Formation of Ligno-Suberized Tissues in Jack Pine Resistant to the European Race of *Gremmeniella abietina*

M. Simard, D. Rioux, and G. Laflamme

Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, 1055 du P.E.P.S., P.O. Box 3800, Sainte-Foy, Québec G1V 4C7, Canada.

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ABSTRACT

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A histological study was conducted to provide insights into the defense mechanisms of *Pinus banksiana* resistant to the European (EU) race of *Gremmeniella abietina* in naturally infected sites. At the time of sampling, the only apparent symptom was a blight induced at the tip of the shoots. The identity of *G. abietina* during microscopic examinations was confirmed by an immunogold labeling method. Once the fungus had succeeded in penetrating the bracts through stomata, it invaded the stem cortex and the phloem cells and attained the vascular cambium. The progression of the pathogen to the pith was possible principally through intense colonization of needle traces but also by the invasion of the rays.

Ligno-suberized tissues confining the pathogen within the necrotic area were revealed by histochemical tests. Well-defined boundaries were initiated at the base of healthy needles and at the vascular cambium level. They regularly formed one continuous suberized barrier completely crossing the shoot from one needle to the other. A nonlamellar form of suberin was observed in transmission electron microscopy. Restoration of cambial activities and tissue regeneration following necrophylactic periderm formation were suggested as essential factors in the defense system of *P. banksiana* against the EU race of *G. abietina*. To our knowledge, this is the first demonstration of an anatomical defense mechanism of a conifer against Scleroderris canker.

Additional keyword: compartmentalization.

Scleroderris canker is caused by the fungal pathogen *Gremmeniella abietina* (Lagerberg) Morelet, whose taxonomy has been reassessed by Petrini et al. (48). This serious disease is damaging to several conifer species in Europe, Asia, and North America (24, 32,57,70). On the latter continent, two of the three races defined by Dorworth and Krywienczyk (20) and recognized in the variety *abietina* by Petrini et al. (48) are present: the indigenous North American (NA) race (18,26,32,33,49) and the introduced European (EU) race (19,25,32,35,57). The NA race will infect only pine shoots covered with snow during the winter, with damage restricted to seedlings or lower branches of trees (38). It is found particularly on indigenous species such as red pine (*Pinus resinosa* Ait.) and jack pine (*P. banksiana* Lamb.) (23,49,61). The EU race is not restricted to the shoots in the snow because infection can occur at any height in the tree, usually starting on lower shoots and progressing upward in the crown of the tree, killing trees of all ages. It damages red pine and Scots pine (*P. sylvestris* L.) (31,57).

Up to now, infection processes by *G. abietina* in nonresistant pine have been described most particularly in their early stages (43,60,69). Briefly, the fungus infects pine through stomata of short shoot bracts, sparsely colonizes bract tissues by late summer or fall, and finally, during winter, crosses the periderm and colonizes the short shoot and surrounding cortical tissue (43). The following spring, external symptoms appear: needle bases on infected branches turn a yellow or brown-red color. Biochemical studies revealed that *G. abietina* in culture media can produce pectinase, hemicellulase, and xylanase (45,47), whereas another study demonstrated the ability of colonizing hyphae in *P. resinosa* seedlings to degrade cellulose and pectin and showed that phenol-

oxidases secreted by the pathogen might also be involved in host cell wall degradation during early stages of colonization (69).

Early investigations of Scleroderris attack on jack pine in the United States and Canada showed that *P. banksiana* has a rather low resistance to the NA race (54). Subsequently, differences in susceptibility among jack pine provenances were found when the occurrence of Scleroderris infection was reduced but not stopped, using seed sources from climatically adapted trees (14,64,68). Recently, a high level of resistance of jack pine to the EU race of *G. abietina* was observed for the first time by Laflamme and Blais (34). In fact, no mortality occurred in jack pine plots, whereas up to 100% of the seedlings in red pine plots were killed 10 years after planting. These plots were established among red pine plantations in which incidence of the disease and mortality caused by this race was very high. The pathogen caused only a tip blight limited to less than 2 cm on *P. banksiana* shoots.

Practically nothing is known concerning defense reactions of conifers against Scleroderris canker. The present histological study attempts to elucidate the mechanisms of resistance of *P. banksiana* against the EU race of *G. abietina* and concomitantly provides additional details about the process of colonization of this pathogen.

MATERIALS AND METHODS

Sampling. Approximately 100 shoots with tip blight of jack pine were sampled at different times to cover the growing season: twice in June 1998, once at the end of August of the same year, and twice in July 1999. To account for potential environmental variation, the samples were taken at two different sites as described by Laflamme and Blais (34). Briefly, plots of 100 jack pines were planted in naturally infected plantations of red pines located 200 km northwest of Montréal, QC, Canada. The two plantations are located at the following longitude-latitude: 46°53'N-75°16'W and 46°39'N-75°16'W. Seedlings were planted in 1985 and 1986 under infected red pines measuring 3 to 4 m and 7 to 8 m, with

Corresponding author: D. Rioux; E-mail address: drioux@cfl.forestry.ca

infection up to 3 and 5 m, respectively. Fifteen shoots of jack pine without any symptoms were used as controls. At the time of sampling, the disease was first identified in the field by the observation of cryptopycnidia according to Cauchon and Lachance (15), and the race was confirmed with soluble protein electrophoresis (4,48). Twenty-seven shoot tips of the 1998 sampling that were not processed for microscopy were cut longitudinally in their center and measured to determine the length of necrosis of the infected shoots (Fig. 1).

Tissue processing for microscopy. Samples for tissue processing were collected from three different parts of the shoot: the necrotic zone at the tip; the transition zone ranging from the first healthy needle to the unaffected part; and finally the healthy zone (Fig. 1). They were treated according to one of the following methods: (i) Unfixed fresh samples were embedded in Tissue-Tek O.C.T. Compound (Miles Scientific, Slough, UK) at -20°C . Serial sections (30 μm each) covering a distance of 2 to 3 mm were obtained with a Histostat cryomicrotome (Reichert, Vienna, Austria) and were used for histochemical tests. (ii) Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) under vacuum for 2 h, rinsed in the same buffer, and postfixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol series, and embedded in Epon 812 according to standard procedures. In light microscopy, sections (approximately 1 μm) were obtained with an Ultracut E microtome (Reichert-Jung, Vienna, Austria), stained with 2% toluidine blue O followed by 1% safranin O (52), and observed with a Polyvar light microscope (Reichert-Jung). In transmission electron microscopy (TEM), ultrathin sections were contrasted with uranyl acetate followed by lead citrate and examined with a Philips 300 (Eindhoven, the Netherlands) by TEM (80 kV). (iii) Samples were fixed in 4% formalin diluted in 0.1 M phosphate buffer (pH 7.4), dehydrated in ethanol series, and embedded in Paraplast (Oxford Labware, St. Louis). Longitudinal sections (5 μm) were obtained with a rotary microtome (Jung 2035; Heidelberg, Germany), deparaffined, and processed as described below.

Histochemistry. To detect lignin and suberin, sections were treated with phloroglucinol-HCl and examined under either bright-field or violet-light excitation (5); the latter was obtained using a BP 330-380 exciter filter with a DS 420 separator mirror and an LP 418 barrier filter. Parallel stainings with Sudan IV (58) were carried out on some sections as additional evidence of suberin deposition in affected tissue.

Gold-complexed probes. To confirm the presence of the pathogen, a polyclonal antiserum raised against mycelium of the EU race of *G. abietina* (anti-GA) (46) was used as described in Ylimartimo et al. (69). Briefly, the sections were floated on a drop of 0.1 M glycine in 0.01 M phosphate buffered saline (PBS) (pH 7.4) for 30 min and on normal goat serum for 10 min, incubated with the anti-GA containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 for 3 h at room temperature, and finally with a gold-labeled goat-anti-rabbit antibody diluted in Tris-HCl-BSA-Tween 20 for 1 h.

The EC 3.2.1.91 exoglucanase (provided by C. Breuil, University of British Columbia, Vancouver), having an affinity for β -(1,4)-D-glucans, was gold-complexed and used according to Nicole et al. (42). Sections were floated on a PBS drop (pH 6.5) containing 0.02% polyethylene glycol 20000 for 10 min and incubated with the exoglucanase-gold complex for 30 min.

Specificity of labeling was tested by (i) incubating the sections with the antibody or the enzyme previously adsorbed, respectively, with the antigen or the substrate; and (ii) omitting the primary antibody in the procedure.

RESULTS

In the two plots of jack pines naturally infected by the EU race of *G. abietina*, no noticeable differences were observed concerning the behavior of either the pathogen or the host.

Anatomy of healthy shoots. The anatomy appeared typical in symptomless trees. The bark tissues were composed of rounded gray or dark-stained parenchyma cells, resin canals containing well-formed epithelial cells, and sieve cells at times compressed. Two or three layers of vascular cambium cells were visible. The xylem contained typical tracheids, uniseriate ray cells, and transverse and longitudinal resin ducts. Pith cells disclosed a normal appearance, although empty intercellular spaces were observed when leaf traces were discernible. No fungal cells were observed within the tissues (Fig. 2A).

Infected stem, necrotic zone: fungal colonization. Symptoms of the infection by the EU race of *G. abietina* on *P. banksiana* were characterized by a very short tip blight (Fig. 1) on which it was often possible to observe pycnidia and cryptopycnidia under the bark surface. The mean length measured from necrotic brown tip to the healthy green part (Fig. 1) was 0.86 cm.

At the light microscopy level, numerous fungal cells were observed in the necrotic area of the infected shoot. Although most host walls were usually still discernible, there was an intense degradation of the epithelial cells of resin canals in the bark (Fig. 2B) and the xylem (Fig. 2C), as well as collapse and necrosis of parenchyma cells disclosing accumulation of lignin-like substances as detected with the phloroglucinol-HCl test. It was also possible to observe the presence of cryptopycnidia in the outside bark (Fig. 2B). The invaded cambial cells were greatly damaged (Fig. 2C). Even though the xylem tissues seemed less affected by the pathogen, tracheids close to areas of severely affected cambium contained numerous fungal cells, whereas the rays were greatly altered and penetrated by the pathogen on all their length (Fig. 2C). In addition, the fungus had apparently spread easily through the entire length of all the needle traces, shown by the large number of fungal cells and the intensity of host cell alterations in these regions (Fig. 2D). Many fungal cells were also noted between and within degraded pith cells (Fig. 2E).

At the TEM level, the presence of *G. abietina* was confirmed by positive labeling with the anti-GA polyclonal antibody (Fig. 3A). All fungal cells found inside the plant tissue were positive. In the cortex, many fungal cells had either penetrated within parenchyma cells (Fig. 3A) or were apparently attached by their extracellular matrix to the host cell wall, the latter frequently showing evidence of alteration. The content of the cortical cells appeared abnormal, with only remnants of cytoplasm together with crystal bodies or very dense and opaque cytoplasm. Epithelial cells of the cortical resin canals were greatly altered, as well as the casing cells and the lumen of the canal (Fig. 3B). Fungal cells were seen in intercellular spaces and within the disorganized cells of the phloem (data not shown), and around crushed cambial cells that contained dense cytoplasm (Fig. 3C). Hypertrophied cells corresponding to newly differentiated xylem elements were intensely invaded by the fungus and contained abundant rod-shaped crystals (Fig. 3C). The pathogen passage from one tracheid to another was mainly observed across pit membranes (Fig. 3C). The invasion progressed from the vascular cambium to the pith mainly through ray cells where pathogen cells were often found affixed to host walls. Severely disorganized ray cells disclosed either an electron opaque content (Fig. 3C) or were nearly empty. Inside the pith, fungal cells were observed intra- or intercellularly. Intercellular hyphae were surrounded by an opaque layer attached to host walls (data not shown).

Infected stem, necrotic zone: host response. Although the necrotic zone displayed a brownish color and features of advanced necrosis, intense resin exudation was noted at the time of tissue processing. With the toluidine-safranin staining method, it was possible to distinguish on the same section, at the vascular cambium level, two clearly demarcated reactions (Fig. 4A). Thus, a part of the cambial zone showed opaque and mostly crushed cells filled with phenol-like components, whereas the other paler part suggested the possibility of a still functional condition although

cells were rather disorganized. This could be interpreted as an incipient barrier zone formation. Although many noticeable anatomical alterations occurred at the vascular cambium level, some xylem tracheids along the needle trace displayed very dense contents and suberized walls typical to tissues pertaining to barrier zone formation (Fig. 2D). In some places, noticeable gaps were observed in the cambial zone that might have corresponded to recently differentiated resin canals (Fig. 4B). There was a noticeable increase in the number and the shape of crystal structures in or close to the infected cambial cells (Fig. 4C). At times, sclereid-like cells were observed among the altered pith cells (Fig. 4D).

Infected stem, transition zone: host response. Major host responses occurred in the transition zone, notably a barrier zone that was initiated where the first healthy needle from the shoot tip emerged from the stem (Fig. 5). A well-defined suberized boundary was formed along the necrotic area, reaching the cambium and at times crossing the pith area. This barrier was observed to merge with other boundaries that originated from the base of other needles located downward on the shoot, resulting in the formation of a closed compartment confining the pathogen to the invaded zone. Thus, according to the time after infection and the level of sectioning, three different patterns of suberin formation were observed. These are presented schematically in Figure 5A, B, and C. In part A of the diagram, two independent barriers were formed but they never completely crossed the twig. Only a few shoots that were sampled in June belong to this group (Fig. 5A). In the B reaction type, one barrier merged with another barrier at the level of the xylem or the pith area, forming a cup-shaped confinement for the pathogen. Depending on the level of cross sections taken out of the transition zone, transversal patterns of the suberin formation varied from one, two, or even more well-defined barriers

separating the necrotic tissues from the healthy ones (Fig. 5B; 1 to 3). Most of the samples were members of this defined group. In the C reaction type, only one needle was involved in the boundary zone formation that completely crossed the twig. Only a few shoots that were sampled at any time during the season were members of this group (Fig. 5C). These results were supported by examination of serial paraffin-embedded or frozen sections for all the distance covering the transition zone (2 to 3 mm).

Externally, a short tip blight appeared as a symptom (Fig. 6A), while it is possible to observe on a freshly cut cross section obtained from a sample collected in an area similar to that of level 2 in Figure 5B, compartmentalization barriers separated the brown necrotic zone from the healthy green ones (Fig. 6B). Similar frozen sections observed under violet-light excitation after phloroglucinol-HCl staining confirmed the presence of suberin within the cortex and the phloem bordering the cambial zone. A representative section (Fig. 6C) corresponding to that of level 1, Figure 5B, clearly showed a light blue fluorescence boundary surrounding several layers of healthy tissue that were thus completely isolated from the necrotic phloem and xylem. Similar results were obtained with the Sudan IV staining (data not shown). As seen by the red color obtained after the phloroglucinol-HCl test, a lignin deposit was observed on each side of the suberized barrier. In fact, not only did a band of lignin externally separate the necrotic tissues from the suberized ones, but a thin band of lignin formed internally and delimited more or less continuously the healthy tissues from the suberized ones (Fig. 6D). Observations of a similar zone after toluidine-safranin staining showed a quite prominent necrophylactic periderm that extended across the inner bark and reached the level of the vascular cambium (Fig. 7A). At the proximity of the cambial zone, inside the compartmentalized reactive area, several cells in a state of intense division were often ob-

Fig. 1. Longitudinal section of a shoot tip showing invaded tissues divided into a brown necrotic zone and an underlying transition area, with these two zones being followed by sound tissues always associated with healthy needles. Bar = 500 µm.

Fig. 2. A, Section from a control shoot of jack pine showing the vascular cambium (arrowheads), resin canals (arrows), ray cells, phloem (P), and cortex cells (C), and no fungal cells except externally to the bark. **B to E**, Cross sections within the necrotic zone from samples collected in September 1998. **B**, The cortex contains numerous collapsed cells, degraded epithelial cells of resin canals (arrow), and a cryptopycnidium in the outer bark (arrowhead). **C**, The cambium (arrowheads) shows extensive damage and many of its cells are filled with phenolic-like compounds. Fungal cells are particularly discernible in altered ray cells (R), resin canals (Rc), and tracheids (T) close to the altered vascular cambium. **D**, Cells within and adjacent to a needle trace (arrows) are heavily colonized by *Gremmeniella abietina*. Some cells with dense contents and suberized walls (arrowheads) are likely part of a small barrier zone formed over colonized tissues. **E**, Fungal cells (arrowheads) are shown within and between necrotic pith cells. Bars **A and B**, 50 μ m, and **C to E**, 10 μ m.

Fig. 3. Transmission electron microscopy of samples collected in the necrotic zone in June 1998. **A**, Immunogold labeling with the anti-*Gremmeniella abietina* is intense over the fungal cell (F), whereas a very low level of nonspecific labeling occurs elsewhere. This cortical cell has an altered electron-opaque content and shows wall degradation (arrowheads). **B**, A xylem resin canal displays altered epithelial cells (E) and a fungal cell (F) within a casing cell (C) and within the canal lumen (L). **C**, An area including the phloem (P), the vascular cambium (Vc), and xylem (X) cells that are highly disorganized and invaded by fungal cells (F). Many hypertrophied host cells are filled with many rod-shaped crystals (arrowheads). Fungal cells are seen along ray cells (R) and within tracheids where a pit membrane is crossed by the pathogen (arrow). Bars = 1 µm.

served (Fig. 7B). These cells had a rectangular outline disclosing large nuclei and rather dense contents.

With samples taken from areas corresponding to that of level 3 in Figure 5B, several types of host cell responses to infection

could be observed in any given section (Fig. 7C). Thus, it was possible to observe necrotic areas separated by recently formed xylem and hypertrophied phloem. Ray cells within the new xylem were prominent with very dense content. A band of meristematic-

Fig. 4. A, B, and D, Light microscopy and **C,** transmission electron microscopy observations of anatomical changes in the necrotic zone. Samples were collected in the fall of 1998, except for **B**, collected in June. **A**, Two different groups of altered cells are shown in the cambial zone (between arrows): the cells in the right part are opaque and intensely stained, whereas those on the left are less stained and seemingly representative of an incipient barrier zone formation. P = phloem, X = xylem. **B**, Traumatic resin canals (arrows) recently differentiated and located in the phloic-cambial zone. **C**, Cells within the altered vascular cambium are abundantly filled with crystals (arrowheads). Another form of suberized crystal (arrow) is located in dense and suberized phloem cell. F = fungal cell. **D**, Altered pith area (Pi) containing sclereid-like cells (arrowheads). X = xylem. Bars **A** and **B**, 10 μ m, **C**, 1 μ m, and **D**, 50 μ m.

like cells bordered the new tissues and this band was located just below the suberized tissues formed at the upper level. In certain places, a ligno-suberized boundary was still present at both pith and vascular cambium levels (Fig. 7C). Meristematic-like cells were found adjacent to or under the suberized area in the pith (Fig. 7D) and in some places between the regenerated phloem and xylem and remnants of the xylem cells present at the time of tissue invasion (Fig. 7E). In most cases, newly formed xylem tracheids adjacent to the tracheids present at the time of invasion were interspersed among the particularly numerous, large, globoid and heavily stained cells. The new vascular cambium zone was discernible outward from the newly formed xylem (Fig. 6E). In some other regions, however, the vascular cambium was hardly distinguishable (Fig. 6F). At the ultrastructural level, the meristematic-like cells contained a well-defined nucleus, vacuoles, and numerous plastids and lipid bodies. The heterochromatin appeared condensed and the cytoplasm more dense in the case of the first row of meristematic-like cells adjacent to the suberized barrier (Fig. 8A). Suberized cells displayed a very dense cytoplasm (Fig. 8B and C) and, as expected, suberin layers were devoid of significant labeling after use of the exoglucanase-gold complex. How-

ever, the usual lamellar structure of suberin in TEM was hardly discernable (Fig. 8C), even at higher magnifications.

Infected stem, healthy zone: host response. The healthy zone was quite similar to the control samples, except in certain places, the vascular cambium was difficult to discern (Fig. 8D) compared with other areas with a normal appearance (Fig. 8E). Epithelial cells of the resin canals and ray cells displayed a living gray cytoplasm but they were frequently filled with dark globules. In the cortex and the pith, cells with opaque and granulated contents were more numerous than in the controls.

DISCUSSION

To our knowledge, this study is the first to report the invasion of *G. abietina* in this pine species, *P. banksiana*, and it is the first to demonstrate a defense mechanism of a conifer against *Scleroderma* canker. Based on the reconstruction of serial sections, present observations of naturally infected jack pine clearly showed that the formation of a ligno-suberized barrier in the transition zone is the major component limiting the progression of the EU race of *G. abietina*. This type of host response was particularly extensive when the pathogen reached the vascular cambium and spread through xylem rays toward the pith. At the beginning of infection, a new periderm, generally called a necrophylactic periderm, was initiated in the bark along the upper part of a healthy needle. The vascular cambium reacted by laying down a reaction zone, the cells of which were intensely impregnated with lignin and suberin. In that sense, such reaction zone formation seemed to be concomitant with the pattern of progression of the pathogen, except that it was initiated along the trace of a healthy needle rather than an invaded one. The suberin, not studied in detail in conifer species, was also observed in the pith, an area rarely reported with suberin in defense mechanisms of trees. However, we do not exclude the possibility that cells producing reactions could originate from the periphery of the pith.

The formation of defensive tissues in the transition area shows many points of similarity with reaction zones already described in conifers, particularly when one considers pine reactions to rust infection. For instance, concerning fusiform rust, necrophylactic periderm formation was observed mainly in the bark of pine having some degree of resistance to the disease (27,65). Comparable defensive zones were described by other investigators who did not mention, however, that these tissues were likely suberized (28,29,39). Differentiation of a necrophylactic periderm was also described as a response to white pine blister rust (63). Such periderms were also reported to be effective in pine against other diseases (1,3,17,37).

Necrophylactic periderm formation is a well-known reaction of angiosperm trees against different types of damage (reviews; 8,9, 22). Such defensive reactions have been associated with anatomical responses that occur in the xylem in response to injuries or infections (10), which are generally grouped together under the name compartmentalization (reviews; 13,44,59). To our knowledge, this is the first description of the formation of continuous suberized tissues completely crossing the shoot from one needle to the other. If one considers only the continuity of defensive suberized tissues from the original periderm to the pith, present results obtained with jack pine may resemble those described in carnation (*Dianthus caryophyllus*) (2) and in balsam poplar (*Populus balsamifera*) (50) in response to inoculation with wilt-causing pathogens. In this last study, similarly to our results, a band of lignified cells was observed on both sides of the suberized reaction zone, but the significance of this feature is not yet known.

Mullick (40) described a lignified, nonsuberized impervious tissue that is formed just before the differentiation of the necrophylactic periderm in the bark following injuries or infections. Using a more sensitive technique to detect suberin, Biggs (5,7) was able to show that the cells of the impervious tissue in many

Fig. 5. Diagrammatic representation of three types of reactions in the transition zone of *Pinus banksiana* in response to the infection of the European race of *Gremmeniella abietina*. **A**, Two suberized boundaries are initiated along the upper part of healthy needles reaching rapidly the vascular cambium and going downward longitudinally. **B**, Both boundaries cross the xylem and pith areas and join together to form a barrier that circumscribes the necrotic zone. The appearance in cross sections of the suberin pattern at three different levels is represented on the right of the diagram. Section 1, close to the necrotic zone at the level of the first healthy needle; section 2, between the first and the second healthy needles; and section 3, close to the healthy zone, above the junction of the two boundaries. **C**, Only one needle is involved in the formation of a single suberized boundary that completely crosses the shoot.

Fig. 6. **A**, Tip blight observed after infection of jack pine by the European race of *Gremmeniella abietina*. **B to F**, Host reactions in the transition zone of infected shoots of jack pine. **B**, Nonstained freshly cut section with two healthy zones (arrows) surrounded by necrotic tissues. **C**, A section stained with phloroglucinol-HCl and observed under violet-light excitation shows the autofluorescence of the suberized boundary (arrows) delimiting healthy (H) and necrotic (Nc) tissues. Note that suberin is also shown in some xylem tracheids (arrowheads). **D**, A section of the same shoot as in **C** cut at a lower level, stained with phloroglucinol-HCl, and observed under brightfield light that reveals the presence of lignified cells internally (arrowheads) and externally (arrows) to the suberized reaction zone. **E**, Xylem elements regenerated after the beginning of infection are seen among hypertrophied barrier zone cells with dense contents. Arrows indicate when reacting cells were first laid down by the cambium. Arrowheads = vascular cambium. **F**, Vascular cambium cells are hardly distinguishable (arrows) in areas where nondifferentiated globoid cells are shown adjacent to the xylem that was present at the time of the infection. Note that some suberized tracheids have a dense content (arrowheads). Sampling period **A to D**, July 1999, and **E and F**, June 1998. Bars **B to D**, 200 μ m, and **E and F**, 20 μ m.

Fig. 7. Host reactions in the transition zone observed at the light microscopy level. The sampling was carried out in September 1998. **A**, The necrophylactic periderm (arrow) is observed in continuity with other suberized cells (arrowheads) located at the vascular cambium level and along the needle trace (bottom left). A gap (*) between suberized cells and the xylem that likely was formed during tissue processing for microscopy. *l* = intensely lignified tissue. **B**, Meristematic-like cells (arrows) present at the vascular cambium level between invaded xylem (X) and nondifferentiated globoid cells (arrowhead). **C**, A composite of a section showing two separated islands of newly formed xylem (Nw) and necrotic tissues (Nc). Parts of the suberized boundary can be observed at the pith and the vascular cambium levels (arrows). **D**, Meristematic-like cells (arrow) are shown in the pith area bordering suberized cells (arrowheads). **E**, A band of meristematic-like cells (arrows) separates the newly formed tissue (Nw) from that present at the time of infection. Bars **A**, **B**, **D**, and **E**, 20 μ m, and **C**, 100 μ m.

Fig. 8. **A to C,** Transmission electron microscopy observations from samples collected in the transition zone in September 1998. **A,** Good preservation of meristematic-like cells showing the presence of well-defined nucleus (N), large vacuoles (V), numerous plastids (P), and lipid bodies (L). M1 = Meristematic cells adjacent to the suberized barrier. **B to C,** Cells in the suberized boundary containing electron-opaque cytoplasm. Walls are labeled with the exoglucanase-gold complex, except for the suberized layers (arrowheads) that appeared mildly electron-dense and without any typical lamellar structure. **D to E,** Observations of infected stems of jack pine in the healthy zone sampled in June 1998. Sections were obtained just below the naked-eye visible reaction zone. **D,** In some places, the vascular cambium is hardly discernible and some cells (arrows) resemble wounded cells of the transition zone in that they accumulate globulous compounds. Swollen ray cells (arrowheads) are obvious and are filled with dark globules. **E,** Generally, the vascular cambium (arrow) is of normal appearance. Epithelial cells of resin canals display a dense cytoplasm, and cortex parenchyma cells with granulated contents seem more numerous than in the controls. Bars **A and B,** 1 μ m, **C,** 0.5 μ m, and **D and E,** 10 μ m.

broad-leaved trees also contained a thin internal layer that was clearly suberized. Although the formation of the impervious tissue seems a prerequisite to necrophylactic periderm differentiation (5,6,40), we failed to distinguish a distinctly suberized layer in the impervious lignified tissue. Even if it were present at the time of sampling, this layer would have been difficult to detect under epifluorescence due to the overcasting of the intense autofluorescence of suberin in the well-developed necrophylactic periderm that may easily mask such details from immediately adjacent cells.

Cells with suberized walls all appeared to be in a moribund state, and suberin deposition was probably their final metabolic function, as suggested elsewhere (12,30,50). In the present study, suberin layers had the same electron-opacity as that observed in many other studies (12,50,51,53,55,56). However, contrary to these studies, a nonlamellar form of suberin was observed in *P. banksiana* species. As proposed by Litvay and Krahmer (36), the suberin layer would be composed of alternating lamellae of phenolics and waxes where phenols appear electron-dense and waxes, electron-lucent. Because the observations of suberized layers in *P. banksiana* represent one of the first reports of this occurrence in conifers, it might thus be possible that the proportion of waxes is lower than that found in lamellar suberin in angiosperm trees.

In a few of our specimens, the suberized layers were discontinuous (Fig. 5A) around the infected xylem and pith tissues. These specimens were all collected early in the season; it is, therefore, likely that if they had been sampled later in the growing season, boundaries could have been found continuous. However, other deterrent mechanisms might also have contributed to limiting the progression of the pathogen. For instance, it is well known that in conifers, and particularly in pines, copious amounts of resin are frequently produced in response to wounding or infection (1,3,17,37,66,67), which is assumed an important defense mechanism. The presence of resin was easily noticeable in our samples, especially those collected in the necrotic area of the shoot. Changes reported in the present study may therefore not be exclusive of other defense mechanisms that occur in *P. banksiana*, and it is likely that many other important biochemical or biophysical events may have occurred in *P. banksiana* without necessarily resulting in anatomical changes detectable in microscopy. For instance, the production of phytoalexins can often play an important role in the cascade of events in tree defense (21,44) and constitutive monoterpenes appear to provide resistance to Scleroderris canker in certain cases (62).

In the transition zone, the vascular cambium was at times difficult to recognize. Many large cells, apparently in an intensive state of division, were present in the region that normally would correspond to the cambium. As proposed by Jewell and Speirs (29), it is plausible that these meristematic-like cells, formed following the killing of the cambial cells, subsequently gave rise to new cambium and other layers of reactive tissues that we consistently observed.

Using immunogold labeling, we confirmed that all fungal cells observed belonged to *G. abietina* species. This was essential because the samples originated from naturally infected plantations. The antibody used has been tested with *Sirococcus strobilinus*, a pathogen in conifers with many similarities to Scleroderris canker, and no immunoreactive sites in its proteins have been detected, whereas only a weak positive reaction was obtained with a species of *Fusarium* isolated from Scots pine (46). The present study provides specific details about the mode of progression of the EU race of *G. abietina* that intensely invades *P. banksiana* before the launching of its defense mechanisms. Our results show a severe colonization of needle trace cells that often allowed the invasion of the pith, a type of invasion already reported for other pine diseases (27,29,37,39). Furthermore, present observations demonstrate that the pathogen can spread quite easily in the vascular cambium, within parenchyma cells of rays and those surrounding

resin canals. *G. abietina* produces enzymes capable of hydrolyzing hemicellulose xylan (47) and pectinic compounds (45). Using different cytochemical probes at the TEM level, Ylimartimo et al. (69) have shown that the NA race of *G. abietina*, in the sensitive host *P. resinosa*, is able to degrade cellulose and that phenoloxidases produced by this pathogen are likely involved in lignin degradation. Moreover, they strongly suggested that the easily discernible extracellular sheath is directly involved in degradation of host tissues and might thus facilitate the diffusion of such enzymes.

Many observations suggest that impervious tissue, necrophylactic periderms (40), or barrier zones (13,44,59) were formed as nonspecific responses to injury regardless of the causal agent. The genetic control of suberin deposition in wounded bark tissues has been demonstrated for peach by Biggs et al. (11). *P. banksiana* has the ability to produce suberized boundaries when infected by the EU race of *G. abietina*, but the question is why such defense mechanisms are absent in trees of this species infected by the NA race? This difference in tissue reactions may be pivotal to explain the host-pathogen relationship in such diseases. Several possibilities may be proposed to explain such a difference (16). For example, the NA race could produce inhibitors or enzymes capable of interfering with defense processes such as the restoration of the vascular cambium and of continuous bands of phellogen as suggested by Mullick (40,41). It may be possible that the NA race is insensitive to constitutive or induced defensive chemicals produced by *P. banksiana*. At this stage, the present study does not allow us to define the phenomenon but it is also clear that a similar study with both races in a controlled environment might give more insight into these possibilities.

Our study suggests that the formation of ligno-suberized boundaries followed by the restoration of cambial activity and tissue regeneration are the essential factors in the defense system of *P. banksiana* against the EU race of *G. abietina*. Such resistance against the EU race has been observed with *P. contorta*, and a study is now in progress to determine if similar defense mechanisms also occur with this species. As reported by Laflamme and Blais (34), one has to consider that even if jack pine expresses such resistance in the field, it nevertheless represents a potential source of inoculum that can be disseminated to nearby sensitive coniferous species, such as red pine, because *G. abietina* produced fruiting bodies on the dead or dying tip.

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