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Effects of *Rhizoctonia* infection and drought on peroxidase and chitinase activity in Norway spruce (*Picea abies*)

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Seedlings of Norway spruce were exposed to fungal infection and drought in order to investigate differences in their stress responses on the enzymatic level. Six-week-old seedlings were infected with the root rot fungus *Rhizoctonia*, or subjected to drought, respectively. Changes at the enzymatic level were more rapid and significantly higher in infected plants in comparison with drought-stressed spruce plants. *Rhizoctonia* infection resulted in early local and systemic increase in peroxidase and chitinase activity. The most prominent isoforms responding were highly basic peroxidases and chitinases (pI 9–9.5) and several acidic chitinases (pI3–4). An increased intensity of similar peroxidase isoforms was found in drought-affected plants. Two peroxidase isoforms (with pI < 9) accumulated exclusively in response to drought. These results suggest that at an early stage of infection and drought stress, the two stresses can be distinguished by the temporal appearance and isoform profile of peroxidases and chitinases. Changes in enzyme activity appeared before changes in physiological parameters, thus these isoform profiles could be used as early markers of stress conditions in spruce.

Introduction

Biotic stress caused by pathogen infection triggers the expression of defense-related genes and induces local and systemic host responses (Ryals et al. 1996, Hammerschmidt 1999). Abiotic stress as drought affects defense-related genes and antioxidative enzymes as well (Schwanz et al. 1996a, b, Polidoros et al. 2001), and increases plant resistance to insects and pathogenic fungi (Dunn and Lorio 1993, Croise et al. 2001). In conifers, induced stress responses include changes in the composition and flow of resins (Phillips and Croteau 1999), as well as changes in anatomy of wood and phloem (Franceschi et al. 2000). However, our knowledge about the molecular basis of stress responses in conifers is still scarce. A better understanding of the different defense pathways could be pertinent to identify markers of different kinds of stresses.

Stress responses include increased lignification, accumulation of phytoalexins and production of defense-related proteins (e.g. chitinases, peroxidases, and glucanase) as discussed by Grayer and Harborne (1994) and Hammerschmidt (1999). Plant peroxidases are involved in cell wall lignin synthesis, as well as signalling, and production of toxic oxygen radicals during stress (Polle et al. 1994, Baumbusch et al. 1998, Mensen et al. 1998). The plant chitin hydrolysing chitinases degrade fungal cell walls, thereby inhibiting fungal growth. The expression of peroxidase and chitinase also is closely associated with non-defensive features, such as normal development and morphogenesis (Campa 1991, Collinge et al. 1993).

In Norway spruce seedlings many isoforms of peroxidase and chitinase accumulate after pathogen infection. In spruce roots, the output from a gene encoding a

Abbreviations – CHAPS, 3-[(3-cholamidopropyl) dimethyl amino]-1-propane-sulphonate; 4-MU-$(GlcNAc)_2$, 4-methylumbelliferyl-β-d-*N,N′*-diacetyl-chitobioside; PAS, Periodic acid Schiff's; Spi2, Spruce pathogen induced number 2.

basic peroxidase (Spi2, spruce pathogen induced no. 2) rapidly increases after an infection by pathogenic oomycete (*Pythium dimorphum*) as shown by Fossdal et al. (2001). In transgenic Norway spruce seedlings, expression of this gene led to increased peroxidase activity and in transgenic tobacco plants to increased resistance against pathogens (Elfstrand et al. 2001, 2002). The production of peroxidase also increases in the stem of spruce trees after fungal infection (*Heterobasidion annosum*), and is localized to cell walls close to the site of pathogen invasion (Asiegbu et al. 1994, Johansson et al. 1994). Drought stress also affects the activity of peroxidases and chitinases. Mild drought increases the peroxidase activity in conifer needles, and thereby protects plant cells against oxygen radicals (Kronfuss et al. 1996, Alonso et al. 2001). Thus, it is likely that peroxidases and chitinases play important roles in coniferous resistance reactions against pathogens and drought.

Pathogen infection, which is a common stress factor in conifer breeding, might appear similar to that induced by abiotic stress. It is not easy to distinguish visually between symptoms of infection, drought and other stresses in seedlings. We are interested in if and how pathogenic and abiotic stresses are distinguished at the molecular level, and if these stresses evoke similar defense pathways in spruce. In this study *Rhizoctonia* sp. is employed as the biotic agent. This is a common fungal pathogen that causes root rot in nursery-grown conifer seedlings. The aim of the study is to compare effects of *Rhizoctonia* infection and drought-stress on peroxidase and chitinase activity, and on the physiological and cellular status in roots and needles.

Materials and methods

Plant material

Seeds of Norway spruce (*Picea abies* [L.] Karst.) were incubated in the dark on moist filter paper for a week before germlings were transferred to sterile Cone-tainer plastic pots (diameter 2.5 cm, depth 12 cm, volume 49 ml; Stuewe and Sons, Inc., Corvallis, OR, USA) with glass beads (0.7 mm; Kebo, Oslo, Norway) as the growth substrate. The seedling pots were placed in separate trays (200 seedling pots in each tray) in climate chambers. Five trays were arranged in each chamber, kept under constant conditions (250 $\mu mol\, m^{-2}\, s^{-1}$; Kolor Arc Daylight MBID 250/TH; General Electric, Budapest, Hungary; 20 h day/4 h night) at 22–24°C, and automatically watered twice a day with a complete nutrient solution, containing (m*M*): NO_3-N, 10.5; NH_4-N, 1.0; K, 4.0; Ca, 4.0; P, 1.0; S, 1.5; Mg, 1.2; Na, 1.7; Fe, 0.07; Mn, 0.03; B, 0.05; Cu, 0.005; Zn, 0.005; Mo, 0.0008. The electrical conductivity (EC) of the solution was $1.6\, mS\, cm^{-1}$, and pH ranged from 5.5 to 6.0. The plants were 6 weeks old, approximately 50 mm tall, and had a stem thickness of 1.5 mm when the experiments started.

Pathogen and drought treatments

We employed as our fungal pathogen the uninucleate *Rhizoctonia* sp., which is involved in root dieback in nursery-grown conifer seedlings (Venn et al. 1986; Hietala et al. 1994). The *Rhizoctonia* (strain no. 83-111/1 N) was first cultured on malt extract agar (1.25% malt extract, 2% agar, w/v) for 1 week, and then sub-cultured in liquid malt (1.25% w/v) medium for 10 days, and homogenized in 250 ml plant nutrient solution with an ultra-Turrax T25 homogenizer (IKA® Works, Inc., Wilmington, NC, USA). Spruce seedlings were inoculated by injecting 4 ml ($20\, mg\, ml^{-1}$) of the fungal homogenate into the growth substrate. Control plants were injected with the same amount of plant-nutrient solution, but without the fungus. Drought conditions were induced by withdrawal of watering. Root and needle samples were collected 2, 4, 8, 10 and 16 days after treatment started. Control samples were collected on the day of inoculation, and on subsequent sampling days.

Protein extraction

From 10 seedlings, pooled samples of roots and needles, respectively, were frozen in liquid nitrogen and stored at −80°C. Total proteins were extracted from 0.3 to 0.4 g plant and fungal tissue in 1.5 × low-pH buffer (0.001 *M* sodium citrate, pH 5.0) with 3% CHAPS (3-[(3-cholamidopropyl) dimethyl amino]-1-propane-sulphonate; Sigma-Aldrich, St. Louis, MO, USA). Insoluble material was removed by centrifugation twice at 9300 *g* for 20 min Protein concentration was measured with the Bradford (1976) method, using BSA as the standard protein.

Isoelectric focusing and visualization of peroxidase and chitinase activity

To detect peroxidase and chitinase isoforms, non-denaturing isoelectric focusing (IEF) gels (10% polyacrylamide gels, 1 mm thick) using broad-range ampholines (pH 3.5–10; Amersham Biosciences, Piscataway, NJ, USA) were prepared according to the manufacturer's instructions. Approximately 5 µg total protein was loaded for each sample and isoelectric focusing was run at 4°C and 20 W for 2.5 h with a LKB 2117 Multiphor system (LKB, Bromma, Sweden). Peroxidase isoform activity was detected according to the method described by Kerby and Somerville 1989), using 3-amino-9-ethylcarbazole (carbazol; Sigma-Aldrich) and hydrogen peroxide (VWR International AS, Oslo, Norway) as substrates.

Detection of chitinase isoform activity was performed as described by Pan et al. 1991); the gels attached to the supporting glass plates were incubated in 0.1 *M* sodium acetate buffer (pH 5) for 5 min, and then covered with a 7.5% polyacrylamide (ratio of acrylamide to *N, N′*-methylene-bisacrylamide, 100 : 2.6) overlay gel containing 0.04% (m/v) glycol chitin in 0.1 *M* sodium acetate buffer (pH 5.0). The gels were first incubated under moist

conditions at 37°C for 2.5 h, after which the overlay gels were incubated in freshly prepared 0.01% (m/v) fluorescent brightener 28 (Calcofluor white M2R; Sigma-Aldrich) in 0.5 *M* Tris-HCl (pH 8.9) at room temperature for 5 min. Finally, the overlay gels were washed overnight in distilled water at 4°C. Chitinase isoforms were visualized as cleared zones by placing the overlay gels on a UV light source.

Enzyme activity analysis

Total protein extracts from roots and needles were assayed for peroxidase enzyme activity according to Mensen et al. (1998) using guaiacol (10 m*M*; Sigma-Aldrich) and hydrogen peroxide (5 m*M*) as substrates. The oxidation of guaiacol was monitored and absorbance measured at 470 nm over the span of 2 min. Chitinolytic activity was determined according to Brurberg et al. (1996) using 4-methylumbelliferone $(GlcNAc)_2$ (Amersham BioSciences) as substrate. The amount of g-moieties (4-MU; fluorescent at excitation wavelength 380 nm, emitting at 460 nm, pH > 8) released was determined with a Hoefer DyNA 200 Fluorometer using a 4-MU solution as standard (Hoefer Scientific Instruments, San Francisco, CA, USA). The activities were determined as the mean of two independent measurements using the linear phase of the enzyme reactions.

Statistics

In each experiment two trays of plants were inoculated with the pathogen, two trays were subjected to drought and one tray was used as a control. The experiments were repeated twice. Data from the enzyme activity analysis and physiological measurements were subjected to one-way analysis of variance (ANOVA), using the general linear models (GLM) procedure of the SAS software package (SAS Institute Inc., Cary, NC, USA). Treatment means ($P < 0.05$), were separated using LSD at $P = 0.05$.

Gas exchange measurements

Needle net photosynthesis was measured in an open gas exchange system (CIRAS-1; PP Systems, Hitchin, Herts., UK) on whole seedlings placed inside a cylindrical needle chamber (Model PLC-C). Air temperature in the chamber was kept at 22°C with 0.5 kPa vapour pressure deficit, CO_2 partial pressure 35 Pa and photon flux density 250 μmol m^{-2} s^{-1}. Average needle area was calculated from the regression: needle area (cm^2) = 0.22 SL (shoot length) + 4.49, based on measurements with a Li-Cor area meter (Model LI-3100; Li-Cor Inc., Lincoln, NE, USA). Photosynthetic rate was measured at mid-day on four seedlings per treatment.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany) employing the saturation pulse method. The actinic photosynthetic photon flux density was 250 μmol m^{-2} s^{-1}. Chlorophyll fluorescence was measured on 16 seedlings per treatment and sampling date. In the dark-adapted seedlings maximal PSII fluorescence (F_v/F_m) was measured after 3.5 h in darkness with a white saturating pulse (750 μmol m^{-2} s^{-1}), lasting 0.8 s. The fixed distance to needles was adjusted prior to conducting measurements to avoid both overload and weak signals during data collection. After 6 h of light (at 250 μmol m^{-2} s^{-1}), fluorescence was measured at the same constant photon flux density. We used an external halogen lamp in combination with a leaf clip holder, and fine-tuned the flux density using a quantum sensor attached to the holder. The standard user run file no. 1 was applied with a saturation pulse of 1100 μmol m^{-2} s^{-1} lasting 0.8 s. In light-adapted seedlings, the PSII quantum yield ($\Delta F/F_m'$) values were calculated using the equation $\Delta F/F_m' = F_m' - F_t)/F_m'$ (Genty et al. 1989).

Water content measurements

The water content in roots and shoots was calculated as percentage of dry weight: (fresh weight – dry weight)/dry weight based on measurements of nine plants per treatment and sampling date and after the samples had been dried at 105°C for 48 h.

Root ion leakage measurements

Roots from three seedlings per treatment were separated from shoots, rinsed and placed in glass vials containing 10 ml Milli-Q water. After shaking the sample for 12 h at room temperature, electrolyte conductivity was measured (CDM210 conductivity meter, Radiometer A/S, Brønshøy, Denmark). Samples were then placed in boiling water for 30 min, stored overnight at room temperature, and electrolyte leakage measured 24 h later. Relative ion leakage was calculated as: 100 × (conductivity before boiling)/(conductivity after boiling).

Microscopy

Needles and lateral roots of three plants per treatment and sampling day were cut into 5 mm pieces. These samples were fixed in paraformaldehyde (2%) and glutaraldehyde (1.25%) in L-piperazine-*N*-*N*′-bis (2-ethane sulphonic) acid buffer (50 m*M*, pH 7.2) for 12 h at room temperature, washed in the same buffer (3 × 15 min), dehydrated in an ethanol series (70–80–90–96–4 × 100%; 15 min in each), and infiltrated (in a resin : ethanol series 1 : 3, 1 : 2, 1 : 1, 100%; 12 h in each) and embedded with L. R. White acrylic resin (polymerization 24 h at 60°C; TAAB Laboratories, Aldermaston Berkshire, UK). Semi-thin cross-sections (1.5 μm) were cut with a diamond knife using an ultramicrotome (Ultracut E. Reichert Jung; C. Reichert A.G., Vienna, Austria), and dried onto gelatin-coated glass slides. Sections were stained with either Stevenel's blue (Del Cerro et al. 1980) or Schiff's periodic acid carbohydrate stain

(Hotchkiss 1948; Nagy et al. 2000), mounted with immersion oil and imaged in a Leitz Aristoplan light microscope (Ernst Leitz Gmbh, Wetzlar, Germany). All reagents were from Sigma-Aldrich unless otherwise noted.

Results

Temporal changes of peroxidase and chitinase isoforms in roots and needles

IEF with carbazol and hydrogen peroxide as substrates indicated the presence of more than 12 peroxidase isoforms in the roots, and more than eight isoforms in the needles of healthy seedlings (Fig. 1A and B). Following infection of the roots with *Rhizoctonia*, a fungus causing root dieback disease, we found increased peroxidase activity, both in roots and needles. In infected roots, the intensity of a broad, highly basic peroxidase band (pI, approximately 9.0–9.5) increased after 2 days (Fig. 1A). In needles of pathogen-infected seedlings, a similar band increased in intensity, but only after day 4 (Fig. 1B). Two days after drought treatment started, the roots showed increased peroxidase activity, in the same highly basic peroxidase band (pI, approximately 9.5), as well as in two other less basic bands (pI, approximately 8.0–8.5; Fig. 1A). There was no increase in needle peroxidase activity after drought treatment (Fig. 1B).

Using IEF with an overlay gel containing chitin as a substrate, we found more than eight chitinase isoforms, both in roots and needles of healthy seedlings (Fig. 1C and D). Two days after pathogen infection, there was an increase in the chitinolytic activity of all the chitinase isoforms present prior to infection. The most prominent chitinases were either highly basic (pH, approximately 9–10) or highly acidic (pI, approximately 3–4; Fig. 1C and D). One highly acidic chitinase (pI, approximately 3) that was not present at the start of the experiment appeared 2 days after infection. We observed a similar but less pronounced isoform profile in needles and the observed changes took place 4 days after infection. After 8 days of drought, we observed a general increase in the chitinolytic activity in roots and only a weak increase in needles (Fig. 1C and D).

Quantitative changes in peroxidase and chitinase activity

In addition to IEF we also made quantitative measurements of total enzyme activity. Pathogen infection led to an increase in peroxidase and chitinase activity, both in roots and needles (Fig. 2). Enzyme activity increased in roots 4 days after infection (Fig. 2A and C). Increased enzyme activity was also pronounced in needles, and 2 days after pathogen infection, there was a two- to three-fold increase in relative peroxidase and chitinase activity in comparison with the control (Fig. 2B and D; $P < 0.1$). In the roots of drought-treated seedlings, we observed a 2.5- to three-fold increase in relative activity of both peroxidases and chitinases after day 8 (Fig. 2A and C;

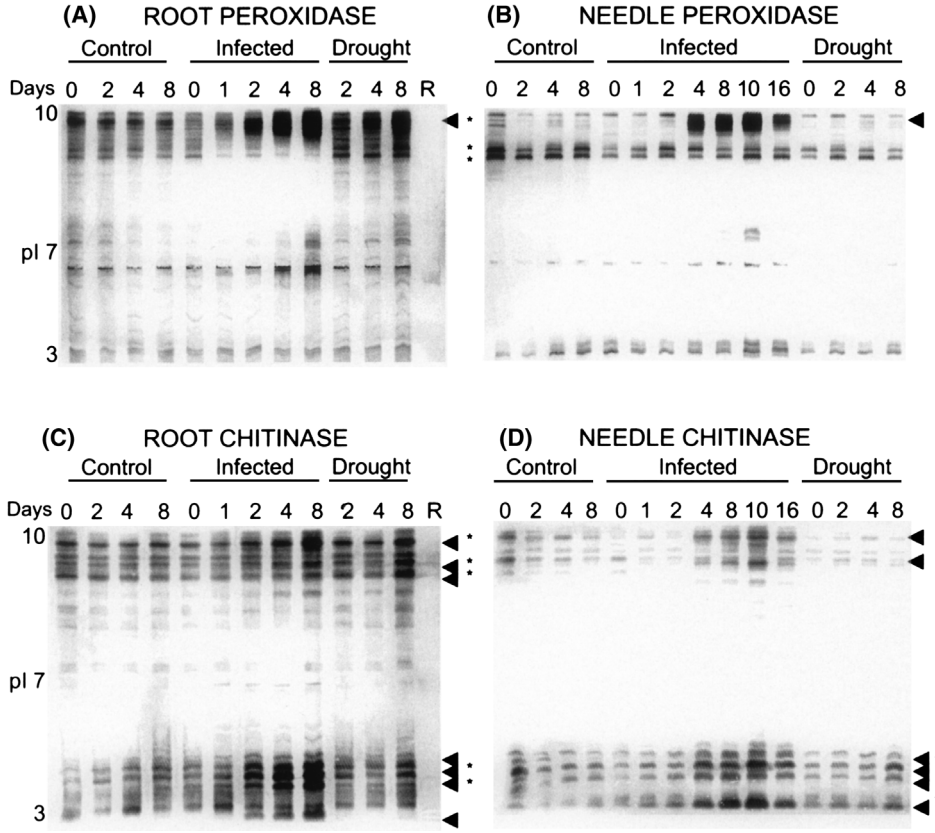

Fig. 1. Peroxidase and chitinase isoforms from roots and needles of *Rhizoctonia*-infected and drought-treated Norway spruce seedlings at the start (0) and 2–16 days after treatment. (A) Peroxidase isoforms in roots; (B) peroxidase isoforms in needles; (C) chitinase isoforms in roots; (D) chitinase isoforms in needles. In each lane 5 μg total protein, extracted from pooled samples from 10 seedlings, were applied. The gels presented are representative of two independent replicas of the experiment. Arrowheads indicate isoforms with increased activities in infected material and * indicates isoforms with increased activity in drought-treated seedlings. R, *Rhizoctonia*, indicates proteins extracted from pure fungal material.

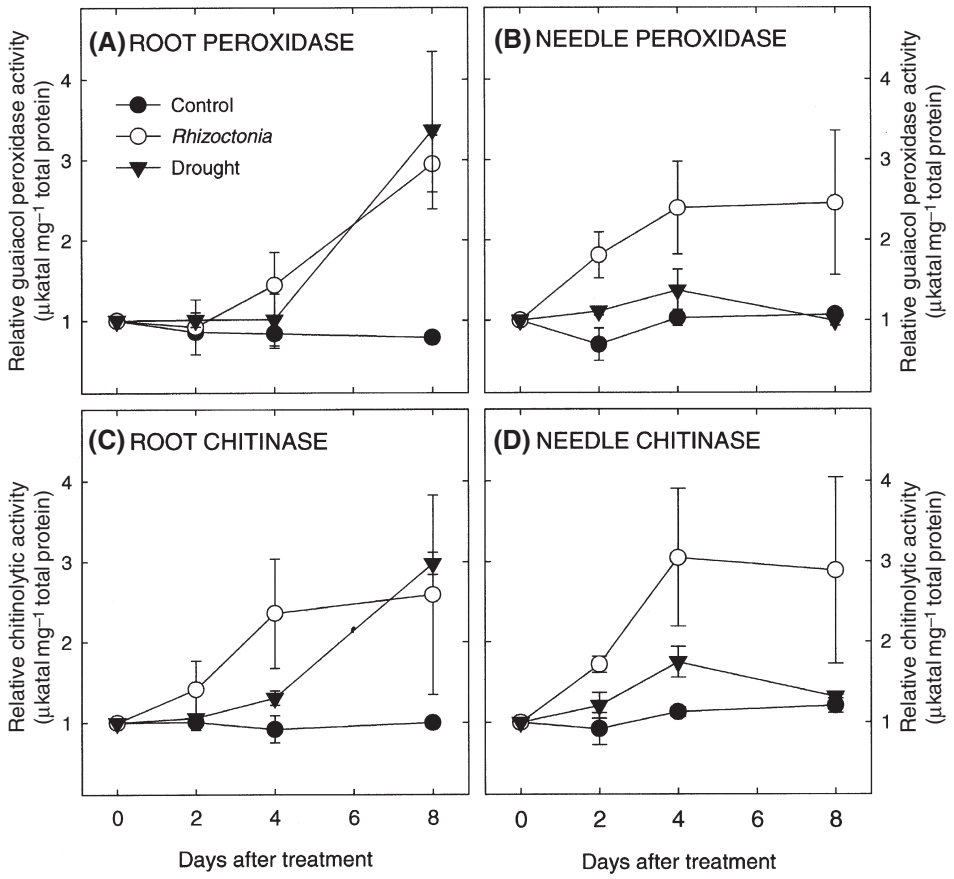

Fig. 2. Peroxidase and chitinase activity in roots and needles of Norway spruce seedlings infected with *Rhizoctonia* sp. (○) or treated with drought (▼) as compared to the control (●). (A) Peroxidase activity in roots; (B) peroxidase activity in needles; (C) chitinase activity in roots; (D) chitinase activity in needles. Protein was extracted from pooled samples from 10 seedlings, and each data point represents the average of two independent replicates ± SE. One unit of peroxidase and chitinase activity was defined as equivalent to an increase of one absorption unit min^{-1}, and the relative activity at the start of the experiment was then set as a reference value 1. Data are presented as relative enzyme activity μkatal mg^{-1} total protein.

$P < 0.08$). There were no significant changes in peroxidase or chitinase activity in the needles of drought-treated seedlings (Fig. 2B and D).

Physiological status of the stressed plants

We monitored the physiological status of plants during fungal infection and drought treatment by measuring needle net photosynthetic rate, needle transpiration, chlorophyll fluorescence, water content and root ion leakage.

Pathogen infection caused only a slight reduction in photosynthetic rate, transpiration and chlorophyll fluorescence compared with controls, and only on day 8 did we see a significant difference between pathogen-infected seedlings and the controls and the drought-treated seedlings (Fig. 3A–D; $P < 0.01$ for both cases). There was a significant reduction in CO_2 uptake 4 days after the start of drought treatment, and after 8 days the photosynthetic rate was close to zero as compared with the controls (Fig. 3A; $P < 0.01$). Similarly, both transpiration and the quantum yield of photosystem II were reduced by drought after 4 days (Fig. 3B and C; $P < 0.01$), and this was intensified further by day 8 ($P < 0.01$) in comparison with the controls. Chlorophyll fluorescence of dark-adapted plants in both pathogen-infected and drought-stressed plants remained stable and did not change significantly throughout the experiment before day 10 (Fig. 3D).

Root water content was relatively stable until day 3, thereafter decreasing slightly in infected plants and strongly in drought-treated plants (Fig. 3E), indicating a similar mild drought stress for both types of stress at earlier times. Root ion leakage increased continuously throughout the experiment, in both pathogen-infected seedlings and drought-stressed seedlings, reaching a level about 55 and 80% higher than in control plants, respectively (Fig. 3F; $P < 0.02$ in both cases). The control samples showed more than 50% leakage, which could be explained by the 12 h long incubation.

Cellular changes in roots and needles following pathogen infection and drought stress

One day after infection we observed fungal hyphae in the intercellular spaces of the outer cortical epidermal and parenchymal cell layers of lateral root tips. The hyphal invasion gradually became more extensive, and by day 4 the fungus was growing in the vascular cylinder of the root apical meristem (Fig. 4A and B). A characteristic feature of the infected roots was the disintegration of root tip cells and the disappearance of a distinct nucleus. In the drought-treated seedlings, the roots became increasingly shrunken in appearance over time, with

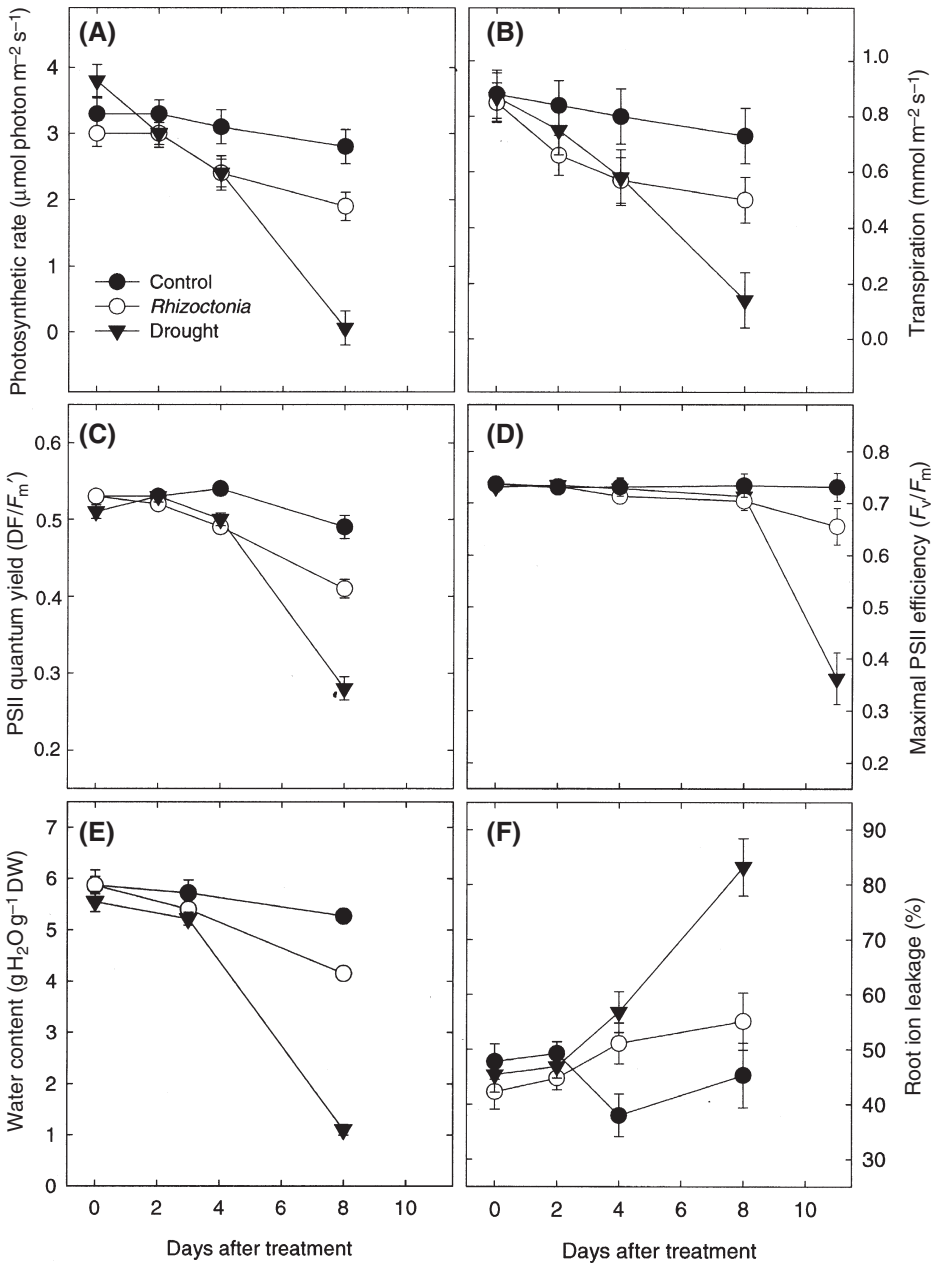

Fig. 3. Physiological measurements in Norway spruce seedlings during drought treatment (▼) and after root inoculation with *Rhizoctonia* (○) compared with the control (●). (A) Needle net photosynthetic rate measured on n = 4 seedlings per treatment; (B) needle transpiration rate measured on n = 4 seedlings per treatment; (C) needle PSII quantum yield measured on n = 16 seedlings per treatment; (D) maximal PSII efficiency (F_v/F_m) measured after 3.5 h in darkness on n = 16 seedling per treatment; (E) root water content; data points represent the average measurements of n = 3 seedlings; (F) relative root ion leakage measured on n = 3 seedlings per treatment. Each data point represents the average of two independent experiments ± SE.

large inclusions of browning phenolics accumulating in the outer cortical cells after 4–8 days (Fig. 4C).

Seven days after the start of the experiment, the shoots of the drought-stressed plants became somewhat wilted, whereas the fungal-infected plants showed no signs of wilting. We observed starch granules in needle mesophyll cells of both control and fungal-infected plants, whereas in drought-treated plants starch granules had completely disappeared by day 8 (Fig. 4D–F).

Discussion

It is likely that the constitutive chitinase and peroxidase activity is involved in the defense against pathogens normally present in both the phyllosphere and the rhizosphere. Our study shows that up to eight chitinase and 12 peroxidase isoforms are constitutively present in roots and needles of healthy spruce plants. The amount of isoforms fits well with the protein expression/activity profile found in non-stressed 1-week-old seedlings (Sharma et al. 1993, Fossdal et al. 2001) and in 35-year-old trees (Nagy, unpublished). The high number of peroxidase and chitinase isoforms constitutively present probably represents a standing defense against pathogens and other stresses (Campa 1991, Collinge et al. 1993).

In *Rhizoctonia*-infected roots we observed increased levels of peroxidase (primarily basic ones) and chitinase

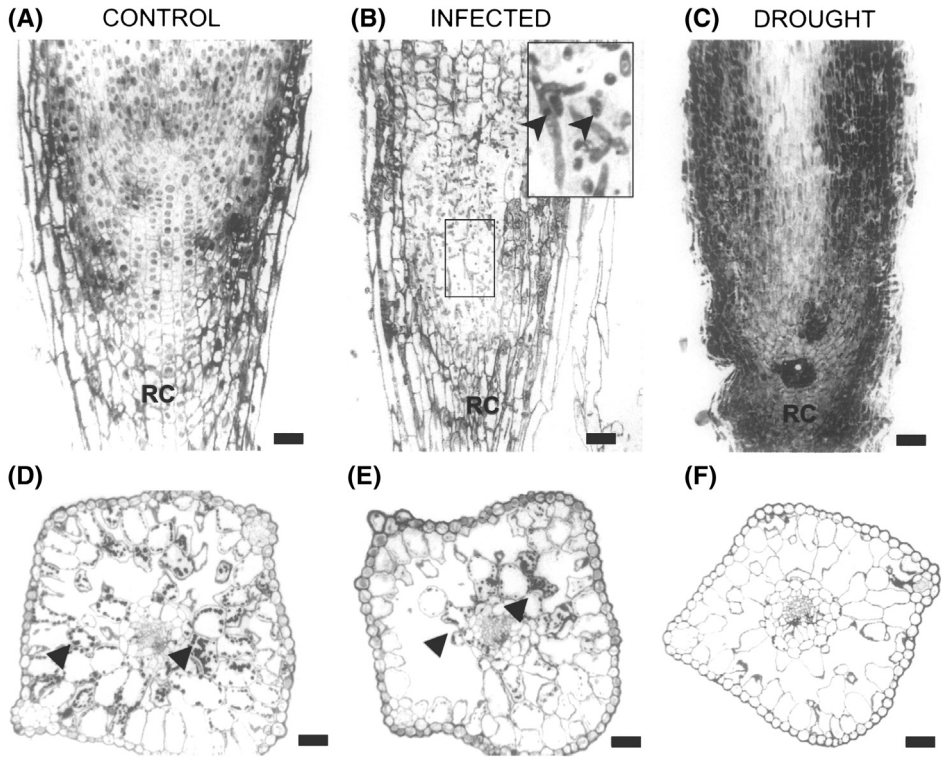

Fig. 4. Light microscopy images of pathogen-infected and drought-stressed roots and needles. (A) Untreated, control root tip on day 4; (B) *Rhizoctonia*-infected root tip with high magnification insert of hyphae on day 4; (C) drought-stressed root tip on day 4; (D) control needle on day 8; (E) needle from *Rhizoctonia*-infected plant on day 8; (F) needle from drought plant on day 8. The panel is a representative of observations made on three seedlings per treatment and control. RC, root cap; arrowheads, starch grains in needle mesophyll; scale bars = 80 μm.

(both basic and acidic) isoforms. The observed rise in isoform activities was also reflected by an increase in total enzyme activities. Thus, elevated total activity can be attributed to both the increase of the individual isoforms mentioned above and novel isoforms appearing. The discrepancy in peroxidase activity at the second day of the experiment can be explained by variations in the host-response at an early stage of infection. In addition, it might be partially due to the application of different substrates as the isoform pattern and total peroxidase activity was estimated by using carbazol and guaiacol, respectively. It is likely that the broad peroxidase band represents several peroxidase species, because elicitors from fungal pathogens may induce several cell-wall-associated peroxidases (Mensen et al. 1998). The predicted peroxidase protein encoded by the Spi 2 gene recorded by Fossdal et al. (2001) is probably included within this broad peroxidase band (pI, 9.0–9.5). Our findings indicate that peroxidases and chitinases contribute to local host-defense responses in Norway spruce seedlings, whereas the increase of similar isoforms of peroxidase and chitinase in needles after pathogen infection indicate a systemic host response. These results indicate that the production of chitinases and peroxidases is part of the general systemically induced defense response regarded as a reminiscence of the increased PR-protein levels found in angiosperms.

In certain respects, pathogens and drought caused similar responses in Norway spruce seedlings, whereas the responses differed in some other aspects. Reduced height growth, needle discoloration and partial destruction of the root system are visual characteristics connected to *Rhizoctonia* infections (Hietala 1997). The *Rhizoctonia* species used in our study primarily penetrated the apical region of lateral roots, colonizing the root tip meristem and vascular cylinder, but not the woody parts of the root. This mode of infection may damage normal water transport and uptake in a way that is analogous to mild drought stress. This may explain similarities in the enzymatic responses to pathogen infection and drought stress. In the present study, peroxidase and chitinase activities increased to a similar extent in roots after drought and pathogen attack. The increase after pathogen infection, however, appeared earlier and stronger than after drought. We found that basic isoforms of peroxidase (pI approximately 9.5), similar to those induced by pathogen infection, increased in roots of drought-stressed seedlings. The drought-stressed roots also accumulated two less basic isoforms ($pI < 9$), which are absent during fungal infection. As some isoforms are activated by drought only, it is likely that they are drought specific, not being involved in the defense against pathogens.

This study demonstrates that spruce seedlings subjected to pathogens and drought showed slight reductions in photosynthetic activity, transpiration, water content, and root ion leakage in the beginning of the experiment. Further treatment led to a significant loss of root and needle function mainly in drought-stressed plants, and towards the end of the experiment drought treatment severely affected needle starch reserves as well. It is likely that reduced gas exchange and quantum yield

lead to reduced CO_2 assimilation. Interestingly, an enzymatic increase in needles was only observed after pathogen infection; drought did not induce any significant changes in needles with respect to enzyme activity. This may be because prolonged drought inhibited or delayed the metabolism necessary to increase enzyme activity or transmit signals (Hsiao 1973).

Our study shows that *Rhizoctonia* infection caused early local and systemic host responses at the enzymatic level. The symptoms of this infection, however, commonly do not show conspicuous symptoms, and may be easily confused with drought. As the changes in enzyme activity appear before changes in physiological parameters, these enzymatic reactions can be used as early markers of stress conditions in spruce. Furthermore, combined results of our drought and infection experiments demonstrate that the two modes of stress can be distinguished at an early stage of development. It is important to note that both drought and infection are detected on the molecular level, and that they can be distinguished by their temporal appearance and isoform profiles. Furthermore, since drought stress mobilizes some defenses similar to those of pathogen infection, mildly drought-stressed plants may be more resistant to *Rhizoctonia*, or other pathogenic infections. In future studies it would be interesting to examine whether mild drought, or signalling substances such as salicylic acid and jasmonic acid (Kozlowski and Metraux 1998, Kozlowski et al. 1999, Franceschi et al. 2002), or abscisic acid (Hsiao 1973) associated with pathogen defenses and drought could render the plants more resistant to fungal attacks.

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References

Alonso R, Elvira S, Castillo FJ, Gimeno BS (2001) Interactive effects of ozone and drought stress on pigments and activities of antioxidative enzymes in *Pinus halepensis*. Plant Cell Environ 24: 905–916

Asiegbu FO, Daniel G, Johansson M (1994) Defense-related reactions of seedling roots of Norway spruce to infection by *Heterobasidion annosum* (Fr.) Bref. Physiol Mol Plant Pathol 45: 1–19

Baumbusch LO, Eiblmeier M, Schnitzler JP, Heller W, Sandermann H, Polle A (1998) Interactive effects of ozone and UV-B radiation on antioxidants in spruce (*Picea abies*) and pine (*Pinus sylvestris*) needles. Physiol Plant 104: 248–254

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brurberg MB, Nes IF, Eijsink VGH (1996) Comparative studies of chitinases A and B from *Serratia marcescens*. Microbiol-UK 142: 1581–1589

Campa A (1991) Biological roles of plant peroxidases: known and potential functions. In: Everse J, Everse KE, Grisham MB (eds) Peroxidases in Chemistry and Biology. CRC Press, Inc., Boca Raton, FL, Vol. II., pp 25–47

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3: 31–40

Croise L, Lieutier F, Cochard H, Dreyer E (2001) Effects of drought stress and high-density stem inoculations with *Leptographium wingfieldii* on hydraulic properties of young Scots pine trees. Tree Physiol 21: 427–436

Del Cerro M, Cogen J, Del Cerro C (1980) Stevenel's blue, an excellent stain for optical microscopical study of plastic embedded tissues. Microscopica Acta 83: 117–121

Dunn JP, Lorio PL (1993) Modified water regimes affect photosynthesis, xylem water potential, cambial growth, and resistance of juvenile *Pinus taeda* L. to *Dendroctonus frontalis* (Coleoptera, Scolytidae) Environ Entomol 22: 948–957

Elfstrand M, Fossdal CG, Sitbon F, Olsson O, Lönneborg A, von Arnold S (2001) Overexpression of the endogenous peroxidase-like gene spi 2 in transgenic Norway spruce plants results in increased total peroxidase activity and reduced growth. Plant Cell Rep 20: 596–603

Elfstrand M, Sitbon F, Lapierre C, Bottin A, von Arnold S (2002) Altered lignin structure and resistance to pathogens in spi 2-expressing tobacco plants. Planta 214: 708–716

Fossdal CG, Sharma P, Lönneborg A (2001) Isolation of the first putative plant peroxidase cDNA from a conifer and the local and systemic accumulation of related proteins upon pathogen infection. Plant Mol Biol 47: 423–435

Franceschi VR, Krekling T, Christiansen E (2002) Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. Am J Bot 89: 578–586

Franceschi VR, Krokene P, Krekling T, Christiansen E (2000) Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). Am J Bot 87: 314–326

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochem Biophys Acta 990: 87–92

Grayer RJ, Harborne JB (1994) A survey of antifungal compounds from higher plants, 1982–93. Phytochemistry 37: 19–42

Hammerschmidt R (1999) Induced disease resistance: how do induced plants stop pathogens? Physiol Mol Plant Pathol 55: 77–84

Hietala AM (1997) The mode of infection of a pathogenic uninucleate *Rhizoctonia sp.* in conifer seedling roots. Can J For Res 27: 471–480

Hietala AM, Sen R, Lilja A (1994) Anamorphic and teleomorphic characteristics of a uninucleate *Rhizoctonia* Sp isolated from the roots of nursery grown conifer seedlings. Mycol Res 98: 1044–1050

Hotchkiss RD (1948) A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparation. Arch Biochem 16: 131–141

Hsiao TC (1973) Plant responses to water stress. Ann Rev Plant Physiol 24: 519–570

Johansson M, Lundgren L, Asiegbu FO (1994) Initial interactions in living bark and sapwood of conifers infected by root rot fungi. In: Aamlid D (ed) Proceedings from the SNS Meeting in Forest Pathology. SNS, Biri, Norway, pp 12–16

Kerby K, Somerville S (1989) Enhancement of specific intercellular peroxidases following inoculation of barley with *Erysiphe graminis* f. sp hordei. Physiol Mol Plant Pathol 35: 323–337

Kozlowski G, Buchala A, Metraux JP (1999) Methyl jasmonate protects Norway spruce (*Picea abies* (L.) Karst.) seedlings against *Pythium ultimum* Trow. Physiol Mol Plant Pathol 55: 53–58

Kozlowski G, Metraux JP (1998) Infection of Norway spruce (*Picea abies* (L.) Karst.) seedlings with *Pythium irregulare* Buism. & *Pythium ultimum* Trow: Histological and biochemical responses. Eur J Plant Pathol 104: 225–234

Kronfuss G, Wieser G, Havranek WM, Polle A (1996) Effects of ozone and mild drought stress on total and apoplastic guaiacol peroxidase and lipid peroxidation in current-year needles of young Norway spruce (*Picea abies* (L.) Karst). J Plant Physiol 148: 203–206

Mensen R, Hager A, Salzer P (1998) Elicitor-induced changes of wall-bound and secreted peroxidase activities in suspension-cultured spruce (*Picea abies*) cells are attenuated by auxins. Physiol Plant 102: 539–546

Nagy NE, Franceschi VR, Solheim H, Krekling T, Christiansen E (2000) Wound-induced traumatic resin duct development in

stems of Norway spruce (Pinaceae): anatomy and cytochemical traits. Am J Bot 87: 302–313
Pan SQ, Ye XS, Kuc JA (1991) Technique for detection of chitinase, beta-1,3-glucanase, and protein patterns after a single separation using polyacrylamide-gel electrophoresis or isoelectrofocusing. Phytopathology 81: 970–974
Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. Trends Plant Sci 4: 184–190
Polidoros AN, Mylona PV, Scandalios JG (2001) Transgenic tobacco plants expressing the maize Cat2 gene have altered catalase levels that affect plant–pathogen interactions and resistance to oxidative stress. Transgenic Res 10: 555–569
Polle A, Otter T, Seifert F (1994) Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L., Karsten). Plant Physiol 106: 53–60
Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819
Schwanz P, Haberle KH, Polle A (1996a) Interactive effects of elevated CO_2, ozone and drought stress on the activities of antioxidative enzymes in needles of Norway spruce trees (*Picea abies*, [L.] Karsten) grown with luxurious N-supply. J Plant Physiol 148: 351–355
Schwanz P, Picon C, Vivin P, Dreyer E, Guehl JM, Polle A (1996b) Responses of antioxidative systems to drought stress in pedunculate oak and maritime pine as modulated by elevated CO_2. Plant Physiol 110: 393–402
Sharma P, Børja D, Stougard P, Lönneborg A (1993) PR-proteins accumulating in spruce roots infected with a pathogenic *Pythium sp*. isolate include chitinases, chitosanases, and β-1, 3-glucanases. Physiol Mol Plant Pathol 43: 57–67
Venn K, Sandvik M, Langerud BR (1986) Nursery routines, growth media and pathogens affect growth and root dieback in Norway spruce seedlings. Meddel Norsk Inst Skogforsk 39: 313–328

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