Weerasinghe et al. 2005, Adomas et al. 2008). Additionally, only a few studies have been reported on saprotrophic colonization of plants (Asieg bu et al. 1999, Adomas et al. 2008, Sun et al. 2011). The present study investigated the response of conifer tissues challenged with trophically diverse fungal species. The objective was to compare pH modulation, changes in ROS activation, glutathione, and, eventually host cell death in root cells of Pinus sylvestris L. challenged by a pathogenic, a saprotrophic and an ECM fungus. Pinus sylvestris was used as a model plant to determine whether or not cell death is a common host response to challenge by a wide range of fungi. Additionally, Czmymek et al. (2007) reported that initial colonization and penetration of Arabidopsis roots by Fusarium oxysporum E. F. Sm. & Swingle occurred primarily in the meristematic zone of tap root tips and emerging lateral roots. Root tips are comprised of cells in very different states within a very short distance, including meristematic and elongating cells, and cells undergoing various kinds of differentiation (Scheres et al. 2002). Root tips are also a zone of active ROS production (Liszsky et al. 2004). Therefore, the precise determination of the physiological changes occurring in the various parts of the root tip will provide information to better understand the interaction between P. sylvestris and fungi representing different trophic strategies.

**Materials and methods**

**Fungal isolates and plant infection assays**

Three isolates of fungi, characterized by different types of trophic interactions with Scots pine, P. sylvestris, were used. Fusarium oxysporum is known for causing host cell death and disease in gymnosperms and angiosperms. Hefeloma crustuliniforme (Bull.) Quél. develops mycorrhizal symbioses with different hosts, including P. sylvestris. Trichoderma harzianum Rifai is a common soil and litter fungus. Fungal isolates were maintained on malt extract agar (Difco, Detroit, MI, USA) at 24 °C and stored at 4 °C. The fungal isolates (pathogen and mycorrhizal fungi) were also cyclically inoculated with the host. All fungal isolates were obtained from the collection of the Laboratory of Root System Pathology, Institute of Dendrology, Kórnik, Poland. Seeds of P. sylvestris used in the study originally came from the provenance of Bolewice (52°28′N and 16°03′E).

Seeds of P. sylvestris were germinated on 0.6% water agar (w/v; Difco) in the dark after surface sterilization (3 min in 0.2% HgCl\(_2\) (Polish Chemical Reagents, Gliwice, Poland), followed by washing three times for 5 min in sterile distilled water). Afterwards, five seeds, for each time point used in the study, were placed on Hoagland medium (Sigma, St Louis, MO, USA) in 14 cm diameter Petri dishes covered with a filter paper and incubated for 1 week in an environmental chamber under light emitted by fluorescent tubes (Osram L36/W77 Flora; 100 µEm\(^{-2}\) s\(^{-1}\) 16 h a day, 60% relative humidity at 24 ± 2 °C day to night temperature ratio. Half of Hoagland’s medium was
removed from each Petri dish in order to make a space for the aerial portions of the developing seedlings. The other half of the medium in the Petri dish was covered with sterile filter paper to allow for sufficient nutrient supply but to prevent roots from growing into medium and ensure immediate contact with fungal inoculum once it was applied. One week later, the filter paper, now covered with fungal inocula of each respective fungus, was placed over entire *P. sylvestris* roots (as illustrated in Figure S1 available as Supplementary Data at *Tree Physiology* Online). Control plants consisted of seedlings of the same age treated in the same way but without fungi. Roots were subjected to analysis after 2, 4, 6, 12, 24, 48, 72 h and 10 days of incubation.

**Colonization of host tissues**

Penetration efficiency of each fungus was estimated using harvested pieces of *P. sylvestris* seedling roots. Roots were fixed in 4% paraformaldehyde and then dehydrated in an ascending alcohol series and embedded in paraffin (Sigma). Sections (8 μm), made with a microtome (Microm, Carl Zeiss, Jena, Germany), were placed on glass microscope slides. After depa- raffinization in xylene and incubation in an ethanol series, root sections were stained in 0.05% of toluidine blue O (Sigma) in 1% tetraborate buffer at pH 4.4. Sections were then dehydrated and mounted in Entellan (Merck, Darmstadt, Germany) using a cover slip. Observations were made with a Carl Zeiss Axioscope 20. The percentage of *P. sylvestris* root cells colonized by fungus relative to all observed cells determined the penetration efficiency or cells surrounded by fungal hyphae.

**pH measurement**

Roots of *P. sylvestris* seedlings were submerged in 8 μM SNARF-1 (5-(and-6)-chloromethyl SNARF-1 acetate; Molecular Probes, Carlsbad, CA, USA) after incubation with fungal isolates for each of the designated time points. The SNARF-1 solution was prepared as described by Diéguez-Uribondo et al. (2008), using 0.02% Pluronic F-127 dispersion agent (Molecular Probes). After incubation for 45 min at 21 °C in 10 mM of 2-(N-morpholino)ethanesulfonic acid (MES) buffer, roots were washed twice in distilled water and observed under a confocal microscope (TCS SP5 II Leica, Wetzlar, Germany) using an argon laser at 488 nm for excitation and emission channel sensors at 500–550 nm.

The intracellular pH was determined by measuring the fluorescence of BCECF (Sakano et al. 1992) at the excitation wavelength of 480 nm and an emission wavelength was 525 nm. Fluorescence images were analysed using a confocal microscope (Leica TCS SP5 II). Since BCECF fluorescence decreases linearly with a decreasing pH, we were able to use this method to monitor relative pH values and the acidification of the cytosol.

Acidification of host cells during the interaction with the different fungi was monitored by carboxy 5-(and-6)-carboxy-2',7'-dichlorofluorescein (DCFDA; Invitrogen), which passively diffuses into the cells and, once cleaved of acetate group, works as a fluorescent pH sensor. The stock solution was prepared by dissolving DCFDA-AM in DMSO to a concentration of 10 mM. Roots were immersed in 10 μM carboxy DCFDA in 10 mM MES buffer (pH 5.0) for 1 h at 20 °C. After incubation, roots were washed twice in 10 mM MES buffer for 20 min each time and observed under a confocal microscope (Leica TCS SP5 II) using an argon laser at 488 nm for excitation and emission channel sensors at 500–550 nm.

**Quantitative estimation of ROS**

**Superoxide radical** The amount of O$_2^-$ was detected according to Doke (1983) using a colorimetric procedure based on the reduction of nitrotetrazolium blue (NBT) (Sigma). Root fragments were placed in 3 ml of a reaction mixture consisting of 0.05 M potassium phosphate buffer (pH 7.8), 0.05% of NBT and 10 mM Na$_3$N$_3$, and incubated in the dark for 30 min. Subsequently, 2 ml of solution was heated in a water bath at 85 °C for 15 min. After cooling, absorbance was measured at 580 nm with a Shimadzu UV-2501PC spectrophotometer (Shimadzu, Nakagyo-ku, Kyoto, Japan). The amount of O$_2^-$ was expressed as ΔA per root fresh weight (g).

**Hydrogen peroxide radical** Root fragments harvested at different times post-inoculation were ground into powder with liquid nitrogen using a mortar and pestle, and then homogenized in 5 ml of 5% trichloroacetic acid (TCA) and centrifuged (26,000 g) at 4 °C for 20 min. The resulting supernatant was used for the analysis. The amount of H$_2$O$_2$ was determined following the procedure described by Sagisaka (1976). The reaction mixture consisted of 0.5 ml of supernatant, 1.5 ml of 50% TCA, 0.4 ml of 10 mM iron-ammonium sulfate and 0.2 ml of 2.5 M potassium thiocyanate. The reaction mixture containing 0.5 ml of 5% TCA instead of supernatant was used as the control. Absorbance was measured at 480 nm with a Shimadzu UV-2501PC spectrophotometer. The amount of H$_2$O$_2$ was defined as micrograms of H$_2$O$_2$ per root fresh weight (g).

**Measurement of intra- and extracellular oxidative activity**

Roots challenged with different fungi were incubated for 20 min in 20 μM 2,7-dichlorofluorescein diacetate (HDCF-DA; Sigma; 20 mM stock in DMSO). Roots were subsequently
imaged for not more than 5 min with a Leica TCS SP5 II confocal microscope using the 488-nm line of the argon laser. Extracellular release of ROS was determined by using the fluorogenic reagent OxyBURST green H2HFF (dihydro-2,4,5,6,7-hexafluorofluorescein)-BSA (Invitrogen). Roots were incubated in a solution of 100 µg ml⁻¹ OxyBURST and fluorescence was recorded using a Leica TCS SP5 II microscope with the same imaging parameters as described for HDCF-DA.

Glutathione assays

Reduced and oxidized forms of glutathione were estimated following the procedure described by Smith (1985). Roots of P. sylvestris challenged with different fungi were homogenized in 5% (w/v) sulfosalicylic acid over an ice bath. Samples were then centrifuged at 10,000 g for 20 min. Afterwards, 1 ml of supernatant was mixed with 1.5 ml of 0.5 M K-phosphate buffer at pH 7.5 to neutralize and evaluate total glutathione (reduced form (GSH) + oxidized form (GSSG)). To determine the amount of GSSG, 1 ml of neutralized supernatant was treated with 0.2 ml of 2-vinylpyridine for 1.5 h at 25 °C. Samples used to determine both total and oxidized forms of glutathione were then twice extracted with 5 ml of diethylether. The assay solution was prepared by mixing 0.5 ml of 0.1 M sodium phosphate buffer (at pH 7.5) with additional 5 mM EDTA, 0.2 ml of 6 mM 5,5’-dithiobis-(2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH, 0.1 ml (1 unit) of glutathione reductase type III (Sigma-Aldrich) and 0.1 ml of extract. The change in absorbance was monitored at 412 nm at 25 °C with a UV-2501 PC spectrophotometer (Shimadzu). A GSH standard (Sigma-Aldrich) was used to prepare a standard curve.

Cell death

Nuclear DNA fragmentation

To examine DNA fragmentation in situ, a TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed using an in situ cell death detection kit (Fluorescein; Roche Applied Science, Penzberg, Germany) according to the instruction manual. Root segments were fixed in 4% paraformaldehyde in 10 mM phosphate-buff-
ed saline (PBS) and then dehydrated in an ethanol series. The speed of colonization degree was similar for the patho-
genic fungus, F. oxysporum, and the saprotroph, T. harzianum, being discernible within 4 h after inoculation (Table 1). In con-
trast, colonization of roots by the ECM fungus, H. crustulini-
forme, was much slower and began to be discernible 24 h after inoculation. The studied fungi also exhibited specific patterns of establishment in different root zones. The pathogenic fungus was observed more frequently in the apical, meristematic portion of the root at 4 h (P < 0.01) and 6 h (P < 0.01) compared with the zone of cell elongation. In contrast, the sapro-
trophic and ECM fungi colonized the elongation zone of host roots more frequently than the root meristematic apex. Significant differences in colonization efficiency were detected in the preferred root zone at 4 h (P < 0.01) and 10 days (P < 0.05) for T. harzianum and at 10 days (P < 0.001) for H. crustuliforme.

Evans blue methods

Cell death, which is defined as loss of integrity of the plasma membrane, was determined using the Evans blue method (Baker and Mock 1994). Roots of P. sylves-
tris seedlings that had been challenged by the different fungi were submerged in 0.25% (w/v) Evans blue solution (Sigma) for 20 min. After washing twice with distilled water, the roots were observed under an Axioskop 20 microscope (Carl Zeiss). The percentage of dead cells was calculated as the number of stained cortical cells taken from one optical layer divided by the number of all cells from the same optical layer × 100. Thirty cells from one optical layer were inspected for each root piece and the average of five root pieces from different seedlings was reported for each time point.

Statistical analysis

To quantify pH estimation and colonization efficiency, the percentage of cells stained with the respective analytical dye was calculated as the number of stained cortical cells taken from one optical layer divided by the number of all cells from the same optical layer × 100.

The percentages of colonized cells, cells expressing acid or alkaline pH, and dead cells were transformed before analysis according to the C.I. Bliss equation (arcsin square root) to ensure normality of distribution and equal variance. A one-way analysis of variance (ANOVA) was used, followed by a Tukey’s test, to compare the colonization efficiency of the different fungi. Regression analysis was used to examine the effects of apoplast and cellular pH (alkaline and acidic), O₂⁻, H₂O₂, glutathione contents (GSSG + GSH), oxidized (GSSG) and reduced form of glutathione (GSH) on colonization degree and cell death. Pearson’s coefficient was applied to indicate a correlation between the studied variables. All statistical analyses were conducted using Statistica version 8.0 (StatSoft, Tulsa, OK, USA).
An increase in the number of *P. sylvestris* root apical cells with alkaline apoplasts was observed only in the presence of the pathogenic and saprotrophic fungi after 24 and 48 h, respectively (Figure 1a). The host root elongation zone exhibited a slightly different response with the highest percentage of cells with an alkaline apoplast being observed 12 h after inoculation of roots with all three fungi (Figure 1b). This did not

**pH dynamics**

An increase in the number of *P. sylvestris* root apical cells with alkaline apoplasts was observed only in the presence of the pathogenic and saprotrophic fungi after 24 and 48 h,
appear, however, to be a factor influencing colonization or cell death (Figure 2a and b). In contrast, acidification of the apoplast of cells explained 61.4% of the variation in the level of host colonization of the elongation zone by the ECM fungus (Table 2). The regression equation describing the relationship is \( y = 14.71 - 0.78 \times \) percentage of cells with acidic pH (Figure 2c). The number of cells characterized by acidic apoplasts in the elongation zone of roots inoculated with the ECM fungus gradually decreased from the early stage of interaction and was positively correlated with levels of the reduced form of glutathione (see Table S6 available as Supplementary Data at *Tree Physiology* Online). The percentage of cells with an acidic apoplast was also correlated with the level of cell death in the elongation zone of root inoculated with *T. harzianum* (Table 3). The best fit regression equation obtained was \( y = 23.21 - 0.82 \times \) percentage of cells with acidic pH (Figure 2d). During the interaction of *P. sylvestris* roots with the saprotroph, the number of cells with acidic apoplast was negatively correlated with levels of \( \text{H}_2\text{O}_2 \) but positively correlated with levels of GSH in the meristematic zone (see Table S3 available as Supplementary Data at *Tree Physiology* Online). In the elongation zone, however, the number of cells with acidic apoplast was positively correlated with the number of cells with acidic cytoplasm versus alkaline pH (see Table S4 available as Supplementary Data at *Tree Physiology* Online).

There were statistical differences between the percentage of cells with alkaline cellular pH in the two studied zones (2.00 ± 1.24 and 0.67 ± 0.67 in meristematic and elongation zones, respectively; mean ± SE) of roots from control, uninoculated roots. After 2 h, a greater percentage of cells with acidic cytoplasm were observed in the meristematic zone than in the elongation zone of roots inoculated with *F. oxysporum* and *T. harzianum* (Figures 2e and 3a). Exposure of roots to the ECM fungus caused a minor increase in the number of alkaline cells after 4, 12 h and 10 days. Pathogenic and saprotrophic fungi also induced a modest alkalization of cells in the elongation zone in early stages of the interaction (2 and 4 h) (Figure 2f). Conversely, *H. crustuliniforme* induced cellular alkalization in a higher number of root cells in the elongation zone in the early stage of the interaction (Figure 3b).

The two root zones (meristematic and elongation) in control roots had comparable numbers of cells with acidic cytoplasm pH (5.19 ± 2.81 and 0.67 ± 0.67 in the meristematic and elongation zones, respectively; mean ± SE) (Figure 3c and d). The pathogenic fungus *F. oxysporum* induced a minor acidification of the cytoplasm of plant cells in the meristematic zone compared with the saprotrophic and ECM fungi. The pathogenic fungus increased acidification of cells in the meristematic zone 6 and 12 h after inoculation (Figure 3c) and the number of acidified cells explained 55.6% of the variance (Table 2) in colonization by *F. oxysporum* \((y = 64.19 - 0.75 \times \) percentage of cells with acidic cytoplasm). In the elongation zone, saprotrophic and pathogenic fungi caused early acidification (2-h post-inoculation) (Figure 2g). At the later stages of the interaction, cytoplasmic pH was not affected by the pathogen,
while saprotrophic and ECM fungus increase the number of cells with alkaline cytoplasm pH in the elongation zone at 48 h after inoculation (Figure 2h). Acidification of the cell cytoplasm significantly accounted for the variation in the level of colonization degree ($R^2 = 0.678$, $y = 68.32 - 0.82 \times$ percentage of cells with acidic cytoplasm) and cell death ($R^2 = 0.545$, $y = 34.55 - 0.74 \times$ percentage of cells with acidic cytoplasm) only in roots infected by F. oxysporum (Tables 2 and 3). The percentage of cells with acidic cytoplasm was also positively correlated with the oxidized form of glutathione in the meristematic zone of P. sylvestris roots inoculated with F. oxysporum (see Table S1 available as Supplementary Data at Tree Physiology Online).

**Table 2.** Regression analysis with colonization degree as a dependent variable and the following studied features as independent variables: apoplast pH (alkaline and acidic), cellular pH (alkaline and acidic), $O_2$, $H_2O_2$, oxidized (GSSG) and reduced form of glutathione (GSH), redox status (represented by GSH : GSSG ratio) and cell death in P. sylvestris root exposed to F. oxysporum, T. harzianum and H. crustuliniforme. Values of $R^2$ are significant (bold type) at: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. ns, not significant.

<table>
<thead>
<tr>
<th>Species</th>
<th>Zone</th>
<th>Apoplast pH (alkaline)</th>
<th>Apoplast pH (acidic)</th>
<th>Cellular pH (alkaline)</th>
<th>Cellular pH (acidic)</th>
<th>$O_2^-$</th>
<th>$H_2O_2$</th>
<th>GSSG</th>
<th>GSH</th>
<th>Redox status</th>
<th>Cell death</th>
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<td>F. oxysporum</td>
<td>Apical</td>
<td>0.068 ns</td>
<td>0.347 ns</td>
<td>0.238 ns</td>
<td>0.556**</td>
<td>0.262 ns</td>
<td>0.364 ns</td>
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<td>0.508*</td>
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<td>0.678*</td>
<td>0.126 ns</td>
<td>0.533*</td>
<td>0.116 ns</td>
<td>0.133 ns</td>
<td>0.041 ns</td>
<td>0.822**</td>
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<td>0.210 ns</td>
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<td>0.224 ns</td>
<td>0.335 ns</td>
<td>0.270 ns</td>
<td>0.714**</td>
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**Table 3.** Regression analysis with cell death as a dependent variable and the following studied features as independent variables: apoplast pH (alkaline and acidic), cellular pH (alkaline and acidic), $O_2$, $H_2O_2$, oxidized (GSSG) and reduced form of glutathione (GSH), redox status (represented by GSH : GSSG ratio) in P. sylvestris root exposed to F. oxysporum, T. harzianum and H. crustuliniforme. Values of $R^2$ are significant (bold type) at: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. ns, not significant.

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<th>GSH</th>
<th>Redox status</th>
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<td>H. crustuliniforme</td>
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<td>0.107 ns</td>
<td>0.890 ns</td>
<td>0.084 ns</td>
</tr>
<tr>
<td></td>
<td>Elongation</td>
<td>0.032 ns</td>
<td>0.264 ns</td>
<td>0.002 ns</td>
<td>0.089 ns</td>
<td>0.006 ns</td>
<td>0.900***</td>
<td>0.224 ns</td>
<td>0.335 ns</td>
<td>0.169 ns</td>
</tr>
</tbody>
</table>

while saprotrophic and ECM fungus increase the number of cells with alkaline cytoplasm pH in the elongation zone at 48 h after inoculation (Figure 2h). Acidification of the cell cytoplasm significantly accounted for the variation in the level of colonization degree ($R^2 = 0.678$, $y = 68.32 - 0.82 \times$ percentage of cells with acidic cytoplasm) and cell death ($R^2 = 0.545$, $y = 34.55 - 0.74 \times$ percentage of cells with acidic cytoplasm) only in roots infected by F. oxysporum (Tables 2 and 3). The percentage of cells with acidic cytoplasm was also positively correlated with the oxidized form of glutathione in the meristematic zone of P. sylvestris roots inoculated with F. oxysporum (see Table S1 available as Supplementary Data at Tree Physiology Online).

**Reactive oxygen species production**

Results indicate that the pattern of $O_2^-$ changes in the two root zones was different when they were inoculated with fungi with different trophic strategies (Figure 4a and b). The meristematic zone of roots exhibited similar levels of $O_2^-$ accumulation in response to F. oxysporum and T. harzianum but lower levels in response to H. crustuliniforme compared with the levels induced by the other two fungi. An exception was at 48 h when inoculation with H. crustuliniforme induced a significant increase in $O_2^-$ (Figure 4a). The elongation zone of roots, however, exhibited a greater accumulation of $O_2^-$ in response to F. oxysporum and T. harzianum compared with the meristematic zone (Figure 4B).

Accumulation of $H_2O_2$ in the presence of the pathogenic and saprotrophic fungi was the highest in both the meristematic and elongation root zones at 2, 4 and 6 h after inoculation (Figure 4c and d). Differences in $H_2O_2$ accumulation induced in the meristematic zone by the pathogen and mycorrhizal fungi compared with the saprotrophic fungus were also observed after 10 days. The pathogen exhibited a reduced level of $H_2O_2$ in the elongation zone at 10 days compared with the mycorrhizal and saprotrophic fungi. The amount of $H_2O_2$ was a significant variable explaining the level of colonization of the elongation zone by H. crustuliniforme and F. oxysporum but had an opposite influence. The regression equation for colonization by F. oxysporum was $R^2 = 0.533$, $y = 83.61 - 0.73 \times H_2O_2$, while the equation for H. crustuliniforme was $R^2 = 0.613$, $y = -3.63 + 0.78 \times H_2O_2$ (Table 2). Moreover, the level of $H_2O_2$ was also a factor explaining cell death in the elongation zone induced by F. oxysporum ($R^2 = 0.775$, $y = 45.31 - 0.88 \times H_2O_2$) and H. crustuliniforme ($R^2 = 0.900$, $y = 2.59 + 0.95 \times H_2O_2$) and cell death in the meristematic zone induced by F. oxysporum ($R^2 = 0.564$, $y = 54.62 - 0.75 \times H_2O_2$) (Table 3). The amount of $H_2O_2$ was also positively correlated with GSH levels in the meristematic zone of roots inoculated with F. oxysporum (see Table S1 available as Supplementary Data at Tree Physiology Online).
To determine the level of ROS in the apoplast, the OxyBURST reagent was used since the intensity of the reaction depends on the amount of ROS but not on other factors such as pH. A low reaction was observed in the apoplast of root cells inoculated with the pathogenic fungus and an even lower level reaction was seen in roots inoculated with the ECM fungus. No induction of apoplastic ROS was observed in roots inoculated with the saprotrophic fungus.

**Glutathione dynamics**

The pathogenic fungus, *F. oxysporum*, induced twice the level of glutathione accumulation and high redox potential in the meristematic zone of roots compared with the control, uninoculated roots at 2 h after inoculation. At later stages (24, 48, 72 h and 10 days) the glutathione levels dropped and were much lower (Figure 5). In contrast, a higher accumulation of glutathione and redox potential was observed, relative to the control roots, in the elongation zone of roots after inoculation with the pathogenic fungus. Completely different host responses were observed after roots were inoculated with the saprotrophic fungus *T. harzianum*. A low glutathione concentration was observed in the meristematic zone compared with the concentration in the zone of elongation. A significant level of the reduced form of glutathione was observed in roots inoculated with *T. harzianum* after 4, 6, 12 and 48 h, with an extremely low concentration at 24 h. Redox status was also statistically significant at all the time intervals except for 12 h. The ECM fungus, *H. crustuliniforme*, induced fewer changes in the meristematic zone compared with the elongation zone during the course of the experiment. Differences in the ratio of the oxidized and reduced forms of glutathione, however, were evident in the meristematic zone after 4 h and 10 days and after 6 h in the zone of elongation. Glutathione concentration was positively correlated with the level of colonization of the apical zone of roots exposed to *F. oxysporum* (Table 2).

**Cell death**

The meristematic zone of *P. sylvestris* roots responded more strongly than the elongation zone to the presence of all fungi and cell death was more frequently observed in this zone than in
the elongation zone (Figure 6). The highest percentage of dead cells was observed in roots inoculated with *F. oxysporum* and the smallest amount of cell death was observed in roots inoculated with *H. crustuliniforme*. Roots inoculated with *T. harzianum* exhibited intermediate levels of cell death. Surprisingly, the two root zones did not react differently when inoculated with *F. oxysporum*. Inoculation of the roots with the pathogenic fungus resulted in the death of a few cells in the meristematic and elongation zones during the early stages of the interaction (2–6 h) (Figure 6). An increasing percentage of dead cells was observed, however, at 12 h and the later time points. The level of cell death in roots inoculated with the saprotrophic fungus was similar to the response to the pathogenic fungus in the meristematic zone, though an increase in the percentage of dead cells was observed at 48 h. In contrast, inoculation of roots with *T. harzianum* resulted in a response similar to that observed for the ECM fungus. Cell death was the most significant factor explaining the degree of root colonization by all of the studied fungi and accounted for 71.4–93% of the variance (Table 2).

**Figure 4.** The level of superoxide (*O*₂⁻) in meristematic (a) and elongation (b) zones of roots and *H₂O₂* in meristematic (c) and elongation (d) zones of *P. sylvestris* root at different time points following inoculation with either *F. oxysporum* (open circles), *T. harzianum* (open squares) or *H. crustuliniforme* (open rhombuses), and in control (solid lines). Data represent the mean ± SE.

**Discussion**

The successful establishment of a necrotrophic pathogen in host tissues is dependent on the destruction of host cells. This is in contrast with an ECM fungus, where success is defined by the establishment of a stable and mutually beneficial relationship with the host. The physiological and biochemical responses of conifer roots exposed to fungi exhibiting different trophic strategies are still not well understood. The comparison of events occurring in *P. sylvestris* roots in response to inoculation with *F. oxysporum*, *T. harzianum* and *H. crustuliniforme* confirmed the involvement of pH, ROS and glutathione in host cell death and, subsequently, success in colonization of host tissues.

Determining the entry points of pathogens, as well as mycorrhizal fungi, into roots is important, since it may reveal whether different plant host reactions in tissues or cells influence colonization (Felle et al. 2009) or if any root regions can be infected. *Fusarium oxysporum* preferentially colonized meristematic zone of roots, which is in agreement with the previous finding of Rodriguez-Gálvez and Mendgen (1995). In contrast, saprotrophic
and mycorrhizal fungi preferentially colonized the elongation zone. Colonization of the meristematic zone could imply possible active disruption of intracellular activity in response to a necrotrophic pathogen. This phenomenon has been widely reported in plant hosts challenged with a necrotrophic pathogen, which obtains nutrients after killing host tissues (Asiegbu et al. 1994, Mayer et al. 2001). The level of colonization, however, was strongly correlated with host cell death regardless of which type of fungi was used to inoculate the roots. Asiegbu et al. (1999) reported that colonization of *P. sylvestris* was associated with necrosis when the plant host was exposed to a necrotrophic and some saprotrophic fungi, as well as to the mycorrhiza-associated bacterium *Pseudomonas fluorescens*. Saprotrophs are not expected to be harmful for the plant root through their behaviour as endophytic fungi (Evans et al. 2003). *Trichoderma* species have been shown to protect plants from pathogens rather than cause injury (Yedidia et al. 2003). The strong host response to *T. harzianum* observed in our study, characterized by the fast accumulation of $\text{H}_2\text{O}_2$, which was high at all time points of the experiment, and the high rate of mortality in the meristematic zone, may have been due to the density of the inoculums utilized. This was also suggested in the study of *Phlebiopsis gigantea* (Fr.) Jülich by Asiegbu et al. (1999). *Trichoderma viride* Pers. has been described as a pathogen of pepper and tomato (Menzies 1993). Another saprotrophic wood-decaying fungus was also reported to cause necrosis of *P. sylvestris* roots, but the pattern of infection was different from that observed for the necrotrophic fungus *Heterobasidion annosum* (Fr.) Bref. (Sun et al. 2011), which the authors attributed to different signals being induced in the host by saprotrophic vs pathogenic fungi. Nonetheless, host cell death was shown to be necessary for proliferation during mutualistic symbiosis with barley as well (Deshmukh et al. 2006).

Host cell death during colonization by an ECM fungus, as occurred in our study, may be the result of stress since it was not typical root zone for colonization by an ECM fungus. Some level of cell death during colonization of *P. sylvestris* roots by an ECM fungus, however, was observed by Adomas et al. (2008).
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Baptista et al. Pathogen exposure was responsible for restricting fungal growth. The regulation of ROS by ECM fungi do not invade plant cells but rather surround the reactions that prevent deeper invasion of tissues by the fungus, however, ROS production may induce host defense a rapid defense reaction. During the interaction with an ECM the early stage of interaction with the ECM hyphae of observed cell death of root cells in response to colonization by an ECM fungus, compared with the elongation zone, interaction indicates though ROS accumulation was observed in the meristematic zone of roots inoculated with a pathogenic fungus, and the elongation zone of roots challenged by T. harzianum, which may indicate a defense reaction in root zones more heavily colonized by a fungus. Certain levels of glutathione seem to be important in the synthesis of pathogen-related molecules and disease resistance, but this specific level could be defined by host conditions and specific fungi (Böttcher et al. 2009, Su et al. 2011, Noctor et al. 2012).

The dynamics of host pH, as influenced by fungi, may play an important role in the establishment of fungi in host tissues (Dieguez-Urbeondo et al. 2008). Billon-Grand et al. (2012) observed a decrease in pH during the initial colonization of Helianthus by Sclerotinia sclerotium (Lib.) de Bary and Botrytis cinerea Pers., which the authors linked with glucose consumption. In the present studies, an increase in the number of host cells displaying an acidic pH in the early stage of interaction (2–12 h) was observed in both root zones of roots challenged by F. oxysporum. A decreased percentage of cells with acidic cytoplasm was associated with higher levels of colonization by F. oxysporum. Acidification of the cytoplasm of host cells during interactions with pathogens is connected with defense reactions (Felle 1996, 1998, 2001). Kacprzak et al. (2001) observed slight increases in the pH of conifer tissue (P. sylvestris and Picea abies) in response to Fusarium solani (Mart.) Sacc. and F. oxysporum (Fr.) Sacc., but these changes did not affect pathogen infection. In contrast, studies utilizing Colletotrichum spp. have shown that alkalization of the cytoplasm of plant host cells facilitated fungal virulence (Prusky and Yakoby 2003, Alkan et al. 2008). Apart from limiting the magnitude of host defense activation, fungi can also modulate pH to enhance the activity of depolymerizing enzymes or other metabolites (Aleandri et al. 2008) and/or enhance colonization by decreasing the activity of enzymatic defense proteins constitutively expressed or induced by the plant. In the present study, acidification of the cell apoplast was observed in the meristematic zone of roots, after which the number of cells with acidic cytoplasm decreased. This may ensure proper conditions for the activity of enzymes related to virulence. The meristematic zone was also characterized by a higher level of colonization by the pathogenic fungus, compared with the elongation zone,
where acidification of the apoplast was not observed. Acidic pH in the apoplast was shown to play a role in the virulence of *F. oxysporum* (Caracuel et al. 2003).

Although alkalization of host cytoplasm has been observed in the presence of symbiotic fungi (*Felle* 1996, 1998, 2001), alkalization of cytoplasm in response to ECM fungus was not observed in the present study. Instead, a fairly high number of cells with acidic cytoplasm were observed in the meristematic zone of roots during the first 3 days following inoculation with the ECM fungus. The meristematic zone of roots is not typically colonized by this type of fungus. In contrast, an increased percentage of cells with alkaline cytoplasm was observed in the early stage of the interaction (2–12 h) and then again after 10 days in the elongation zone of *P. sylvestris* roots inoculated with the ECM fungus. Hyphae were also observed more frequently in this zone. Additionally, there was an increase in the number of cells with acidic cytoplasm between 24 and 72 h. Acidification of cell cytoplasm is considered as a prerequisite for gene activation (*He et al.* 1998) and is often connected with host defense response. An increase in the percentage of cells with acidic cytoplasm was observed in the present study in response to the presence of saprotrophic and ECM fungi, which may reflect the activation of defense reactions in response to the fungi. *Trichoderma* species have been observed to endophytic or pathogenic in different plant hosts (*Harman et al.* 2004) and are thus able to induce host defense responses (*Yedidia et al.* 2003).

In conclusion, the comparison of events occurring in different root zones infected with *F. oxysporum*, *T. harzianum* and *H. crus-tuliniforme* provides evidence that cell death may play a role in the invasion of either the elongation or the meristematic zone with fungal colonization. Cell death in roots, however, may be regulated differently in response to different fungi. The data obtained serve as a mode and responses may vary under normal soil conditions where many factors can operate together. Development of such a model under controlled conditions is necessary to form a basis for understanding the nature of plant–fungal interactions.

**Supplementary data**

Supplementary data for this article are available at Tree Physiology Online.

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**Conflict of interest**

None declared.

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**References**


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