Spatial Patterns in Hyphal Growth and Substrate Exploitation within Norway Spruce Stems Colonized by the Pathogenic White-Rot Fungus *Heterobasidion parviporum*^{∇†}

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In Norway spruce, a fungistatic reaction zone with a high pH and enrichment of phenolics is formed in the sapwood facing heartwood colonized by the white-rot fungus *Heterobasidion parviporum*. Fungal penetration of the reaction zone eventually results in expansion of this xylem defense. To obtain information about mechanisms operating upon heartwood and reaction zone colonization by the pathogen, hyphal growth and wood degradation were investigated using real-time PCR, microscopy, and comparative wood density analysis of naturally colonized trees with extensive stem decay. The hyphae associated with delignified wood at stump level were devoid of any extracellular matrix, whereas incipient decay at the top of decay columns was characterized by a carbohydrate-rich hyphal sheath attaching hyphae to tracheid walls. The amount of pathogen DNA peaked in aniline wood, a narrow darkened tissue at the colony border apparently representing a compromised region of the reaction zone. Vigorous production of pathogen conidiophores occurred in this region. Colonization of aniline wood was characterized by hyphal growth within polyphenolic lumen deposits in tracheids and rays, and the hyphae were fully encased in a carbohydrate-rich extracellular matrix. Together, these data indicate that the interaction of the fungus with the reaction zone involves a local concentration of fungal biomass that forms an efficient translocation channel for nutrients. Finally, the enhanced production of the hyphal sheath may be instrumental in lateral expansion of the decay column beyond the reaction zone boundary.

To grow to great heights, trees continually replace their water- and nutrient-conducting elements. Older elements, such as the heartwood that is formed in many trees, gradually become nonconductive. In contrast to the living sapwood, heartwood lacks active defense mechanisms against microbes. However, lignin, the polymer coating cell wall polysaccharides, is highly resistant to microbial degradation. In fact, white-rot fungi, besides having evolved the ability to tolerate or detoxify the secondary metabolites accumulating in heartwood, are the only organisms capable of efficiently degrading lignin. Following establishment in the heartwood of living trees, the colonies of pathogenic white-rot fungi expand and eventually also threaten the conductive sapwood.

The white-rot fungus *Heterobasidion annosum* sensu lato, composed of three species with overlapping geographic distributions and host ranges in Europe (23), is the most important pathogen of Norway spruce (*Picea abies* L. Karst) in boreal forests. Primary infection of Norway spruce stands by *H. annosum* sensu lato takes place through fresh thinning stumps or wounds on roots and at the base of the stem. Basidiospores landing on these entrance points give rise to mycelia which colonize the root systems, and eventually the fungus spreads into the stem heartwood. At sites infested with *Heterobasidion parviporum*, a species primarily restricted to Norway spruce,

roots of saplings can become infected by the fungus after around 10 years of growth (25). Stem colonization usually initiates only after the heartwood has started to develop, which in Norway spruce takes place in trees 25 to 40 years old (17). Due to relatively rapid axial spread within heartwood, the decay column caused by *H. annosum* sensu lato often is up to 10 m high in the stems of mature Norway spruce trees.

In response to sapwood challenge by an expanding heartwood-based colony of *H. annosum* sensu lato, Norway spruce forms a so-called reaction zone (RZ) in the border area between healthy sapwood and colonized heartwood. This xylem defense is characterized by high pH due to increased carbonate content and enrichment of phenolic compounds, particularly lignans, some of which have shown antifungal properties in bioassays (14, 30, 31). Although several wood decay fungi are able to eventually penetrate the RZ regions formed in trees, the strategies employed by fungi to breach these unique defense barriers are poorly understood (24). The purpose of this study was to obtain information about the mechanisms operating in heartwood colonization and expansion of the decay column via penetration of the RZ. To do this, we examined spatial growth of *H. parviporum* and the associated substrate exploitation patterns within naturally colonized mature stems of Norway spruce.

MATERIALS AND METHODS

Sampling. Two mature Norway spruce trees growing at a forest site in Ås in southern Norway were examined in detail in this study. The trees, designated trees 4 and 9, had been naturally infected by *Heterobasidion* and had extensive decay columns at the time of sampling. After felling, 3-cm-thick stem disks were cut with a chain saw at 1-m intervals up to a height where visual signs of decay

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were no longer observed, 4 m in tree 4 and 7 m in tree 9. Two disks were cut at each height, one for fungal isolation and observation of conidium formation and the other for isolation of DNA, microscopy, and comparative wood density analyses. The latter disk was stored at -20°C until it was processed further. The two disks were cut next to each other, and the sides were marked so that corresponding points on the disks could be located. For fungal isolation, pieces of decayed wood were excised and placed on malt extract agar (1% malt extract, 1.5% agar). After this the disks were wrapped in moist newspaper, and the formation of conidia by *H. annosum* sensu lato was observed after incubation for 5 days at room temperature. Based on mating tests (15), both spruce trees were colonized by *H. parviporum*. Mycelial interaction between the isolates from spruce and test strains of *Heterobasidion* species resulted in the formation of clamp connections with the homokaryotic test strains of *H. parviporum* but not with the homokaryotic test strains of *H. annosum*. Somatic incompatibility tests (35) with the heterokaryotic isolates obtained from the two trees showed that trees 4 and 9 were colonized by different genotypes of the species. In pairings between isolates obtained from different heights of a decay column in each tree, the colonies showed somatic compatibility and fused together. In contrast, confrontation of isolates obtained from the two trees showed somatic incompatibility and formation of a demarcation line between the colonies.

Determination of the RZ area and localization of conidiophores. The position of the RZ on the wood disks analyzed was determined by spraying the disks with a 0.3% solution of 2,6-dichlorophenol. This chemical stains the RZ blue-green due to an elevated pH, while surrounding areas (sapwood or heartwood, either sound or decayed) with a lower pH turn reddish (14). The production of conidiophores was employed to verify the area colonized by *Heterobasidion* at each sampling height. A transparency sheet with grid lines (1 by 1 cm) was attached to the surface of a disk, and the presence of conidia was recorded for each grid cell using a compound microscope (16).

DNA isolation. A 1-cm-thick section covering a radius from the center of the pith to the living sapwood was removed from area of interest on a frozen disk. To allow spatial sampling, this section was then divided into 5-mm-wide samples that were processed separately for DNA isolation. For selected subregions at the edge of decay columns, more intensive sampling (2-mm-wide samples) was used. The samples were first ground in liquid nitrogen using a mortar and pestle. After this the samples were transferred to 2-ml Eppendorf tubes, and the final grinding (twice for 1 min each time at the maximum speed) was performed using liquid nitrogen-chilled samples and a Retsch 300 mill (Retsch GmbH, Haan, Germany) with the aid of a 100-mg steel bead.

Twenty-milligram aliquots of the ground powder from each sample were subjected to DNA isolation with a DNeasy plant mini kit (Qiagen, Hilden, Germany) used according to the manufacturer's instructions; 5 ng of a pGEM plasmid (pGEM-3Z vector; Promega, Madison, WI) was incorporated into the lysis buffer for normalization (7).

Real-time PCR. Real-time PCR detection of *H. parviporum* and the reference pGEM DNA was performed with TaqMan universal PCR master mixture (4304437; Applied Biosystems) as described in our previous study (39). The lacase primer-probe set used for detection of *H. parviporum* shows high specificity for this species (13).

To construct standard curves, DNA isolated with a DNeasy plant mini kit (Qiagen) from a pure culture of the *H. parviporum* strain obtained from tree 4 was quantified by using a Versafuor fluorometer (Bio-Rad, Hercules, CA) and a PicoGreen DNA quantification kit (Molecular Probes, Eugene, OR). To mimic the PCR conditions used for the experimental samples, standard curves were prepared using a DNase I-treated DNA solution obtained from tree 4. The pathogen DNA standard curve samples (1,000, 100, 10, and 1 pg of DNA) were all spiked with 10 pg of pGEM DNA. The reference DNA standard curve samples (10, 1, 0.1, and 0.01 pg) of pGEM DNA were all spiked with 33 pg of *H. parviporum* DNA. For experimental samples, serial dilutions (undiluted and diluted 1/10, 1/100, and 1/1,000) were used as templates for real-time PCR. *H. parviporum* and Norway spruce DNA were used as negative controls for the pGEM marker, while pGEM and Norway spruce DNA served as negative controls for the pathogen marker. Real-time detection of fluorescence emission was performed with an ABI PRISM 7700 (Applied Biosystems) by using the PCR conditions previously described (39).

Calculation of fungal colonization levels. Standard curves for *H. parviporum* and the reference pGEM plasmid were constructed based on the relationship of threshold cycle (C_T) values and known host and pathogen DNA concentrations; the C_T values were plotted against log-transformed amounts of DNA, and linear regression equations were calculated for the quantification of DNA pools by interpolation for unknown samples.

ITS rRNA gene sequence analysis. To determine whether fungi other than *H. parviporum* were present, selected DNA samples were also subjected to internal

transcribed spacer (ITS) rRNA gene-targeted PCR, followed by gel electrophoresis and sequence analysis of the PCR products. Samples obtained from decayed heartwood, the dark brown to blue wood neighboring the RZ ("aniline wood" [31]), and the RZ zone were included. Amplification was carried out with primers ITS1-F and ITS4 (11) using 50- μl reaction mixtures containing HotStar-Taq DNA polymerase (Qiagen) according to the manufacturer's instructions. After gel electrophoresis, the amplicons from each reaction were purified and sequenced in both directions with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Contigs were assembled with Seqman software (Lasergene; DNASTAR Inc., Madison, WI) and queried against ITS sequences in the GenBank database.

Microscopy. Specimens used for microscopic observations were made from a strip of wood (1 cm thick, spanning from the pith to the living sapwood) which was removed from each of the stem disks. From the strip, 5-mm-wide wood blocks were cut from the RZ (70 to 100 mm from the pith) and from the zone with advanced decay (30 to 50 mm from the pith). The specimens were fixed in paraformaldehyde (2%) and glutaraldehyde (1.25%) in L-piperazine-*N,N'*-bis(2-ethanesulfonic) acid buffer (50 mM, pH 7.2) for 12 h at room temperature.

The wood blocks were embedded in L.R. White resin, and cross sections (1.5 μm) were cut using an LKB 2128 Ultratome (Leica Microsystems, Germany) as previously described by Nagy et al. (20). Some samples were frozen in Tissue-tec, and cryosections (thickness, 20 μm) were cut with a Leitz cryostat microtome at -18°C . Both resin sections and cryosections were dried on superfrost Plus glass slides (Menzel-Gläzer, Germany). These sections, which were used for routine observations, were stained with Stevenel's blue (8). For assessment of wood delignification, the sections were stained with safranin-astra blue counterstaining. This method is a general method for assessment of wood delignification by white-rot fungi; astra blue stains cellulose blue in the absence of lignin, and safranin stains lignin regardless of whether cellulose is present (33). Periodic acid-Schiff (PAS) staining was used to identify carbohydrate-rich compounds.

Unstained sections (both L.R. White resin sections and cryosections) were examined for autofluorescence of phenolic compounds as described by Franceschi et al. (9), using a Leitz Aristoplan microscope operated in epifluorescence mode. Blue light at 450 to 490 nm was used for excitation, and a long-band-pass filter (≥ 520 nm) was used for visualization of induced fluorescence. For localization of polyphenolic deposits, an ethanolic solution of 2% ferric chloride was used. This reagent produces a greenish color upon reaction with phenolics (10).

Wood density and growth ring analyses. To obtain reference data for substrate exploitation, wood density and growth ring data were collected for trees 4 and 9. These analyses were performed using the same heights and radial positions as those utilized to determine the DNA-based colonization profiles of *H. parviporum*. The reflected light intensity method implemented in WinDendro (26) was used to assess ring width on the radial surface of pith-to-bark samples. Wood density was determined from computed X-ray images obtained for pith-to-bark samples by using a medical X-ray tomograph (Siemens Somatom Emotion single slice computed tomography [CT] scanner with Syngo software). The images were computed from the measured CT values which conform to the DICOM standard (32) and were analyzed with the ImageJ software (<http://rsb.info.nih.gov/ij/>). A 1-mm-wide slice was used. The CT values were translated into basic wood density by using a function that was calibrated against gravimetric basic wood density. The calibration material consisted of 19 cubic specimens (with a volume of 10 to 60 cm^3) from 11 different tree species with densities ranging from 320 to 600 kg m^{-3} . The specimens were acclimatized to $\sim 12\%$ moisture content in an environment at 20°C with 65% relative air humidity (18) for 2 weeks before CT values were determined. The following function was calibrated: $\rho_b = 820.18 + 0.792 \cdot \text{CT}$ ($R^2 = 0.99$; root mean squared error, ± 9.1), where ρ_b is the basic wood density and CT is the CT value at an $\sim 12\%$ moisture content. The relationship between CT values and basic density was perfectly linear in the range analyzed.

The impact of *H. parviporum* on wood density was assessed by comparing the observed wood density with a predicted wood density for intact wood prior to fungal decomposition. The predicted value was estimated by using model 15 of Molteberg and Høibø (19) based on a site index and data for year ring width, horizontal and vertical position within the stem, tree height, and diameter at breast height. Since there are considerable residual errors connected to such models, we adjusted the predicted wood density so that density estimates for sound sapwood were at a level similar to the measured density. Taking into account all the heights examined, an average difference between the predicted density and the measured density in intact sapwood was calculated for each tree. This adjustment factor, 75 and 117 kg for trees 4 and 9, respectively, was then subtracted from the predicted wood density values for both heartwood and intact sapwood. All calculations were done with the SAS programming language (28).

Figure 1 continues on the following pages

FIG. 2. Characteristics of advanced decay in inner heartwood in basal stem regions and incipient decay at the top of the stem decay column in Norway spruce wood colonized by *H. parviporum*. (A) Production of conidiophores by *H. parviporum* in the inner heartwood upon advanced decay. Note the frequent localization of white conidiophores in terminal latewood (arrows). (B and C) Delignified earlywood (B) (tree 9) and latewood (C) (tree 4) areas located within inner heartwood at stump height. Sections were stained using safranin-astra blue counterstaining, and astra blue incorporation indicated selective delignification. Note the clefts extending deep into the S2 wall and hyphae (arrows) growing on the S3 wall (C). (D) Safranin-astra blue-counterstained earlywood section from incipient decay at the top of the decay column, showing hyphal sheaths attaching flattened hyphae to the tracheid wall and fragments of hyphal sheaths (arrow). (A) Scale bar = 2 mm. (B to D) Scale bars = 10 μ m.

RESULTS

Visual characteristics of wood and extent of colonization indicated by spatial production of conidiophores. In both trees the interface between colonized heartwood and the pale green to yellow RZ was characterized by dark brown to blue wood, referred to below as aniline wood. In both trees, the RZ displaying an elevated pH was narrower and the associated aniline wood was broader at the base of the stem than at positions higher in the decay column (Fig. 1A and B; see Fig. S1 in the supplemental material).

In disks showing the formation of an RZ, conidiophores characteristic of *Heterobasidion* were present throughout the entire heartwood. However, in the inner heartwood with ad-

vanced decay the conidiophores were sparse; they occurred particularly in the terminal regions of latewood (Fig. 2A). In contrast, there was often heavy production of conidiophores in a belt-like region (Fig. 3A) at the colony edge bordering the RZ area. The belts, typically 1 to 2 mm wide, were restricted mostly to the aniline wood-associated growth rings closest to the RZ, but in basal stem regions similar formations were occasionally also observed 1 to 2 cm from the RZ toward the pith. No RZ or aniline wood had formed in tree 4 at a height of 4 m and in tree 9 at a height of 7 m. At these heights the area with conidiophores was confined to inner heartwood, and abundant conidiophore production was not observed at the colony edges.

FIG. 1. Characteristics of the *H. parviporum* colonized wood analyzed, impact of colonization on wood density, and amounts of pathogen DNA at different heights (the height is indicated at the bottom right in each panel) in naturally infected stems of mature Norway spruce trees 4 (A) and 9 (B). Filled circles, wood density predicted for wood prior to infection; open circles, actual wood density at the time of harvest. The columns show the normalized amounts of pathogen DNA (per mg wood) obtained from 2-mm-wide samples (heights of 1, 3, and 4 m in tree 9) and 5-mm-wide samples (all other heights in both trees). Two DNA isolation replicates were processed using separate DNA isolation series for heights of 1 to 4 m in tree 4; the average coefficient of variation for the amount of DNA was around 10%. Note that for tree 9, excluding the height of 7 m, the profile of the amount of DNA amount was determined only for the colony border area. For all the heights examined in both trees the entire RZ area was included in the profile of the amount of DNA. The striped columns (heights of 0 and 3 m in panel A and heights of 0 and 4 m in panel B) indicate data for the selected DNA samples subjected to ITS rRNA gene sequence analysis in which only a PCR product from *H. parviporum* was detected. The filled column in panel B (height, 4 m) indicates data for a sample in which a PCR product corresponding to *Ascomyces* sp. was codetected with the *H. parviporum* PCR product. Note that no PCR products were obtained from samples taken from the RZ at the four selected sampling areas. The photographs show the visible characteristics of wood in the areas sampled; both the wood density and DNA amount data are aligned so that their data point positions are compatible with each other and with the photographs. In cases where a RZ was formed, the filled bar in the photograph indicates the position of the RZ having an elevated pH.

FIG. 3. Interaction of *H. parviporum* with RZ formed by Norway spruce. (A) Abundant production of conidiophores by *H. parviporum* in the colonization front bordering the RZ area (arrow) with an elevated pH. (B) Unstained section from the inner border of the RZ showing a ray and a few tracheids occluded with yellow-brown polyphenolic deposits. (C) Unstained transverse resin-embedded microtome section prepared from the aniline wood neighboring RZ wood and viewed under blue light. The deposit-impregnated tracheids emit a bright yellow fluorescence characteristic of polyphenols. Note the neighboring tracheids displaying degradation of compound middle lamellae and hosting hyphae (arrow) and hyphal sheath fragments (arrowhead) on the S3 wall. (D and E) PAS-counterstained sections from aniline wood showing diffusion of red-stained material into the phenol deposit (D, viewed under blue light) and a lumen with hyphal growth within a phenolic deposit and an area clear of the deposit (arrow) (E). (F to H) Unstained and safranin-astaxanthin blue-counterstained sections from a colony edge located in aniline wood-associated earlywood bordering the RZ area. Note the abundant golden brown hyphae (F), differential staining of hyphae due to the presence (red) or absence (blue) of a hyphal sheath (G), and hyphal attachment to the tracheid wall by a red-stained sheath upon formation of a microhypha (arrow) that penetrates the secondary wall (H). (A) Scale bar = 2 mm. (B to H) Scale bars = 10 μ m.

Impact of *H. parviporum* on wood density. We used growth ring analysis to obtain retrospective estimates of wood density in the trees studied prior to decomposition by the pathogen. To examine the impact of *H. parviporum* on wood density, we compared these estimates to the measured density of decayed wood. In both trees the estimated weight losses were greatest at the base of the decay column. In tree 4 modest weight losses in the inner heartwood were recorded for the first 2 m of the decay column (Fig. 1A). In tree 9 distinct weight losses were recorded for most of the heartwood in the first 4 m of the decay column (Fig. 1B).

Extent of colonization as determined by pathogen DNA amount profiles. In the sample standard curves constructed for *H. parviporum* and for the reference pGEM DNA there was a linear relationship between the C_T values and the log-transformed amount of DNA in the ranges from 1,000 to 1 pg DNA and from 10 to 0.01 pg DNA, respectively. The equations for standard curves for *H. parviporum* and pGEM, based on the relationship of the log amounts of template (x) generated from known DNA concentrations and the corresponding C_T values (y) were $y = 36.573 - 3.517x$ ($R^2 = 0.998$) and $y = 25.189 - 3.548x$ ($R^2 = 0.9998$), respectively.

To test for the presence of substances inhibitory to PCR, 10-fold dilution series were prepared for all experimental samples. For both *H. parviporum* and pGEM, differences in C_T values between the undiluted and 10-fold-diluted DNA samples were often well below the expected difference, approximately 3.3 cycles. These observations suggest that substances inhibitory to PCR were present in the undiluted samples and/or that there were excess levels of the template. In contrast, the differences in the corresponding C_T values between the 10- and 100-fold diluted DNA samples were in the expected range, which was also observed for the 10-fold dilutions included in the corresponding standard curves. This compatibility indicates that substances inhibitory to PCR amplification or DNA levels greater than the threshold levels for linear detection in the assays were not present in the diluted samples. The 10-fold-diluted DNA samples were then used to calculate the amounts of *H. parviporum* and pGEM reference DNA present in the experimental samples.

The yields of the reference DNA were comparable for the RZ, incipient decay, and advanced decay. To compare the recovery rates for different isolation series, aliquots of tree 4 samples taken at heights of 1, 2, 3, and 4 m were subjected to DNA isolation using two separate isolation series. The mean covariance for the unnormalized DNA yield of *H. parviporum* between the isolation series was 20.1%, indicating that there was moderate variation in the isolation efficiency between the different isolation series. When the DNA yield of the pathogen in each sample was normalized using a conversion factor based on the recovery of pGEM DNA (a conversion factor calculated by dividing the 5 ng added by the DNA yield for pGEM calculated using the appropriate standard curve formula), the mean covariance for the DNA yield of *H. parviporum* for the two isolation series was reduced to 12.1%. This indicates that variation in the DNA isolation efficiency had similar effects on the two DNA pools; i.e., a lower yield of *H. parviporum* DNA for a sample in isolation series A than in isolation series B coincided with a similar difference in the yield of pGEM DNA.

The shapes of the unnormalized and pGEM-normalized

DNA amount profiles for *H. parviporum* were very similar (data not shown), but due to the moderate variation in DNA extractability the normalized DNA amount data were used as the basis for sample comparison. The shapes of the pathogen DNA amount profiles differed clearly for different heights of the decay column (Fig. 1A and B). In both of the trees examined, the pathogen DNA amount at stump level peaked approximately 20 mm behind the colony margin bordering the RZ. A similar decline in the pathogen DNA amount at the colony frontier was observed at heights of 1 and 3 m in tree 9, while at other heights where colonization was restricted to the RZ the pathogen DNA amount peaked at the colony margin in both trees. At the top of the decay column, at a height of 4 m in tree 4 and at a height of 7 m in tree 9, no detectable RZ had been formed, and the *H. parviporum* DNA amount peaked some distance behind the colony frontier that had not yet reached the heartwood-sapwood border.

ITS rRNA gene sequence analysis. A total of 30 ITS amplicons were sequenced (Fig. 1A and B). A single PCR product with the same *H. parviporum* sequence was obtained from tree 9 at a height of 0 m and from tree 4 at heights of 0 and 3 m using the selected samples taken from decayed heartwood and aniline wood (Fig. 1A and 1B). For tree 9 at a height of 4 m the five innermost samples facing the pith also produced a single PCR product with an *H. parviporum* sequence. In the sample obtained at the interface between aniline wood and the RZ, a very faint PCR product with a sequence corresponding to the sequence of the ascomycete genus *Ascocoryne* was detected together with an *H. parviporum* PCR product (Fig. 1B). For these four sampling sectors no PCR products were obtained from samples obtained from the RZ.

Wood degradation and hyphal growth pattern for advanced and incipient decay. To visualize the degree of delignification, differential staining with safranin and astra blue was employed. Within the inner heartwood advanced decay was characterized by patches with delignified tracheids, as indicated by the incorporation of astra blue into the secondary cell wall. Delignified areas, which appeared to be larger in tree 9 than in tree 4, were found in both early wood and latewood (Fig. 2B and C). In areas with advanced decay the secondary wall had areas devoid of lignin and clefts that extended deep into the S2 layer. Individual hyphae were typically observed on the S3 layer of latewood, and no sheath-like material was visible (Fig. 2C).

At the top of the decay columns with incipient decay, hyphae were restricted primarily to earlywood. Here they were attached particularly to the cell wall corners of tracheids by extracellular material that stained strongly with safranin (Fig. 2D) and PAS (not shown). These hyphae were often half-moon shaped in cross section, with the maximized hyphal surface area oriented toward the tracheid wall. In areas with incipient decay hyphae were also common within rays containing polyphenolic deposits and residual starch granules. Such hyphae were typically circular in cross section and had sheath-like material that surrounded the entire hyphal surface and stained positively with PAS and safranin (not shown). Diffusion of PAS-stained material into the lumen phenolics and areas cleared with this type of deposit was also commonly observed in phenol-filled rays showing hyphal growth (not shown).

Wood degradation and pattern of hyphal growth upon penetration of the RZ. The two trees analyzed had similar wood anatomy and hyphal characteristics in the areas surrounding the RZ. Within the aniline wood bordering the RZ, tracheid lumina and ray cells had various amounts of deposits (Fig. 3B), and occluded tracheids sometimes formed a continuous tangential belt. Under UV light the ray and tracheid lumen deposits emitted a yellow fluorescence characteristic of phenolic compounds (Fig. 3C). These regions stained positively for phenols with ferric chloride as well (data not shown). The hyphae associated with such deposits were typically circular in cross section and encased in a sheath-like material that stained with PAS (Fig. 3D and E) and safranin (not shown). Diffusion of PAS-stained extracellular material into the phenolic deposit (Fig. 3D) and areas devoid of such a deposit (Fig. 3E) were also commonly observed in lumens showing hyphal growth in the deposit. Hyphae that were golden brown and had numerous hyphal tips were observed mostly within earlywood tracheids lacking any lumen deposit (Fig. 3F). The hyphae observed here, some of which were flattened and other of which were circular in cross section, were associated mostly with lumen corners and were partially or fully encased by a sheath-like material that was stained by PAS (not shown) and safranin (Fig. 3G). Cracks in the cell walls of earlywood tracheids were commonly observed in these regions, as were fragments of hyphal sheaths attached to the tracheid cell wall (Fig. 3G). Growth along the longitudinal axis of the tracheids, characterized by adhesion of hyphae to each other (not shown), appeared to be the main direction of growth at the colony frontier. Transverse penetration of the tracheid cell wall was achieved via formation of microhyphae by basal hyphae. These hyphae were attached to the S3 wall by extracellular material that was stained strongly with safranin (Fig. 3H) and PAS (not shown). Most septa in hyphae were simple, but clamp connections were occasionally formed, as were chlamydospore-like structures (not shown).

DISCUSSION

In Norway spruce, challenge of sapwood by a heartwood-based colony of the white-rot fungus *H. parviporum* triggers formation of an RZ. Fungal penetration into the RZ is followed by expansion of this xylem defense to inner sapwood, which first turns into an RZ and eventually becomes part of the decay column. Finally, this process terminates in death of the tree due to a reduced amount of conductive tissue or stem failure. The trees examined here were each colonized by a different heterokaryotic genotype of *H. parviporum* and displayed the features typical of the pathosystem: an extensive stem decay column having a conical form separated from the sapwood by an RZ, a region with an elevated pH and enrichment of phenolics (36). The presence of a single ITS rRNA gene PCR product corresponding to *H. parviporum* in all but one assay sample from advancing decay and the discolored colony border indicates that there was territorial predominance of the pathogen. Hemicelluloses and pectin are the preferred carbon sources of fungi capable of selective delignification (4). Together with lignin, they make up about 55% of the dry mass of Norway spruce heartwood (3). The estimated maximum weight losses at stump level, 35% for tree 9 and 20%

for tree 4, thus indicate that the trees were in the intermediate and relatively early stages of decay, respectively. It is still difficult to evaluate the impact of fungal colonization on wood density in aniline wood due to the substantial amount of extractives present in this region (38).

At the top of the decay columns and at the lateral colony margins bordering the RZ, hyphae were associated mostly with earlywood. In contrast, advanced decay within inner heartwood was characterized by hyphal association with latewood. This pattern probably reflects faster depletion of nutrients from earlywood with thin-walled tracheids. Incipient decay at the top of the decay column was characterized by hyphae attached to the tracheid wall by a carbohydrate-rich sheath, as indicated by its staining with PAS. Similarly stained material was occasionally observed lining the entire S3 wall. The model white-rot fungus *Phanerochaete chrysosporium* possesses β -1,3-1,6-linked glucans as an extracellular sheath but can also excrete glucan into the culture medium (27). Both ligninolytic enzymes and hemicellulases have been immunolocalized in the hyphal sheath of white-rot fungi during decay (12, 27). This matrix is thought to facilitate wood degradation via immobilization of fungal enzymes on the polysaccharide filaments. The sheath may also provide a suitable microenvironment for enzyme action by adjusting the pH at the interface (12). Extracellular glucan has also been proposed to provide a source of hydrogen peroxide that is involved in fungal attack on wood. The generation of this compound within the glucan matrix lining the tracheid wall reduces the risk of toxicity to the fungus (1). The observed absence of a hyphal sheath in areas with advanced decay is compatible with these roles as the oxidative attack on lignin should decline with advancing delignification.

In trees whose decay column was characterized, the intact RZ gradually broadened toward the younger regions of the decay column, while the aniline wood, a dark tissue with fungistatic properties similar to those of the RZ (14, 30), showed the opposite change. Following exposure to air after wood is cut, the originally light green to yellowish RZ becomes bluish to black due to oxidation of phenols (30). Taken as a whole, these observations suggest that the aniline wood represents a region of the RZ that has been compromised, with the discoloration probably resulting from oxidized RZ components. Comparison of the chemical compositions of aniline wood and the RZ would be required to verify their proposed relationship and to evaluate whether a more precise term could be adopted for aniline wood. Like heartwood of Norway spruce formed under normal circumstances without any pathological effects (3), the RZ is also separated from the sapwood by a dry transition zone (30). Infection of conifer sapwood by *H. annosum* sensu lato is known to induce the formation of dry zones ahead of the expanding colony (5, 6), but what triggers the formation and expansion of the RZ remains unknown.

Excluding the stump level, the profiles of the amount of DNA and conidiophore production coincided with the aniline wood closest to the RZ. Hyphae within aniline wood displayed the irregular formation of clamp connections characteristic of heterokaryotic mycelia of the species (17), and the golden brown color has also been observed for *H. annosum* sensu stricto hyphae upon growth within polyphenol-impregnated rays in Scots pine (29). While in areas with incipient decay the carbohydrate-rich material was spatially restricted to the hy-

phal surface facing the tracheid wall, the hyphae were fully encased by a carbohydrate-rich sheath when they were exposed to phenolic lumen material of tracheids and ray cells. In the sheath material of spores of the ascomycete *Colletotrichum graminicola*, a maize anthracnose pathogen, the glycoprotein components bind phenols and allow spore germination under conditions that are otherwise toxic (21, 22). Similarly, a prerequisite for fungal takeover of the RZ is the ability to tolerate exposure to polyphenols. The hyphal sheath that encapsulates *H. parviporum* hyphae upon exposure of hyphae to phenols may thus prevent membrane permeabilization by organosolvents. This would allow maintenance of cellular activities in this phytotoxic environment. It is unclear how common the production of this matrix is in other fungi when they are subjected to such conditions. However, increased production of a hyphal sheath has been observed upon exposure of artificially grown wood decay fungi to an adverse pH and fungicides (37, 38).

Degradation of polyphenols by *H. parviporum* within aniline wood was suggested by the apparent hyphal secretion of carbohydrate material into the surrounding phenolics and the appearance of lumen areas in which the deposit was cleared. This pattern resembled the pattern of degradation of polyphenols by *Ustilina deusta* in the RZ of large-leaved lime (2). Since in general the ligninolytic extracellular peroxidases of white-rot fungi are nonspecific, it could be expected that these enzymes participate in the oxidation of polyphenols associated with RZ. In our recent study we monitored the levels of transcripts of two manganese peroxidases of *H. parviporum* at the basal level of decay columns in Norway spruce stems (39); the three trees studied included tree 4 examined in this study and two additional trees with advanced decay. Later, we examined the transcription of three additional manganese peroxidases in these three trees (unpublished data). None of the five manganese peroxidases was upregulated at the RZ-bordering colony edge. Yet the genes encoding an aryl-alcohol oxidase putatively involved in production of hydrogen peroxide (a cosubstrate required by manganese peroxidases), two laccases, and several P450 monooxygenases were among the genes that were upregulated by *H. parviporum* at the colony edge bordering the RZ (39). As conidiophore production depends on transport of nutrients from assimilative hyphae, the observed dense conidiophore belt within aniline wood suggests that an efficient translocation channel is present at the colony margin. Consistent with this, a myosin binding protein also showed striking upregulation at the colony edge bordering the RZ (39). Myosins are molecular motors that facilitate vesicle transport and in this capacity contribute to both hyphal growth and exo- and endocytosis (34). The possible roles of laccases and H_2O_2 -associated oxidation in detoxification of RZ components, of P450 monooxygenases in intracellular metabolism of the corresponding degradation products, and of the intracellular trafficking upon conversion of RZ as part of the expanding decay column of *H. parviporum* need to be examined in future studies.

In summary, interaction of *H. parviporum* with the inner border of the RZ in stems of Norway spruce involves accumulation of fungal biomass and encapsulation of hyphae in a carbohydrate-rich hyphal sheath upon hyphal growth in polyphenolic lumen material. While the present detailed study was

conducted with two trees, we have verified that these RZ-associated patterns of fungal morphology and spatial conidiophore production occur in several additional trees (unpublished data). To better understand the mechanisms of adaptation of this white-rot fungus to conditions in the heartwood and RZ of Norway spruce, elucidation of the exact composition and roles of the hyphal sheath upon fungal exploitation of lignocellulose and phenolics is a natural area for future investigation.

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REFERENCES

- Barrasa, J. M., A. Gutierrez, V. Escaso, F. Guillen, M. J. Martinez, and A. T. Martinez. 1998. Electron and fluorescence microscopy of extracellular glucan and aryl-alcohol oxidase during wheat straw degradation by *Pleurotus eryngii*. Appl. Environ. Microbiol. 64:325–332.
- Baum, S., and F. W. M. R. Schwarze. 2002. Large-leaved lime (*Tilia platyphyllos*) has a low ability to compartmentalize decay fungi via reaction zone formation. New Phytol. 154:481–490.
- Bertaud, F., and B. Holmbom. 2004. Chemical composition of earlywood and latewood in Norway spruce heartwood, sapwood and transition zone wood. Wood Sci. Technol. 38:245–256.
- Blanchette, R. A. 1984. Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. 48:647–653.
- Coutts, M. P. 1976. The formation of dry zones in the sapwood of conifers. I. Induction of drying in standing trees and logs by *Fomes annosus* and extracts of infected wood. Eur. J. For. Pathol. 6:372–381.
- Coutts, M. P. 1977. The formation of dry zones in the sapwood of conifers. II. The role of living cells in the release of water. Eur. J. For. Pathol. 7:6–12.
- Coyne, K. J., S. M. Handy, E. Demir, E. B. Whereat, D. A. Hutchins, K. J. Portune, M. A. Doblin, and S. C. Cary. 2005. Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. Limnol. Oceanogr. Methods 3:381–391.
- Del Cerro, M., J. Cogen, and C. del Cerro. 1980. Stevenel's blue, an excellent stain for optical microscopical study of plastic embedded tissues. Microsc. Acta 83:117–121.
- Franceschi, V. R., T. Krekling, A. A. Berryman, and E. Christiansen. 1998. Specialized phloem parenchyma cells in Norway spruce (Pinaceae) bark are an important site of defense reactions. Am. J. Bot. 85:601–615.
- Gahan, P. B. 1984. Plant histochemistry and cytochemistry. Academic Press, London, United Kingdom.
- Gardes, M., and T. D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113–118.
- Gutierrez, A., M. J. Martinez, G. Almendros, A. Prieto, F. J. Gonzalez-Vila, and A. T. Martinez. 1995. Hyphal-sheath polysaccharides in fungal deterioration. Sci. Total Environ. 167:315–328.
- Hietala, A. M., M. Eikenes, H. Kvaalen, H. Solheim, and C. G. Fossdal. 2003. Multiplex real-time PCR for monitoring *Heterobasidion annosum* colonization in Norway spruce clones that differ in disease resistance. Appl. Environ. Microbiol. 69:4413–4420.
- Johansson, M., and O. Theander. 1974. Changes in sapwood of roots of Norway spruce attacked by *Fomes annosus*. Part I. Physiol. Plant. 30:218–225.
- Korhonen, K. 1978. Intersterility groups of *Heterobasidion annosum*. Commun. Inst. For. Fenn. 94:1–25.
- Korhonen, K. 2003. Simulated stump treatment experiments for monitoring the efficacy of *Phlebiopsis gigantea* against *Heterobasidion* infection, p. 206–210. In G. Laflamme, J. A. Bérubé, and G. Bussières (ed.), Root and butt rots of forest trees. 10th International Conference on Root and Butt Rots. Natural Resources Canada, Canadian Forest Service, Sainte-Foy, Quebec, Canada.
- Korhonen, K., and J. Stenlid. 1998. Biology of *Heterobasidion annosum*, p. 43–70. In S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann (ed.), *Heterobasidion annosum*—biology, ecology, impact and control. CAB International, Oxford, United Kingdom.
- Kučera, B. 1992. Skandinaviske normer for testing av små feilfrie prøver av heltre. Skogforsk, Norwegian Forest Research Institute, Ås, Norway.

19. Molteberg, D., and O. Høibø. 2007. Modelling of wood density and fibre dimensions in mature Norway spruce. *Can. J. For. Res.* **37**:1373–1389.
20. Nagy, N. E., V. R. Franceschi, H. Solheim, T. Krekling, and E. Christiansen. 2000. Wound-induced traumatic resin duct formation in stems of Norway spruce (Pinaceae): anatomy and cytochemical traits. *Am. J. Bot.* **87**:302–313.
21. Nicholson, R. L., L. G. Butler, and T. N. Asquith. 1986. Glycoproteins from *Colletotrichum graminicola* that bind phenols: implications for survival and virulence of phytopathogenic fungi. *Phytopathology* **76**:1315–1318.
22. Nicholson, R. L., J. Hipskind, and R. M. Hanau. 1989. Protection against phenol toxicity by the spore mucilage of *Colletotrichum graminicola*, an aid to secondary spread. *Physiol. Mol. Plant Pathol.* **35**:243–252.
23. Niemelä, T., and K. Korhonen. 1998. Taxonomy of the genus *Heterobasidion*, p. 27–33. In S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann (ed.), *Heterobasidion annosum*—biology, ecology, impact and control. CAB International, Oxford, United Kingdom.
24. Pearce, R. B. 1996. Tansley review no. 87. Antimicrobial defenses in the wood of living trees. *New Phytol.* **132**:203–233.
25. Piri, T. 2003. Early development of root rot in young Norway spruce planted on sites infected by *Heterobasidion* in southern Finland. *Can. J. For. Res.* **33**:604–611.
26. Regent Instruments Inc. 2002. WinDendro 2002b. Regent Instruments Inc., Quebec, Canada.
27. Ruel, K., and J.-P. Joseleau. 1991. Involvement of an extracellular glucan sheath during degradation of *Populus* wood by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **57**:374–384.
28. SAS Institute Inc. 1990. SAS language: reference, version 6, 1st ed. SAS Institute Inc., Cary, NC.
29. Schwarze, F. W. M. R., J. Engels, and C. Mattheck. 2000. Fungal strategies of wood decay in trees. Springer, Berlin, Germany.
30. Shain, L. 1971. The response of sapwood of Norway spruce to infection by *Fomes annosus*. *Phytopathology* **61**:301–307.
31. Shain, L., and W. E. Hillis. 1971. Phenolic extractives in Norway spruce and their effects on *Fomes annosus*. *Phytopathology* **61**:841–845.
32. Siemens. 2004. Somatom Syngo V.B10B_FP3. DICOM conformance statement. Siemens AG, Berlin, Germany.
33. Srebotnik, K., and K. Messner. 1994. A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi. *Appl. Environ. Microbiol.* **60**:1383–1386.
34. Steinberg, G. 2007. Hyphal growth: a tale of motors, lipids, and the Spitzenkörper. *Eukaryot. Cell* **6**:351–360.
35. Stenlid, J. 1985. Population structure of *Heterobasidion annosum* as determined by somatic incompatibility, sexual incompatibility, and isoenzyme patterns. *Can. J. Bot.* **63**:2268–2273.
36. Stenlid, J., and D. Redfern. 1998. Spread within the tree and stand, p. 125–141. In S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann (ed.), *Heterobasidion annosum*—biology, ecology, impact and control. CAB International, Oxford, United Kingdom.
37. Vesentini, D., D. J. Dickinson, and R. J. Murphy. 2005. The production of extracellular mucilaginous material (ECMM) in two wood-rotting basidiomycetes is affected by growth conditions. *Mycologia* **97**:1163–1170.
38. Vesentini, D., D. J. Dickinson, and R. J. Murphy. 2006. Fungicides affect the production of extracellular mucilaginous material (ECMM) and the peripheral growth unit (PGU) in two wood-rotting basidiomycetes. *Mycol. Res.* **110**:1207–1213.
39. Yakovlev, I., A. M. Hietala, A. Steffenrem, H. Solheim, and C. G. Fossdal. 2008. Identification and analysis of differentially expressed *Heterobasidion parviporum* genes during natural colonization of Norway spruce stems. *Fungal Gen. Biol.* **45**:498–513.