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Induced Responses in Phenolic Metabolism in Two Norway Spruce Clones after Wounding and Inoculations with *Ophiostoma polonicum*, a Bark Beetle-Associated Fungus[1]

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Two Norway spruce (*Picea abies* Karst.) clones, one resistant and the other susceptible to mass inoculation with *Ophiostoma polonicum* Siem., were compared with regard to their phenolic compositions and chalcone synthase (CHS) and stilbene synthase activities of their phloem before and at 6 and 12 d after artificial inoculation with sterile malt agar or *O. polonicum*. In unwounded phloem, the resistant clone differed from the susceptible clone by the presence of taxifolin glycoside, lower concentrations of stilbene glycosides, and higher CHS activity. After inoculation, (+)-catechin concentration and CHS activity dramatically increased around the wound, particularly in the resistant clone. Stilbene synthase activity also increased, but more slowly and to a lower level, whereas the concentrations of stilbenes remained stable. Tanning ability decreased in the susceptible clone, whereas it remained stable in the resistant one. It is proposed that the induced phenolic response of Norway spruce phloem consists of an activation of the phenolic pathway, finally leading to tannins and insoluble polymers. It is suggested that resistance to *O. polonicum* depends on the ability of the tree to easily activate the flavonoid pathway.

The basic mechanism of conifer resistance to bark beetle (Coleoptera; Scolytidae) attacks is a vigorous response that is induced and stimulated by the boring insect and its associated fungi (Reid et al., 1967; Berryman, 1972; Raffa and Berryman, 1982, 1983; Christiansen and Horntvedt, 1983; Christiansen et al., 1987; Lieutier et al., 1988; Långström et al., 1992; Lieutier, 1993). This response is localized in both the phloem and sapwood. In the phloem, it takes place in an elliptical reaction zone surrounding each point of attack. This zone is impregnated with secondary metabolites, such as terpenes and phenols, and gradually turns into a necrotic zone in which aggressors are confined.

A bark beetle population can be successfully established on a tree only when the tree's defenses become exhausted by a sufficiently high number of simultaneous attacks. Resistance of conifers to bark beetle is thus measured as the maximum density of attacks that a tree is able to contain before being overcome (Thalenhorst, 1958; Berryman, 1978; Raffa and Berryman, 1983; Mulock and Christiansen, 1986). Another estimation of the resistance of a tree can be obtained by replacing bark beetle attacks with artificial mass inoculations with fungi isolated from beetles, thus leading to the determination of a threshold of inoculum density (Horntvedt et al., 1983; Raffa and Berryman, 1983; Christiansen, 1985; Solheim et al., 1993). These estimation methods have been used to demonstrate that the tree's resistance depends on its physiological status (Waring and Pitman, 1980, 1983; Mulock and Christiansen, 1986; Långström et al., 1992), but it probably also depends on genetic factors. Because these estimation methods are destructive, they cannot be used for prediction or for breeding programs. For that, markers of tree resistance are a necessity.

The present study is part of a cooperative program aimed at (a) understanding the mechanisms involved in the induced response of Norway spruce (*Picea abies* Karst.) phloem to aggression by *Ophiostoma polonicum* Siem., a highly pathogenic fungus associated with the bark beetle *Ips typographus* L. (Solheim, 1986), and (b) determining genetic markers of Norway spruce resistance to bark beetle attacks. Previous investigations have demonstrated that the phenolic composition of the phloem of pine and spruce changes dramatically during the development of the response, and that the nature of these changes varies from clone to clone. It has also been suggested that flavonoids and stilbenes are involved in the efficacy of the phloem response in arresting the aggressors (Lieutier et al., 1991, 1995), but a direct relation between phenol composition and resistance has not yet been established. This paper presents the first results of a study dealing with between-clone variations of the phenolic metabolism in Norway spruce phloem with respect to a tree's resistance level. It considers the monophenolic and tannin composition and the activities of the key enzymes of the biosynthetic path-

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Abbreviations: CHS, chalcone synthase; STS, stilbene synthase.

ways leading from general phenolic precursors to flavonoids (CHS) or stilbenes (STS).

MATERIALS AND METHODS

Experimental Device

Two clones of Norway spruce (*Picea abies* Karst.) growing at Hogsmark Experimental Farm in Ås, Akershus, Norway, were selected for their highly different resistance to mass inoculation with *Ophiostoma polonicum* Siem.; clone 409 was susceptible, whereas clone 589 was resistant. They originated from seedlings obtained from seeds of selected trees. Rooted cuttings of these seedlings had been planted in 1980, and the resulting trees were about 10 m high in 1993.

On May 13, 1993, one tree of each clone received both eight inoculations of a malt agar culture of *O. polonicum* and eight inoculations of sterile malt agar to serve as a wounded control. The inoculations were made by cutting a hole to the cambium with a 5-mm-diameter cork borer, placing agar in the hole, and returning the bark plug. Six and 12 d later, eight 1-cm × 10-cm longitudinal bark strips from around the inoculation hole were sampled from each tree, four from fungal inoculations and four from sterile ones. Four strips of uninoculated bark were taken similarly at the time of inoculation and after 6 and 12 d. The samples were immediately frozen on dry ice and then freeze-dried in the laboratory. In each category, three samples were used for studies of monophenol and tannin content, whereas the fourth sample was used for the enzymatic studies.

Extraction of Soluble Monophenols and Tannins

A 2.5-mm-wide strip was removed from each edge of the freeze-dried samples, to keep only a central longitudinal 5-mm-wide strip, from which the periderm was also removed. No necrotic zone was visible in the samples. The central strips were divided into subsamples, each 10 mm long and 5 mm wide, and the subsamples located at the same distance from the inoculation hole were pooled to constitute the final samples ready for analyses. Only the three final samples located between 0 and 3 cm from the inoculation points were analyzed. In the unwounded samples, the whole central strip was used for extraction.

The freeze-dried phloem samples were ground, and the powder was washed with pentane to remove resinous compounds, as suggested by Alcubilla (1970). Extractions were then performed at 4°C with 80% methyl alcohol in ultra-pure water, according to a method adapted from Jay-Allemand et al. (1988) for monophenols and as suggested by Scalbert (1992) for tannins. Twenty to 30 mg of phloem powder were put in 2 mL of pentane with sodium metabisulfite (antioxidant). The mixture was placed in a sonicating water bath for 30 min and then centrifuged at 16,350*g* and −5°C for 20 min. The supernatant was discarded and the residue was washed twice again using the same method. Eighty percent methyl alcohol (1.9 mL) was added to the washed powder, with 0.1 mL of vanillyl alcohol (0.05 M) in methanol as an internal standard for the HPLC analysis of monophenols. The mixture was placed in a sonicating water bath for 15 min and centrifuged (16,350*g*, 20 min). The supernatant constituted the final extract.

Analysis of Monophenols and Estimation of the Tanning Ability of the Extract

Monophenol composition was analyzed by reversed-phase HPLC (Waters 600E, photodiode array detector 991), using a 250-mm-long and 4-mm-i.d. column. The stationary phase was C_{18}-grafted silica (Merck, Lichrospher RP18) with 0.005 mm porosity. The mobile phase was a mixture of acetonitrile and 1% acetic acid in ultra-pure water. The following gradient was used (Lindberg et al., 1992): 95% acetic acid solution and 5% acetonitrile for 5 min, a linear increase of acetonitrile up to 25% within 43 min, followed by another linear increase of acetonitrile up to 70% within 10 min, and finally 70% acetonitrile for an additional 10 min. The mobile phase returned to 95% acetic acid solution and 5% acetonitrile in 3 min, and it stayed there for 10 min. The flow rate was 1 mL/min. A 0.1-mL aliquot of the final sample extract to be analyzed was dried under vacuum, and 0.1 mL of 10% methyl alcohol was added to the residue. A 0.02-mL aliquot of this solution was injected. A_{280} was read. Results were expressed in vanillyl alcohol equivalents per g of freeze-dried powder.

Monophenols were characterized using readings of UV spectral characteristics between 240 and 402 nm with the photodiode array detector, co-chromatography in HPLC with various standards, two-dimensional TLC on cellulose plates followed by tests with chemical reagents, and tests for the presence of glycosidic links after acid hydrolysis.

Tanning ability was determined as the protein-binding efficiency of the extract. Agarose gels (1% [w/v], type I, Sigma) were prepared with an acetic acid buffer (50 mM, pH 5) and with BSA (0.1% [w/v], fatty acid-free fraction V, Sigma) and dispensed in 10-mL aliquots into standard Petri dishes (Hagerman, 1987; Peng and Jay-Allemand, 1991). Four 6-mm-diameter wells were made in each dish. An aliquot of the final extract (1 mL) was evaporated, the residue was solubilized in 0.1 mL of ultra-pure water, and 0.02 mL of the latter solution was placed into each well of a Petri dish. After 24 h at 25°C in the dark, the area of each visible BSA precipitation zone was measured. Results were expressed as tanning ability, in tannic acid equivalents per g of freeze-dried powder, from a standard curve prepared with tannic acid (Extrasynthese, Genay, France).

Extraction of CHS and STS and Measurement of the Enzymatic Activities of the Extract

A 5-mm-wide × 10-mm-long central strip prepared as described above from samples located no more than 3 cm from the inoculation point was analyzed. Enzymes were extracted from the ground phloem. The method of extraction and assay was adapted to spruce from that of Claudot and Drouet (1992) for walnut trees and has been described elsewhere (Lacroix, 1994). In short, since CHS and STS have the same physical and chemical properties, the same substrates, and the same requirements (Schröder and Schrö-

der, 1990), they were extracted and assayed simultaneously. Their respective activities were determined after separation of their reaction products by TLC on cellulose plates. Results were expressed in pkat per g of freeze-dried powder and in percentage of the total (CHS + STS) activity due to CHS.

Statistical Methods

Means were expressed with their SD. They were compared by *t* test or analysis of variance using SAS software (SAS Institute, Cary, NC). Differences were taken into account only when they were significant at the 5% level.

RESULTS

Variations in Monophenols and Tannins

The five main monophenols were considered in both unwounded and inoculated phloem. Two were flavonoids, (+)-catechin and taxifolin glycoside, and three were stilbene glycosides, astringin (astringenin glycoside), piceid (resveratrol glycoside), and isorhapontin (isorhapontigenin glycoside). No aglycone was observed. Taxifolin glycoside was detected only in the resistant clone; the other four compounds were present in both clones.

In unwounded phloem, the (+)-catechin concentration and the tanning ability did not differ significantly between the two clones (Figs. 1 and 2). Stilbenes, particularly piceid and isorhapontin, were significantly more concentrated in the susceptible clone than in the resistant one (Fig. 3). Concentrations in unwounded phloem did not vary significantly with sampling date for any compound.

In response to aggressions, the concentration of taxifolin glycoside did not vary consistently. On the other hand, (+)-catechin concentration increased considerably after inoculation in both clones, either with fungus or sterile malt agar, but its increase depended on inoculation type, clone, and location in the reaction zone (Fig. 1). This increase was higher close to the inoculation site and generally higher after fungus inoculation. In response to sterile inoculation it was less extended in the susceptible clone (0–10 mm) than in the resistant one (0–20 mm). However, in response to fungus inoculation on d 12, it extended up to 30 mm in the susceptible clone but only up to 20 mm in the resistant one. In the 10- to 20-mm zone after both fungus and sterile inoculations, it was significantly higher in the resistant than in the susceptible clone. In the 20- to 30-mm zone, it was significantly higher between d 0 and 6 in the resistant clone after fungus inoculation.

At d 6, for the two treatments on both clones, the tanning ability in the 0- to 30-mm zone did not significantly differ from that of unwounded phloem (Fig. 2). At d 12, the tanning ability of the 0- to 20-mm zone of the susceptible clone became significantly lower than that of unwounded phloem, after both fungus and sterile inoculations, whereas it still did not differ in the resistant clone.

Concentrations of the three stilbenes in the susceptible clone tended to decrease slightly after fungus inoculation, especially close to the point of inoculation (Fig. 3). In the resistant clone, no consistent difference was observed.

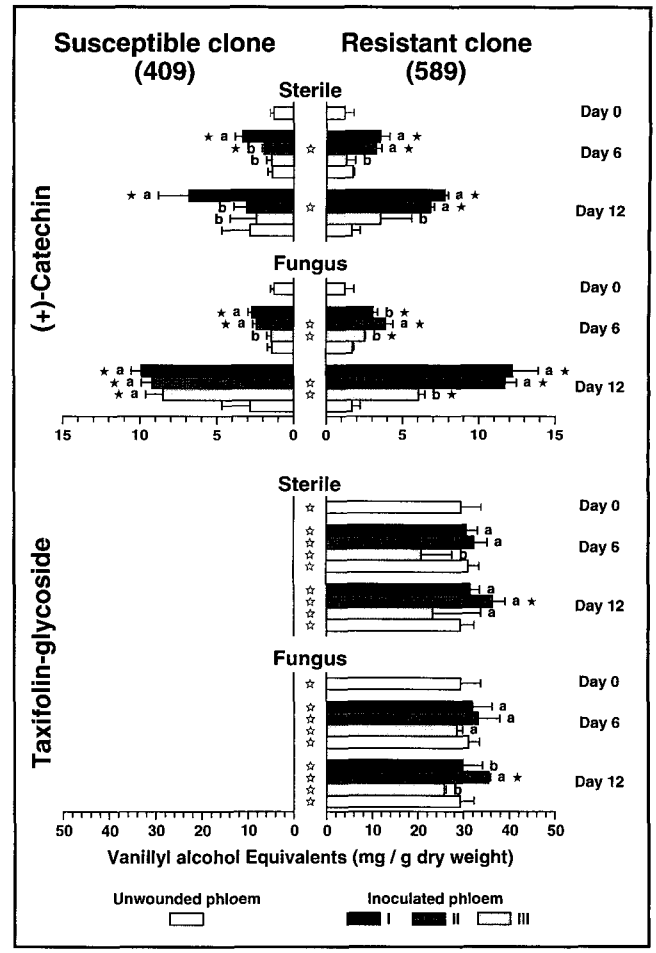

Figure 1. Concentration of flavonoids in unwounded and in inoculated phloem of two Norway spruce clones at different times after inoculation with sterile agar or with *O. polonicum*. I, II, III, 0 to 10, 10 to 20, and 20 to 30 mm from the inoculation site, respectively. Horizontal lines show the SD. In the same group, bars labeled with the same letter do not significantly differ from each other at the 5% level. Values marked with a black star at the end of the bar differed significantly from unwounded phloem. White stars indicate significant differences between clones.

Variations in Enzymatic Activities

In unwounded phloem, CHS activity appeared to be higher in the resistant than in the susceptible clone, whereas the differences between the clones with regard to STS activity were not so evident (Fig. 4). The percentage of the total (CHS + STS) activity due to CHS was around 44% in the resistant clone and around 26% in the susceptible clone (Table I).

After inoculation, either with fungus or sterile malt agar, both metabolic pathways were stimulated in the two clones (Fig. 4). However, CHS activity was stimulated more than STS activity. CHS activity was generally higher in the resistant than in the susceptible clone. It was also more intense close to the inoculation site. STS activity was higher in the resistant clone at d 12 only and did not vary consistently with distance from the inoculation site. In both clones, especially at d 12, enzymatic activities were higher

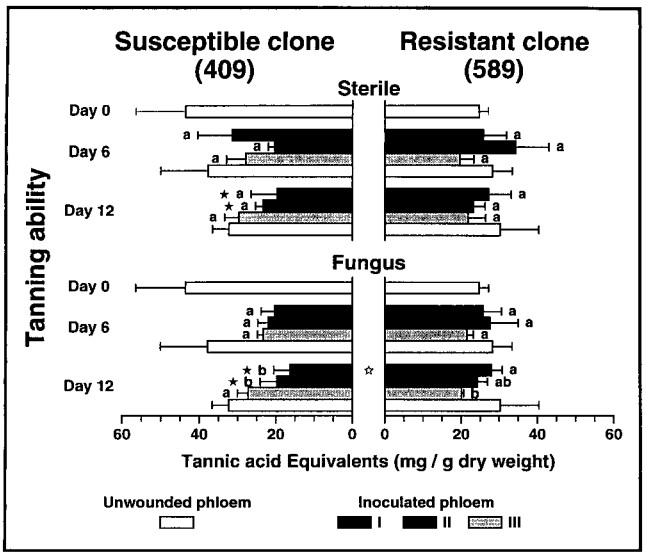

Figure 2. Tanning ability in unwounded and in inoculated phloem of two Norway spruce clones at different times after inoculation with sterile agar or with *O. polonicum*. For the meaning of the symbols, see Figure 1.

after fungus inoculation than after sterile inoculation. After fungus inoculation, both CHS and STS activities increased until d 12. After sterile inoculation, CHS activity reached a maximum at d 6, whereas STS activity constantly increased.

The fraction of the total synthase activity due to CHS increased considerably after inoculation, reaching up to 85% in some cases (Table I). It was higher in the resistant than in the susceptible clone at d 6, but became the same in the two clones at d 12. In each clone, values at d 6 were comparable after sterile or fungus inoculation. At d 12, they strongly decreased for the sterile inoculations, whereas for the fungus inoculations, they increased in the susceptible clone and remained the same or slightly decreased in the resistant clone.

DISCUSSION

Mechanisms of the Induced Phenolic Response

The stimulation of both CHS and STS activities in response to inoculations is in agreement with the observations made by several authors concerning an increase of the expression of genes involved in phenolic metabolism, particularly CHS and STS, after various aggressions (Dixon and Lamb, 1990; Ouchi, 1991; Yoshikawa and Takeuchi, 1991; Schwenkendiek et al., 1992). After sterile inoculation, the maximum of CHS activity preceded the maximum of STS activity (Fig. 4), suggesting two successive stages that depend on the relative importance of the flavonoid and stilbene pathways (Table I). In the case of fungus inoculation, this distinction was not possible, at least during the first 12 d, possibly due to the stimulation of the tree's response by the aggressor. Indeed, CHS and STS activities were higher after fungus inoculation than after sterile inoculation at d 12, whereas both treatments gave similar responses at d 6 (Fig. 4). This agrees with the suggestion that a tree's response is basically a response to wounding that is further amplified by the presence of fungus in the wound (Lieutier, 1993).

The parallel increases in (+)-catechin concentration and CHS activity after inoculations suggest that the increase in (+)-catechin levels was perhaps due to an increase in the de novo rate of its synthesis. On the contrary, even though

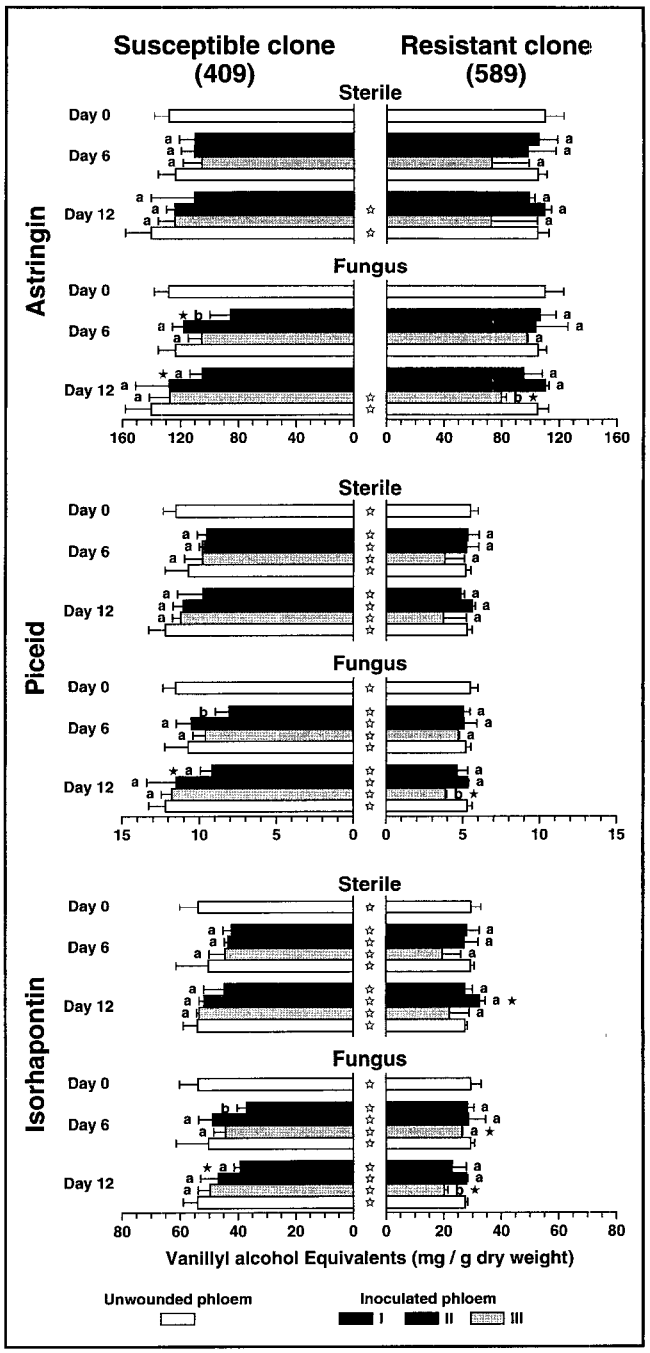

Figure 3. Concentration of stilbenes in unwounded and in inoculated phloem of two Norway spruce clones, at different times after inoculation with sterile agar or with *O. polonicum*. For the meaning of the symbols, see Figure 1.

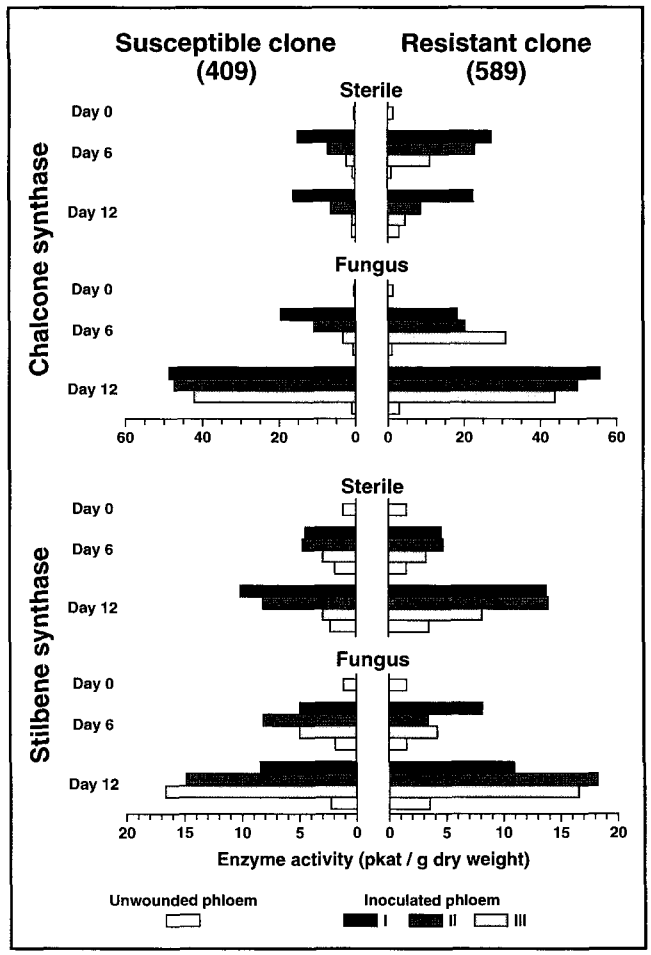

Figure 4. CHS and STS activities in unwounded and in inoculated phloem of two Norway spruce clones, at different times after inoculation with sterile agar or with *O. polonicum*. For the meaning of the symbols, see Figure 1.

STS activity was increased by the inoculations, no corresponding increase in stilbene concentrations was observed (Fig. 3). The failure to accumulate stilbenes could be due to either or both of two factors. First, the newly synthesized stilbenes might be incorporated into tannins and no longer detected as stilbene monomers. Second, the competition of CHS and STS for their common substrate precursor might favor CHS, with a resulting decrease in the amounts of stilbene accumulating in vivo. Consistent with this possibility is the report (Lacroix, 1994) that CHS has a higher affinity than STS for their common precursor. This reasoning could also explain why stilbene accumulated to higher levels in the susceptible clone than in the resistant clone, since the susceptible clone had lower levels of CHS activity than the resistant clone, whereas both had similar levels of STS activity. It should be noted that the CHS and STS activities were measured in vitro under conditions in which substrate concentrations were not limiting; therefore, the measured activities should reflect the amounts of active enzyme present.

The higher level of CHS activity in unwounded phloem of the resistant clone in comparison with the susceptible clone could be related to the need to produce the additional precursor required for taxifolin glycoside synthesis in the resistant clone. The lack of taxifolin glycoside in the susceptible clone indicates that the metabolic pathway leading to this compound is either lacking or inhibited in the susceptible clone. Since only (+)-catechin levels, and not taxifolin glycoside levels, increase in the resistant clone after inoculations, there must be regulation in the utilization of the chalcone produced by CHS for flavonoid synthesis under these conditions.

A general conclusion from these observations is that one response of the Norway spruce to wounding, and to wounding coupled with inoculation by a fungal pathogen, is to stimulate the metabolic pathways leading to (+)-catechin and stilbene phenolic monomers, which then are more gradually converted to tannins and later to insoluble products.

Differences between Clones, and Markers of Norway Spruce Resistance

We suggest that the increased capacity of the resistant clone for activation of the flavonoid biosynthetic pathways in comparison with the susceptible clone is correlated with the level of Norway spruce resistance. This model predicts that one or more products of the flavonoid pathway are involved as defensive agents in resistance, although no specific products with inhibitory properties have been identified to date. Several observations reported in this paper are consistent with this model. One flavonoid derivative (taxifolin glycoside) was present only in the resistant clone, and the synthesis of another flavonoid [(+)-catechin] was stimulated to a greater extent in the resistant clone than in the susceptible clone. On the other hand, stilbenes

Table I. *Percentage of the total CHS plus STS activity due to CHS in unwounded and in inoculated phloem of two Norway spruce clones at different times after inoculation with sterile agar or with O. polonicum*

I, II, III; 0 to 10, 10 to 20, and 20 to 30 mm from the inoculation site, respectively.

Day	Susceptible Clone							Resistant Clone						
	Unwounded phloem	Inoculated phloem						Unwounded phloem	Inoculated phloem					
		Sterile			Fungus				Sterile			Fungus		
		I	II	III	I	II	III		I	II	III	I	II	III
0	21							48						
6	27	77	60	44	80	58	40	39	86	83	77	84	83	85
12	30	62	31	23	85	76	72	45	62	38	35	84	73	73

(piceid and isorhapontin) were present at higher levels in the susceptible clone. Also, the CHS activity (Fig. 4) and the percentage of CHS activity in the total of CHS + STS activities (Table I) were higher in the resistant clone than in the susceptible clone. Thus, it appears that the resistant clone favors the flavonoid pathway, whereas the susceptible clone has a lower capacity for this pathway.

Although the level of (+)-catechin was approximately the same in unwounded phloem of both clones, the increase of (+)-catechin after inoculation was generally more intense and extended farther from the inoculation point in the resistant clone than in the susceptible clone (Fig. 1). Tree resistance could thus be linked to the ability to synthesize (+)-catechin rapidly and to the maintenance of tanning ability, both of which occur in the region closest to the point of inoculation in the resistant clone. According to this model, the flavonoid levels and the tanning ability in the region proximal to the point of inoculation would be inadequate to contain fungal growth at earlier times in the susceptible clone.

Only two clones have been investigated in the present study, which makes it difficult to reliably define markers of tree resistance to *O. polonicum*. Nevertheless, important differences between the two clones suggest good candidates for such a role. In the unwounded phloem, the presence of taxifolin glycoside, a high CHS activity, or a high ratio between CHS and STS activities could be markers of tree resistance, whereas relatively high quantities of piceid and isorhapontin could indicate susceptibility. All of these markers are especially interesting because they can be measured without inoculating trees. However, Lindberg et al. (1992) have reported a negative correlation between the radial extent of *Heterobasidion annosum* growth and the concentration of astringin. In wounded phloem, the concentration of (+)-catechin, the level of CHS activity, and the ratio between CHS and STS activities could also be used as markers.

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