Inducible anatomical defense responses in Norway spruce stems and their possible role in induced resistance

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Summary  Norway spruce (Picea abies (L.) Karst.) trees were preinoculated with a sublethal dose of the blue-stain fungus Ceratocystis polonica Siem. (C. Moreau) 1 to 52 weeks before they were mass inoculated with the same fungus. Trees pretreated 1 week before mass inoculation had similar, severe symptoms of fungal infection as the control trees. Pretreatment 3, 6 or 9 weeks before mass inoculation resulted in effective protection of the trees, reducing pathogenic symptoms by 63–90% relative to the control trees, whereas pretreatment 52 weeks before mass inoculation gave intermediate protection (44–71% reduction in symptoms). Thus, pretreatment induced resistance to the blue-stain fungus in Norway spruce by a process that requires more than 1 week to become activated and protects trees for at least one year after pretreatment. Pretreatment induced formation of traumatic resin ducts (TDs) in the sapwood and swelling and proliferation of polyphenolic parenchyma cells (PP cells) in the phloem. Trees pretreated 3–9 weeks before mass inoculation had more TDs and showed greater swelling of existing PP cells than control trees or trees pretreated 1 week before mass inoculation. We conclude that induced disease resistance in Norway spruce is probably associated with PP cell activation and TD induction, because resistance was enhanced within the same time frame as the induction of these defense responses.

Keywords: Ceratocystis polonica, inducible defenses, Picea abies, polyphenolic parenchyma cells, traumatic resin ducts.

Introduction

Norway spruce (Picea abies (L.) Karsten) is subjected to large-scale outbreaks of the spruce bark beetle Ips typographus (L.) (Christiansen and Bakke 1988, Führer 1996). The beetles transfer phytopathogenic fungi that play an important role in tree killing by enhancing the virulence of each attack (Horntvedt et al. 1983, Christiansen 1985, Solheim 1988). With a high enough attack density, the beetle–fungus complex may overwhelm the defensive capacity of healthy trees, destroying the active phloem, disrupting xylem water transport and eventually killing the tree (Horntvedt et al. 1983, Solheim 1992). The most important fungal associate of I. typographus is the blue-stain fungus Ceratocystis polonica (Siem.) C. Moreau, which is capable of killing healthy Norway spruce trees when artificially mass inoculated into the stem bark (e.g., Horntvedt et al. 1983, Christiansen 1985, Krokene and Solheim 1998).

Recently, we have demonstrated that pretreatment with sublethal fungal inoculations increases the resistance of Norway spruce to subsequent mass inoculation with C. polonica and to mass attack by I. typographus (Christiansen and Krokene 1999, Christiansen et al. 1999b, Krokene et al. 1999). This induced resistance follows dose-response dynamics, is nonsystemic and is restricted to the pretreated stem area, and is nonspecific with respect to the nature of the pretreatment (mechanical wounding or inoculation with different necrotizing fungi protects trees against C. polonica infection) (Krokene et al. 1999, 2001). However, it has not been determined how soon after pretreatment resistance is activated, or how long a tree is protected by a pretreatment.

The exact mechanisms responsible for induced resistance have not been identified, but they probably involve two inducible anatomical defense responses: changes in polyphenol-containing parenchyma cells (PP cells) in the phloem (Franceschi et al. 1998, 2000) and induction of traumatic resin duct (TD) formation in the sapwood (Christiansen et al. 1999a, Nagy et al. 2000). The PP cells represent a major proportion of the living cells in the secondary phloem of Norway spruce and are a primary site of both constitutive and induced defenses (Franceschi et al. 1998, 2000). The dark staining PP cells form concentric layers around the stem and a new 1–2 cells thick layer is differentiated in May–June every growing season (Krekling et al. 2000). In addition, extra PP cells may develop from undifferentiated parenchyma cells later in the season (Krekling et al. 2000). After wounding or infection of the bark, the PP cells alter their phenolic content and expand in size. This response, which is activated within a few days, probably helps to inhibit fungal growth and arrest infections (Franceschi et al. 2000).
Induction of axial TDs in the sapwood is a widespread defense mechanism in conifers against wounding, fungal infection and insect attack (see Christiansen et al. 1999a and references therein). In Norway spruce, TDs are usually formed in tangential layers, as opposed to “normal” axial resin ducts that occasionally are found scattered among the 5–10 rows of tracheids formed at the end of the growing season (Nagy et al. 2000). Induction of TD formation is a slower process than PP cell activation, and about 2–3 weeks are required for the formation of ducts with secretory capacity (Nagy et al. 2000, Krekling et al., unpublished results).

Taken together, the anatomical and chemical features of the PP cells and TDs are likely to have a significant effect on the growth and spread of pathogenic fungi introduced by bark beetles. However, the temporal relationship between resistance to pathogens and induction of these defense structures is unknown. The objectives of the present study were: (1) to determine the onset and duration of induced resistance in Norway spruce; and (2) to elucidate the mechanisms underlying induced resistance by correlating degree of resistance in pretreated trees with quantitative anatomical data on PP cell activation and induction of TD formation.

Materials and methods

Effect of pretreatment on tree resistance

The experiments were carried out in 1997 and 1998 in a plantation of Norway spruce clones at Hogsmark, As, SE Norway. The clonal trees were planted in 1970 in a regular 2 × 2 m array, and were about 15 m high, with a diameter 1.3 m above ground of about 17 cm (see Franceschi et al. 1998 for further description of the stand).

In 1997, one tree from each of Clones 193 and 194 was pretreated with a sublethal dose of C. polonica one (P1), three (P3), six (P6) or nine (P9) weeks before being mass inoculated with the same fungus at a dose that would be lethal to normal trees of similar size and age (Christiansen 1985). One additional tree from each clone was mass inoculated without pretreatment as a control (C). In 1998, five trees from each of seven clones (including the two clones used in 1997) were given the same five pretreatments as in 1997 and later mass inoculated with C. polonica. In addition, we mass inoculated one tree per clone that had been pretreated 52 weeks earlier (P52). Pretreatment and mass inoculations were made using a 5-mm cork borer, as described by Krokene and Solheim (1998). Trees were pretreated at 50 inoculations per m² (~21 inoculations per tree) and mass inoculated at 400 inoculations per m² (~169 inoculations per tree). All inoculations were evenly spaced over a 0.8-m section of the stem from a height of 1.2 to 2 m. Mass inoculation took place on July 15, 1997 and July 7–9, 1998.

Trees were harvested 15 weeks after mass inoculation. Before harvesting, we measured the vertical extension of four of the uppermost and four of the lowermost phloem necroses on each tree. Because necroses tend to coalesce within mass inoculated sections in susceptible trees, necrosis lengths were measured from the center of upper and lower inoculation wounds and away from the inoculation band. Two thin discs (5–10 mm) were cut from within the inoculation band at about 1.4 and 1.8 m height. We measured the area of blue-stained and desiccated sapwood and the proportion of dead and live cambium on each disc (Krokene and Solheim 1998). In 1998, fungus was re-isolated from three mass inoculation points per tree.

Sapwood and phloem anatomy

In 1997, samples for light microscopy studies were collected at the time of pretreatment (two samples outside the inoculated stem section), mass inoculation (two samples inside the section) and felling (two samples inside and one sample 5–10 cm above the section). Samples containing bark and sapwood (2 cm in length and breadth, 1.5 cm deep) were placed directly in fixative (2% paraformaldehyde and 1.25% glutaraldehyde buffered in 50 mM L-piperazine-N,N'-bis(2-ethane sulfonic) acid, pH 7.2). In the laboratory, the periderm was excised while the sample was submerged in fixative, and subsamples (20 mm long, 4 mm wide, 1 mm deep) containing phloem, cambium and sapwood were cut with razor blades. The subsamples were placed in fixative overnight at room temperature, rinsed with 100 mM Hepes buffer (pH 7.0), dehydrated in a graded series of ethanol and embedded in L.R. White acrylic resin (TAAB Laboratories, Aldermaston, Berkshire, U.K.). Cross sections (1 µm thick) were cut with a diamond knife, dried on gelatin-coated slides, stained with Stevenel’s blue (Del Cerro et al. 1980), and mounted with immersion oil. Digital images were recorded at magnifications of ×10 (phloem) and ×4 (xylem) with a Leica DC100 CCD camera mounted on a Leitz Aristoplan photomicroscope, and analyzed using ThumbsPlus image analysis software (V4.02, Ceroius Software, Charlotte, NC).

In the xylem, we measured the width of the last annual growth ring (not including the cambial zone) and the distance from any TDs present to the cambium–sapwood border. To facilitate comparison between sections with varying annual ring widths, the distance from the TDs to the cambial zone was expressed as a proportion of the full ring width. Along 1.1 mm in the tangential direction on each cross section, we measured the proportion of xylem that was covered with TDs (including the epithelial cells lining the ducts). Normal axial resin ducts, which are rare, were not measured.

In the phloem, we measured the proportion of phloem that was covered with PP cells along 0.58 mm in the tangential direction on each section. We included the last three annual layers of PP cells (PP₀, PP₁, and PP₂), plus any extra PP cells found among the sieve cells between the cambial zone and PP₀. We also measured the diameter (radial direction) of 5–7 representative PP cells within each annual layer of PP cells.

Statistical analyses

Data from the 1998 inoculation experiment was subjected to ANOVA, using the general linear models procedure of the SAS software package (1987; SAS Institute, Cary, NC). If
treatments were significantly different ($P < 0.05$), means were separated by LSD at $P = 0.05$. Proportional data were arcsin-transformed and data on necrosis lengths were log-transformed before ANOVA to correct for unequal variance and departures from normality. Data on tree symptoms from the 1997 experiment were not analyzed statistically because there were only two replicates that year (one tree per clone).

**Results**

**Effect of pretreatment on tree resistance**

Trees pretreated with *C. polonica* 3–52 weeks before mass inoculation had less necrotic cambium, less blue-stained sapwood and shorter phloem necroses than untreated control trees (Figures 1 and 2). Trees pretreated 1 week before mass inoculation had symptoms similar to the control trees, and were heavily colonized by the fungus (Figures 1 and 2). There were no significant differences in symptoms in trees pretreated 3–9 weeks before mass inoculation, and these pretreatments seemed to give equally good protection against fungal infection. The results were consistent across study years. Pretreatment 52 weeks before mass inoculation in 1998 seemed to give intermediate protection between C and P3–P9, but the difference between P52 and P3–P9 was significant only in the case of cambium necrosis (Figure 2). There were significant differences in resistance to the fungus among the seven clones used in 1998 (necrotic cambium: $F = 10.41, P = 0.0001$; blue-stained sapwood: $F = 4.63, P = 0.002$; necrosis length: $F = 3.35, P = 0.01$), with Clone 194 (one of the two clones used in 1997) being the most resistant. *Ceratocystis polonica* was re-isolated from 44% of all mass inoculation points and from 71% of all trees.

**Tree resistance and sapwood anatomy**

Of the two clones tested in 1997, Clone 194 showed a greater increase in resistance after pretreatment than Clone 193 (Figure 3). At the time of mass inoculation, TDs (Figure 4B) had formed in trees pretreated 3–9 weeks earlier (referred to as induced trees), but not in control trees or trees pretreated 1 week earlier (non-induced trees) (Figure 3). Traumatic resin duct coverage was negatively correlated with fungal colonization success following mass inoculation (both clones combined ($n = 10$); percent blue-stained sapwood: $r^2 = 0.63, P = 0.006$; percent dead cambium: $r^2 = 0.34, P = 0.08$; necrosis length: $r^2 = 0.56, P = 0.01$). Thus, trees that produced more TDs in response to pretreatment tended to be more resistant to mass inoculation with *C. polonica*. This relationship appeared to be stronger in Clone 194 than in Clone 193, probably because all trees in Clone 193 (including the induced trees) were quite susceptible and showed extensive symptoms of infection (Figure 3).

Because the cambium continues to produce tracheids throughout the summer, TDs were embedded further into the xylem with increasing time after pretreatment (Figures 4B and 4D). The TDs were still within the cambial zone 3 weeks after pretreatment (Figure 4B), and were embedded 23 and 70% into the current annual ring after 6 and 9 weeks, respectively (mean values for both clones). Tree resistance did not appear to depend on the radial distance from the ducts to the inoculation sites on the sapwood surface, because there was no correlation between TD depth and fungal colonization success (both clones combined ($n = 6$); $r^2 = 0.01–0.07$ and $P = 0.61–0.84$ for the various tree symptoms).

All trees produced abundant TDs in response to the mass inoculation, and in many trees, two rings of TDs were observed at the time of felling (Figure 4D). The inner ring was probably induced by the pretreatment and the outer ring by the mass inoculation. The mass inoculation induced more extensive TD production than the pretreatment, resulting in a TD coverage of almost 50% of the sapwood circumference in some trees (Figure 5). Non-induced trees without TDs before mass inoculation also produced TDs after mass inoculation (Figure 5), but they produced fewer TDs than induced trees because most

Figure 1. Symptoms of fungal infection in Norway spruce trees mass inoculated with *Ceratocystis polonica* in 1997. Trees from Clones 193 (hatched bars) and 194 (white bars) were pretreated with a sublethal inoculation of *C. polonica* 1–9 weeks before mass inoculation (P1–P9), or mass inoculated without prior pretreatment (C) ($n = 1$).

Figure 2. Symptoms of fungal infection in Norway spruce trees mass inoculated with *Ceratocystis polonica* in 1998. Trees from seven different clones were pretreated with a sublethal inoculation of *C. polonica* 1–52 weeks before mass inoculation (P1–P52), or mass inoculated without prior pretreatment (C) ($n = 7$). Bars with the same letter were not significantly different (LSD test at $P = 0.05$ following ANOVA).
of the cambium was killed by the fungus. 

**Tree resistance and phloem anatomy**

The annual layers of PP cells formed continuous rings around the phloem circumference, interrupted only by radial rays and an occasional sieve cell (Figure 4A). There were no clear differences in the percentage of the phloem circumference that was covered by PP cells between clones (68 and 71% in Clones 193 and 194, respectively), sampling times (70, 64 and 73% at pretreatment, mass inoculation and felling, respectively), annual layers (65, 71 and 70% for PP_{95}, PP_{96}, and PP_{97}, respectively), or induced and non-induced trees (68 and 70%, respectively). There were, however, notable differences in the number of extra PP cells (Figure 4C) produced between annual layers. The coverage of extra PP cells increased from

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**Figure 3.** Traumatic resin duct (TD) formation in Norway spruce trees at the time of mass inoculation in 1997 and symptoms of fungal infection 15 weeks later (black bars = blue-stained sapwood, hatched bars = dead cambium circumference and white bars = phloem necrosis length). Trees from Clones 193 and 194 were pretreated with a sublethal inoculation of *Ceratocystis polonica* 1–9 weeks before mass inoculation with the same fungus (P1–P9), or mass inoculated without pretreatment (C) (*n* = 1). The TD-coverage was measured as the proportion of xylem that was covered with TDs along 1.1 mm in the tangential direction on two samples per tree.

**Figure 4.** Anatomical responses in sapwood and phloem of Norway spruce after pretreatment inoculation and mass inoculation with *Ceratocystis polonica*. All figures are cross sections. (A) Fresh bark at the time of pretreatment (June 24) showing anatomy typical of untreated samples. Sieve cells (S) and the three most current annual layers of PP cells (PP_{95–97}) can be seen above the cambial zone (CZ). R = radial ray. (B) Sample taken 3 weeks after pretreatment inoculation (July 15), with a layer of large traumatic resin ducts (TD) at the interface of the xylem (X) and cambial zone. (C) Bark sample taken above the mass inoculated stem section 15 weeks after mass inoculation (October 27), showing swelling of PP cells, crushing of the older sieve cell layers, and abundant extra PP cells between the cambial zone and PP_{97} (PP_{ex}) and adjacent to the cambium (arrowheads). (D) Sample taken 15 weeks after mass inoculation (October 27) from a tree that was pretreated 24 weeks earlier. Two distinct layers of large TDs can be seen; the layer close to the cambium (TD_{2}) was probably induced by the mass inoculation, whereas the layer deeper within the xylem (TD_{1}) was probably induced by the pretreatment 9 weeks earlier. Bars = 50 µm (A and C) and 200 µm (B and D).
2.3% of the phloem circumference before mass inoculation to 14.0% after mass inoculation (mean numbers for both clones and all cell layers examined). Most of the extra PP cells observed after mass inoculation were found between the cambial zone and PP97, and the susceptible Clone 193 had many more extra PP cells than the resistant Clone 194 (Figure 6). Only samples taken 5–10 cm above the inoculated section of the stem could be analyzed after mass inoculation, because in Clone 193, the phloem samples taken closer to the inoculation point were destroyed by fungus.

There were no clonal differences in PP cell size within the annual layers at the time of pretreatment (t-tests; PP95: $P = 0.93$; PP96: $P = 0.10$). (The PP97 could not be analyzed because PP cells were not fully differentiated at the time of sampling.) However, at the time of mass inoculation, the PP cells in induced trees had increased in size compared with those in non-induced trees (Figure 7A), and this increase was most pronounced in Clone 193. In this clone, the PP cells in all three annual layers that were examined increased in size, whereas in Clone 194, only the newly formed cells in PP97 had swollen substantially (Figure 7A). After mass inoculation, there was a further increase in PP cell size in induced trees (Figure 4C). Again, the swelling was stronger in Clone 193, where all three PP cell layers responded similarly (Figure 7B), compared with Clone 194 where the response seemed weaker in the older cell layers.

**Discussion**

This study supports the hypothesis that PP cell activation and TD induction play a functional role in induced resistance in Norway spruce. Resistance to blue-stain fungus developed over a time frame that paralleled PP cell activation and TD formation. Furthermore, the extent of TDs produced in response to pretreatment was positively correlated with tree resistance, and there was more extensive swelling of PP cells in trees with increased resistance after pretreatment than in trees that remained more susceptible.

Previous work has suggested that TDs add considerably to a tree’s total resin reservoir, and that induction of TD formation is important for development of induced resistance in Norway spruce (Christiansen et al. 1999a, 1999b). Resin produced after fungal inoculation appears to be more toxic or fungistatic...
than constitutive resin (Solheim 1991), possibly because of inclusion of phenolics (Nagy et al. 2000) or changes in composition of mono- and diterpenes. Our results suggest that depth of TDs within the sapwood is not important for tree resistance, probably because TDs are interconnected with the complex constitutive resin duct network through radial ducts extending from the sapwood to the secondary phloem (Bannan 1936, Nagy et al. 2000).

Formation of TDs is likely important for induced defense because it represents a major investment for the tree that disrupts normal cambium activity, involves complex changes in cell division patterns and differentiation, and puts heavy demands on limited plant resources. For example, TDs covered 50% of the stem circumference in mass inoculated trees in the present study, and may gradually extend several meters from the infection area (Christiansen et al. 1999). However, the relevance of TD formation to resistance to bark beetle attack is not simple, as has been discussed previously (Franceschi et al. 2000). The formation is too slow to deal with rapid mass attacks because beetle attacks may be completed in a few days. However, TD formation may prevent opportunistic pathogens from invading the stem through, e.g., mechanical wounds made by insects, and may also help when bark beetle attacks are interrupted for extended periods by adverse flight conditions, as discussed below.

The PP cells are dynamic and play complex roles in the constitutive and induced defenses of Norway spruce (Franceschi et al. 1998, 2000). Early responses after wounding and infection include mobilization of phenolic bodies and swelling of existing PP cells. Later on, new PP cells may be produced or differentiated (Krekling et al., unpublished results), and an impervious, suberized wound periderm that may arrest and isolate invading organisms develops from older PP cells close to the infection site (Franceschi et al. 2000). Changes in the phenolic content of PP cells occur within a few days of an infection (Brignolas et al. 1995), and the accompanying swelling of cells causes the surrounding dead sieve cells to collapse into dense layers of cell walls (Franceschi et al. 1998, 2000). These layers probably represent a barrier to penetration by invading organisms, and this barrier is reinforced by phenolics that are released from the PP cells and deposited in the cell walls (Franceschi et al. 2000). The PP cells also seem capable of sensing invading organisms, and together with radial ray cells, they are probably involved in radial and circumferential transmission of signals that activate defense responses (e.g., TD formation) in tissues distant from the attack (Franceschi et al. 2000).

Production of extra PP cells between annual PP cell layers after an infection is not part of the normal developmental program of the secondary phloem, but is only observed after wounding or fungal inoculation and appears to be related to induced resistance (Krekling et al., unpublished results). Such cells are produced a considerable distance from the inoculation site and may be quite abundant, covering almost 50% of the phloem circumference in some of our trees. Like TD formation, production and differentiation of extra PP cells is a relatively slow process that probably enhances long-term resistance rather than increasing resistance to an on-going attack (Krekling et al., unpublished results).

The susceptible Clone 193 generated stronger anatomical defense reactions in the bark after fungal inoculations than the resistant Clone 194. Similar observations were made by Krekling et al. (unpublished results), who found that two susceptible Norway spruce clones produced more extra PP cells and TDs in response to fungal inoculations than two resistant clones. In our study, the resistant Clone 194 mainly activated the current annual layer of PP cells after mass inoculation and did not produce many extra PP cells between annual layers. In contrast, the susceptible Clone 193 activated the last three PP cell layers and produced many extra PP cells, but was still unable to defend itself against the mass inoculation. A possible explanation for the stronger response in the susceptible clone is that it suffered more extensive fungal colonization, and thus was more severely wounded than the resistant clone. We speculate that the susceptible clone perceived the infection as a more severe threat than the resistant clone, and therefore mobilized more of its inducible defenses.

The time required to activate induced disease resistance in Norway spruce (i.e., more than 1 week) suggests that the anatomical changes we observed may be an important underlying mechanism. If the increased resistance in pretreated trees resulted primarily from direct activation of defense-related genes in existing cells, which is the case in systemic acquired resistance, we would have expected to see an effect within the first week after pretreatment (Kessmann et al. 1994, Ryals et al. 1996). The much longer process of anatomical changes will eventually have chemical or biochemical consequences, because it involves differentiation and gene activation in specialized cells that produce chemicals (e.g., resins and phenolics) with known defensive properties.

Induced resistance has obvious implications for the interaction between Norway spruce and its major bark beetle pest, Ips typographus, as well as for the population dynamics of the beetle (Christiansen and Krokene 1999, Krokene et al. 1999, Franceschi et al. 2000). Although induced resistance is not rapid enough to prevent death resulting from rapid mass attacks by bark beetles, it can protect trees when attacks are delayed by, e.g., colder periods. The finding that enhanced resistance induced by pretreatment persists for a whole year after pretreatment suggests that sublethal bark beetle attacks in one year may render trees more resistant in the next season. This means that induced resistance may play a role in terminating beetle outbreaks, or in maintaining the beetle population at a low or endemic level.

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