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Mechanical injury and fungal infection induce acquired resistance in Norway spruce

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Summary Norway spruce trees (*Picea abies* (L.) Karst.) pretreated by wounding and fungal infection showed highly enhanced resistance to a subsequent challenge inoculation with the pathogenic bluestain fungus *Ceratocystis polonica* (Siem.) C. Moreau. This is the first time the effectiveness of the constitutive and inducible defenses has been shown to depend on prior wounding and infection in conifers, although such acquired resistance has previously been found in several angiosperms. Trees that were pretreated with a combination of 12 bark wounds (1.6 × 10 cm), four fungal inoculations and four sterile inoculations 1–15 days before mass inoculation with *C. polonica* at 400 inoculations per square meter over a 0.8 m stem section had significantly shorter necroses in the phloem, less bluestained sapwood, and less dead cambium than untreated control trees. Pretreatment with four fungal or sterile inoculations alone did not lead to enhanced resistance. Pretreatment by bark wounding alone seemed to provide an intermediate degree of resistance compared to bark wounding, fungal inoculations and sterile inoculations combined. All trees had a marked increase in the number of resin ducts in the year of inoculation compared with previous years, suggesting that formation of traumatic resin ducts play an important role in the development and maintainance of enhanced resistance.

Keywords: acquired resistance, bluestain fungus, Ceratocystis polonica, conifers, Picea abies, traumatic resin ducts.

Introduction

Conifers have both constitutive and inducible defenses against insect attack and pathogen infection (e.g., Berryman 1972, Christiansen et al. 1987). Constitutive defenses consist of resin ducts or blisters in the phloem and sapwood (Bannan 1936, Berryman 1972) and specialized parenchyma cells in the phloem (Franceschi et al. 1998). Wounding or infection of the phloem induces a hypersensitive response that leads to accumulation of resin and other defensive chemicals in the cells surrounding the attack site, and the formation of necrotic reaction zones (Reid et al. 1967, Raffa 1991, Dangl et al. 1996). In conifers, the effectiveness of these defenses has never been shown to depend on previous exposure to a pathogen. However, in several angiosperms, studies have demonstrated that pathogen infection induces systemic acquired resistance (SAR), a long-lasting, broad-spectrum resistance to subsequent infection (Ross 1961*b*, Ryals et al. 1996), and localized acquired resistance (Ross 1961*a*).

In an experiment with the original objective of characterizing the defense mechanisms of Norway spruce (*Picea abies* (L.) Karst.), we infected 25-year-old clonal trees with the bluestain fungus *Ceratocystis polonica* (Siem.) C. Moreau (Brignolas et al. 1995, Franceschi et al. 1998). This is the most pathogenic fungal associate of the aggressive bark beetle *Ips typographus* L., and can kill healthy Norway spruce trees when inoculated under the bark in sufficiently high doses (Horntvedt et al. 1983, Christiansen 1985*b*). Bark samples for chemical and histological analyses were taken from the trees 1–15 days before they were challenged with a massive infection of *C. polonica*. An interesting and unexpected result of this work was that the tree's resistance to subsequent fungal infection was greatly enhanced by pretreatment consisting of bark sampling and a few fungal and sterile inoculations. Here we present the results from the original experiment and from a follow-up experiment designed to characterize more precisely the pretreatments that gave enhanced resistance.

Material and methods

The experiments were carried out in a plantation of Norway spruce clones at Hogsmark in Ås, Norway. The clonal trees originated from seedlings obtained from seeds of selected mother trees. Rooted cuttings from these seedlings were planted in 1970 in a regular 2 × 2 m array. By 1995 (the start of the first experiment), the trees had reached a diameter at breast height (DBH) of about 14 cm and a height of about 13 m, with some variation among and within clones.

Ceratocystis polonica was used for both pretreatment inoculations and subsequent challenge inoculations. Trees were in-

oculated by removing a bark plug with a 5 mm cork borer, inserting a 5 mm plug with inoculum in the wound, and replacing the bark plug. Inoculum consisted of actively growing mycelium of *C. polonica* (Isolate No. NISK 93-208/115) on malt agar (2% malt, 1.5% agar) or sterile malt agar.

On May 18, 1995, three trees were selected from each of 20 clones. At this initial sampling (Day 0), two ramets were left untreated as controls and one ramet per clone was pretreated in the following manner: 12 sites were randomly marked within a 0.8 m section of the tree bole between 1.2 and 2.0 m above ground. Four of these sites were inoculated, by means of a cork borer, with *C. polonica*, four with sterile agar, and four were used to identify sites for sampling of intact bark. Two bark samples (1.6 cm wide by 10 cm tall) were removed on Day 0 (intact bark), four samples were removed on Day 6 (two each of fungus and sterile inoculated bark), and six samples were removed on Day 12 (two each of fungus inoculated, sterile inoculated, and intact bark).

On June 6, 1996, 15 trees were selected from one of the most susceptible clones used in 1995 (Clone 503). Three trees were randomly assigned to each of five pretreatments designed to show whether it was the sampling injury, fungal inoculations, sterile inoculations or a combination of all three factors that caused enhanced resistance in 1995. The five pretreatments were: (1) the treatment used in 1995; (2) sampling of intact bark on Day 0 (two samples), Day 6 (four samples) and Day 12 (six samples); (3) four inoculations with *C. polonica* on Day 0 and no bark sampling; (4) four inoculations with sterile malt agar on Day 0 and no bark sampling; and (5) control (no inoculation or sampling).

Thirteen to 15 days after initial sampling in both years, all trees were mass-inoculated with *C. polonica* to determine their resistance. Inoculations were evenly spaced using a template over the sampled stem section at a dosage of 400 inoculations per m^2. This dosage has previously been shown to kill susceptible, but not resistant, Norway spruces (Christiansen 1985*a*).

Trees were harvested 14 weeks after mass inoculation, tree height was measured, and two thin discs (about 5 mm thick) were cut from all trees within the inoculation band at heights of 1.45 and 1.75 m. On each disk, we measured the percentage of sapwood that had been bluestained by the fungus and the percentage of dead and live cambium along the circumference (Krokene and Solheim 1998). On the lower disc, we measured annual ring width for the last four years at four cardinal locations. This disc was also examined with the aid of a stereomicroscope for the occurrence of resin ducts in the annual rings of the last five years. The number of ducts was counted over a tangential distance of 2.65 mm on four cardinal locations (i.e., a total of 10.6 mm for each disc). In 1996, we selected one tree with a high and one with a low percentage of bluestained sapwood (a control tree and a tree pretreated with bark sampling, fungus and sterile inoculations, respectively) and counted resin ducts on 12 additional stem discs cut below and up to 10 m above the inoculated part of the stem. Before harvesting, we measured the vertical extension of six of the uppermost and six of the lowermost phloem necroses on each tree (Krokene and Solheim 1998). Because necroses tended to coalesce within mass-inoculated sections, their lengths were measured upward from upper inoculation points and downward from lower inoculation points.

Data were subjected to ANOVA. If treatments were significantly different ($P < 0.05$), means were separated by LSD at $P = 0.05$ (Montgomery 1991). Percentage data were transformed (arcsin y) before analysis to correct for unequal variances and departures from normality. Statistical analyses were performed with the general linear models (GLM) procedure of the SAS software package (Version 6.12, SAS Institute Inc., Cary, NC).

Results

In 1995, fungal infection after mass inoculation was highly variable among the 20 clones, ranging from almost complete penetration of bark and sapwood in some clones to negligible infection in others (range of clone means: 0–66% sapwood bluestaining; 3–60% dead cambium; 0.6–40.8 cm necrosis length). However, the most striking result was that trees that had been pretreated were much more resistant to the subsequent mass inoculation than intact control trees. Sapwood bluestaining, proportion of cambium killed, and length of phloem necroses were significantly less extensive in pretreated trees than in control trees (Figure 1). In particular, there was very little sapwood bluestaining in pretreated trees compared with control trees (on average, 0.5 versus 20.4%).

Fungal infection in pretreated ramets of Clone 503 in 1996 was greater than in pretreated ramets of the same clone in 1995, indicating a strong phenotypic or year-to-year variability in susceptibility. In 1996, percent bluestained sapwood in trees pretreated with bark sampling, fungus and sterile inoculations was 7.2% compared with 0% in 1995. The corresponding values for percent dead cambium were 69.9 versus 8.0%, and for phloem necrosis length 65.2 versus 41.2 mm. In control trees, fungal colonization success was high in both years. Although fungal colonization success was higher in 1996 than in 1995, pretreatment with bark sampling and fungal and sterile inoculations still gave significantly enhanced resistance (Figure 2). There were no significant differences between the

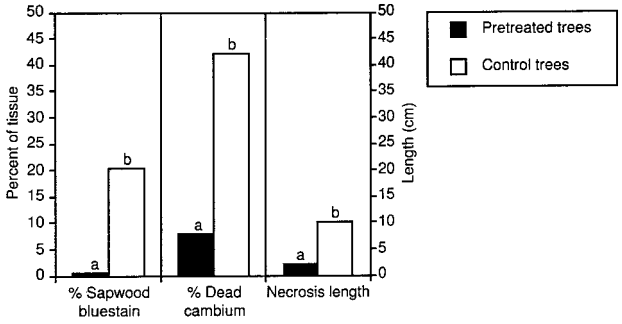

Figure 1. Fungal colonization success of 20 Norway spruce clones 14 weeks after mass inoculation with *Ceratocystis polonica* in 1995. One ramet per clone was pretreated (bark sampling, fungal and sterile inoculations) before mass inoculation, whereas two ramets were left untreated as controls. Within each part of the figure, columns with different letters were significantly different by the LSD test ($P = 0.05$) following two-way ANOVA (Treatment, Clone).

other pretreatments. Neither were there any significant differences in height or annual ring width between trees that were given different treatments.

False rings (*sensu* Fritts 1976) had formed within the current annual ring of sapwood in all trees in 1995, except for some control trees that succumbed very rapidly to fungal infection. There were numerous traumatic resin ducts within this ring of narrow tracheids. In all of these trees, the number of resin ducts in the 1995 annual ring was much higher than in the four previous rings (Figure 3). False rings were found uninterrupted along the periphery of pretreated ramets, whereas in control ramets they were absent or occurred only in regions that had not been colonized by the fungus. Thus, in control ramets, the number of resin ducts in the 1995 annual ring was negatively correlated with percent bluestained sapwood area ($r^2 = 0.71$, $P < 0.00001$, n = 40). In 1996, an increased occurrence of traumatic resin ducts was observed several meters above the inoculated stem section in both the resistant tree (which had 0.3% bluestained sapwood) and the tree that was heavily colonized by fungus (64.6% bluestaining), but in the latter tree no resin ducts were formed immediately above or below the inoculation band.

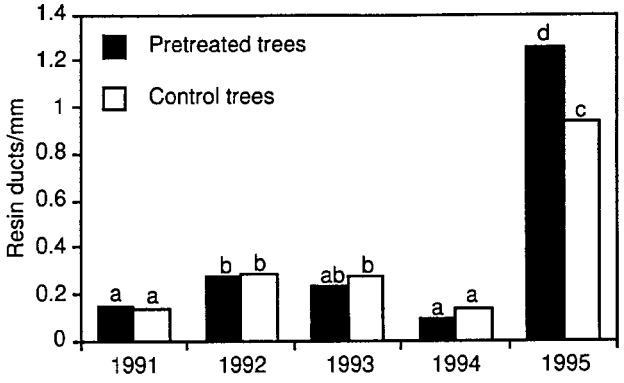

Figure 3. Number of resin ducts at 1.45 m height in the last five annual rings of 20 Norway spruce clones that were mass inoculated with *Ceratocystis polonica* in 1995. One ramet per clone was pretreated (bark sampling, fungal and sterile inoculations) before mass inoculation, whereas two ramets were left untreated as controls. Columns with the same letter were not significantly different according to the LSD test ($P = 0.05$) following ANOVA.

Discussion

In both 1995 and 1996, mechanical injury and fungal infection of Norway spruce trees resulted in enhanced resistance to subsequent mass inoculation with *C. polonica*. Such acquired resistance has previously been observed in several angiosperms (Ross 1961*b*, Görlach et al. 1996, Ryals et al. 1996), but to our knowledge this is its first demonstration in gymnosperms. In previous studies, pines that were subjected to a few fungal inoculations or to sub-lethal bark beetle attacks showed no evidence of enhanced resistance to subsequent inoculations (Paine and Stephen 1987, Cook and Hain 1988).

The combination of mechanical injury and inoculations was the only pretreatment that unequivocally resulted in enhanced resistance in Norway spruce trees. However, mechanical injury alone may have had an intermediate effect, because it reduced the amount of sapwood bluestain compared with the other pretreatments and the control. In several angiosperms, mechanical injury did not induce acquired resistance to microbial pathogens (e.g., Ross 1961*b*, Kuc 1982). Our results indicate that acquired resistance in Norway spruce requires a threshold inductive stimulus to be reached before protection is achieved. Pretreatment with four fungal or sterile inoculations did not seem to enhance resistance; however, if the density of sterile or fungal pretreatment inoculations was increased from about 11 m^{-2} to about 50 m^{-2} there appeared to be a strong positive effect on tree resistance (Evensen 1998).

Because pretreatments and challenge inoculations were applied to the same section of the bole, we could not determine whether the enhanced resistance was localized or systemic. However, the entire inoculated stem section seemed to be protected by the pretreatment, even though only about 5% of the area was injured. The increased number of traumatic resin ducts observed several meters above the inoculation band in trees sampled in 1996 also suggests the occurrence of systemic effects. The formation of traumatic resin ducts was probably not induced by the pretreatment alone, because trees were

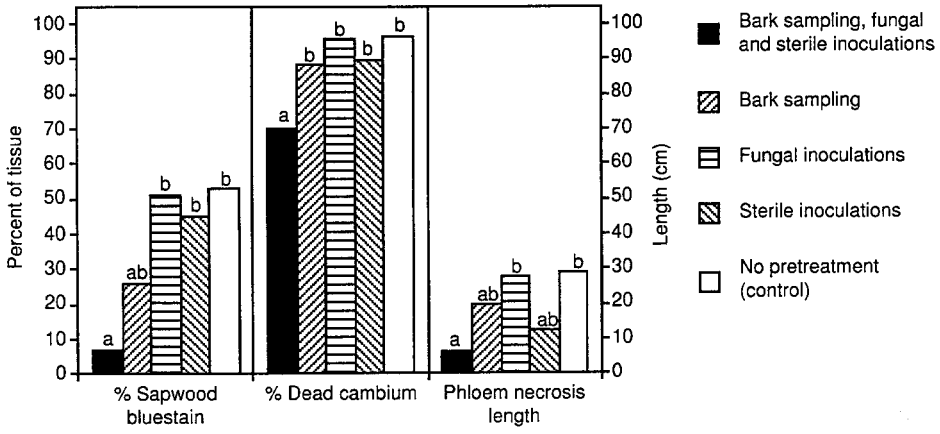

Figure 2. Fungal colonization success in a single clone of Norway spruce (Clone 503) 14 weeks after mass inoculation with *Ceratocystis polonica* in 1996. Three trees were randomly assigned to each of five pretreatments before mass inoculation. Within each part of the figure, columns with different letters were significantly different according to the LSD test ($P = 0.05$) following ANOVA.

mass-inoculated soon after pretreatment and some control trees that were not pretreated also formed traumatic resin ducts. Traumatic resin ducts start to form soon after wounding or infection, and are fully formed after 36 days (Christiansen et al. 1999), unless the fungal pathogen spreads fast enough to kill cells involved in traumatic resin duct formation.

Resistance of Norway spruce to *C. polonica* infection is positively correlated with both constitutive resin flow and resin concentration in reaction zones in the phloem (Christiansen 1985*a*, Horntvedt 1988). Traumatic resin ducts probably have a positive effect on tree resistance by adding to the volume of stored resinous material, and perhaps by providing so-called secondary resin to reaction zones (Christiansen et al. 1999). Secondary resin seems to be more fungistatic than preformed resin (Solheim 1991), probably because it contains additional phenols and tannins produced by phloem parenchyma cells (Brignolas et al. 1995, Franceschi et al. 1998), and possibly by epithelial cells lining the traumatic ducts (Christiansen et al. 1998). Formation of traumatic resin ducts has been reported in several cases where conifers have survived bark beetle attack or fungal infection or both (Berryman 1969, Christiansen and Solheim 1990, Christiansen and Fjone 1993, Kytö et al. 1996, Solheim and Safranyik 1997).

Our next research priority is to clarify whether acquired resistance in Norway spruce can be induced by fungal or sterile inoculations alone, how long the acquired resistance is effective, whether its effect is systemic or localized, and whether the acquired resistance is sufficient to protect trees from mass-attack by aggressive bark beetles.

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