First Extensive Microscopic Study of Butternut Defense Mechanisms Following Inoculation with the Canker Pathogen *Ophiognomonia clavignenti-juglandacearum* Reveals Compartmentalization of Tissue Damage

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ABSTRACT

Ophiognomonia clavignenti-juglandacearum endangers the survival of butternut (*Juglans cinerea*) throughout its native range. While screening for disease resistance, we found that artificial inoculations of 48 butternut seedlings with *O. clavignenti-juglandacearum* induced the expression of external symptoms, but only after a period of dormancy. Before dormancy, compartmentalized tissues such as necrophylactic periderms (NPs) and xylem reaction zones (RZs) contributed to limiting pathogen invasion. Phenols were regularly detected in RZs, often in continuity with NPs during wound closure, and confocal microscopy revealed their presence in parenchyma cells, vessel plugs and cell walls. Vessels were blocked with tyloses and gels, particularly those present in RZs. Suberin was also detected in cells formed over the affected xylem by

the callus at the inoculation point, in a few tylosis walls, and in longitudinal tubes that formed near NPs. Following dormancy, in all inoculated seedlings but one, defensive barriers were breached by *O. clavignenti-juglandacearum* and then additional ones were produced in response to this new invasion. The results of this histopathological study indicate that trees inoculated in selection programs to test butternut canker resistance should go through at least one period of dormancy and that asymptomatic individuals should be dissected to better assess how they defend themselves against *O. clavignenti-juglandacearum*.

Additional keywords: catechins, flavonoids, fluorescence, hybrids, lignin, tannins.

Butternut canker, caused by the fungus *Ophiognomonia clavignenti-juglandacearum* (Nair, Kostichka, & Kuntz) Broders & Boland (previously known as *Sirococcus clavignenti-juglandacearum*), was first reported in the United States in Wisconsin (United States) in 1967 (Renlund 1971) and in the province of Quebec (Canada) in 1990 (Innes and Rainville 1996). The widespread occurrence of diseased and dead trees originally described in Wisconsin suggests that the disease had been active for many years prior to 1967 (Kuntz et al. 1979). Since then, the disease has threatened the survival of butternut (*Juglans cinerea* L.) throughout its native range in North America, to the extent that in Canada butternut has been listed as endangered under the *Species at Risk Act* since 2005 (Environment Canada 2010). In the United States, butternut is considered a species of special concern, meaning that it is not protected under the *Endangered Species Act*, but at the state level, it has received other designations such as threatened or vulnerable (USDA-AGR 2017).

The geographic distribution of three distinct clonal lineages of *O. clavignenti-juglandacearum* in North America suggests that the pathogen was introduced, or has emerged from unknown hosts

or from an avirulent form on butternut, on more than one occasion (Broders et al. 2012). When a tree is infected, conidia are usually produced on small branches in the lower crown, and are spread downward via rainwater and may also be airborne in rain splashes and aerosols (Tisserat and Kuntz 1983, 1984). It has been reported that conidia dispersed as droplets can be disseminated up to about 40 m (Tisserat and Kuntz 1983) to 46 m (Nicholls 1979). New infections occur through various wounds or via natural openings such as leaf scars, lenticels, bark fissures, and around adventitious buds (Ostry 1997; Sinclair and Lyon 2005). Coalescing cankers on the trunk and buttress roots eventually kill the tree. The pathogen can be seedborne on black walnut (*J. nigra*) and butternut (Innes 1998) and dissemination by insects is also strongly suspected (Halik and Bergdahl 2002; Katovich and Ostry 1998).

Occasionally healthy butternut trees or individuals that apparently limit pathogen colonization are found among dying or dead trees (Ostry and Moore 2008). These trees are potentially resistant to butternut canker and should be retained, for instance as seed sources, and eventually exploited in conservation and restoration programs (Ostry and Woeste 2004). Silvicultural guidelines suggest the retention of butternut trees with more than 70% of live crown and less than 20% of the combined circumference of the trunk or root flares affected by cankers, and all trees with at least 50% live crown and no canker on the bole or root flares (Ostry et al. 1994).

In 2010, we started a project aimed at identifying, propagating and testing trees putatively resistant to butternut canker. We located among diseased butternut trees several mature individuals that might be resistant, but their vegetative propagation was very difficult

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to achieve. As an alternative, nuts were collected under these trees and seedlings emerging from them were artificially inoculated with *O. clavignenti-juglandacearum*. As some seedlings showed signs of resistance, stem samples collected from them were examined in microscopy to study their defense mechanisms, which are described in the present paper. To our knowledge, such mechanisms were only briefly reported in a book chapter (Nair 1999) showing for instance that phenols and vessel occlusions were apparently produced to fend off the pathogen.

MATERIALS AND METHODS

Plant material. During our project, 202 trees potentially resistant to butternut canker were located in the province of Quebec among dying or dead trees affected by the pathogen. These trees showed characteristics that met the retention criteria proposed by Ostry et al. (1994). We tried to vegetatively multiply around 100 of these trees using either axillary bud culture, roughly following the method described by Pijut (1997), or cuttings according to Pijut and Moore (2002), but as most of the individuals were mature, these propagation methods were unsuccessful. As an alternative way of testing their resistance, nuts were collected from these butternuts or on the soil under 15 of those trees. Nuts from hybrids with Japanese walnut (*J. cinerea* × *J. ailantifolia*) were also obtained from the public nursery of the Ministère des forêts, de la faune et des parcs in Berthier (QC, Canada). The husk was mechanically removed at this nursery using a device called “The Nut Huller” (Cobjon Nutculture Services, Ottawa, ON, Canada). First, 50 ml of water was poured into plastic containers (6.25 liters) and the depulped nuts were then placed on a support to avoid contact with the liquid. The moisture level, kept around 100%, was measured with sensors (Hobo ProV2, Onset, Bourne, MA) placed inside the containers before sealing and placing them in cold chambers adjusted at 2°C for 123 days.

With a global germination rate of only 17%, we did not obtain enough seedlings to conduct the inoculation assays with some of our material, so additional butternut seedlings were bought from Lafeuillée, a private nursery located in Joliette (QC, Canada). The tree source of nuts from which these seedlings were cultivated in this nursery is unknown. Table 1 lists details about the seedlings used in the study. SAD1, SAD11, SAD14, FRE105, MBGHYD, and JOL1 seedlings were randomly distributed in two greenhouse compartments (blocks) at the Laurentian Forestry Centre (LFC). Each block was set to contain 48 seedlings (four inoculated and four controls from these six sources of seedlings), but in the end, we were short of five controls, so in all there were 91 seedlings (48 inoculated and 43 controls) in the experimental blocks. Additional seedlings from trees SAD19 and ODA1 (Table 1), also potentially resistant to canker, were inoculated and observations about them are presented in the Results.

Tests conducted with four microsatellite markers on the DNA extracted from leaves of butternut trees, including SAD11, SAD19, and ODA1, did not show any sign of hybridization, notably with Japanese walnut (*J. A. McLaughlin* and *G. Halicki Hayden*, Ontario Ministry of Natural Resources and Forestry [OMNRF], unpublished data). These tests included additional putatively

resistant butternut trees originating from Saint-Augustin-de-Desmaures (5), Odanak (11), and Frelighsburg (10), the three sites where the nuts come from in the present study (Table 1), and all of them appeared to be pure butternuts. To ensure that any trace of hybridization was detected, more thorough assays were conducted at the LFC using 2.6 million Ion Torrent sequences (around 160 bp on average) generated using genotyping-by-sequencing (GBS), with the total genomic DNA from 63 trees, including trees SAD11 and ODA1. The GBS method consists in sequencing random regions of the genome cut by two rare restriction enzymes. Those analyzes confirmed that there were no apparent trace of hybridization in trees tested on the three sites mentioned above (N. Isabel and M. Lamothe, LFC, unpublished data).

We also obtained, from the OMNRF, grafted material from putatively resistant butternuts inoculated with *O. clavignenti-juglandacearum*. A few of these scions were examined in microscopy in the present study.

Inoculation and sampling. Seedlings were 20 weeks old when they were inoculated in the greenhouse on 25 to 26 June 2012. Temperatures in the greenhouse were set at 28 and 22°C during the day and night, respectively. Seedlings were well watered and generally fertilized with Plant-Prod’s 20-20-20 (Laval [Qc], Canada) to weekly provide 4 mg of product by seedling. Inoculation was carried out with an isolate of *O. clavignenti-juglandacearum* collected from a seedling from the Lafeuillée nursery that showed symptoms of butternut canker and signs such as hyphal pegs, tiny stomatal columns that may lift and/or rupture the thin bark of small branches (Sinclair and Lyon 2005). Necrotic tissues and hyphal pegs were surface sterilized with 50% ethanol for 30 to 45 s, rinsed in distilled water, and placed onto potato dextrose agar (PDA) containing 50 ppm of streptomycin. The bark was surface disinfected with 70% ethanol 5 cm above the soil level and, at each inoculation site, the bark was removed with a sterilized cork-borer (4.75 mm in diameter). Agar plugs (4 mm in diameter), taken at the edge of actively growing mycelium, were immediately placed onto the exposed cambium and the wound was covered with Parafilm (Bemis NA, Neenah, WI). Controls were treated with uncolonized agar plugs. Scions were wounded (some at two opposite locations on the stem) using three sterilized needles taped together, producing holes approximately 1 mm deep (S. Greifenhagen, OMNRF, personal communication). An agar plug (4 mm in diameter) from the advancing edge of *O. clavignenti-juglandacearum* cultures was placed over the holes, which were then covered with Parafilm.

On 13 August 2012, 42 days postinoculation (dpi), two controls (one with an open wound and the other with a closed wound) and two inoculated seedlings (one with an open wound and the other with a closed wound), all asymptomatic, were prepared for microscopy. To induce dormancy, all the other seedlings, still asymptomatic, were placed in growth chambers at 4°C on 3 December 2012, and at the beginning of January and February 2013, the temperature was reduced by 2°C; finally in mid-February, it was reduced one last time to -2°C. On 1 March 2013, seedlings were returned to the greenhouse at a temperature of 14°C for 48 h before setting the temperature as previously described. After 40 days

TABLE 1. Information about the seedlings inoculated with *Ophiognomonium clavignenti-juglandacearum*

Tree or source of seedlings	Location	UMT Coordinates Zone-Easting-Northing	Resistance
SAD1	Saint-Augustin-de-Desmaures	19-309485-5177488	Potentially
SAD11	Saint-Augustin-de-Desmaures	19T-309458-5177567	Potentially
SAD14	Saint-Augustin-de-Desmaures	19T-309416-5177548	Potentially
FRE105	Frelighsburg	18T-676128-4989882	Potentially
MBGHYB ^a	Botanical Garden, Montreal	18T-612599-5046286	Potentially
JOL1 ^b	Joliette	18T-615956-5104428	Unknown
SAD19	Saint-Augustin-de-Desmaures	19T-308851-5177215	Potentially
ODA1	Odanak	18T-664631-5105833	Potentially

^a Location of four adjacent hybrids (*J. cinerea* × *J. ailantifolia*) from which nuts were obtained.
^b Source trees, from which the nuts were obtained, are unknown. Location and coordinates of the nursery where the seedlings were cultivated.

(290 dpi), samples taken from the stem of the remaining 87 seedlings were prepared for microscopic observations. Reisolations of *O. clavignenti-juglandacearum* were attempted on PDA as previously described.

Tissue processing for microscopy. Transverse sections of the stem of all control and inoculated seedlings were examined at the inoculation point and 1.5 cm above the wound with a SZX16 stereomicroscope (Olympus, Markham, ON, Canada). We selected several of those seedlings for other microscopic observations of their defense reactions and to study what happens when the pathogen succeeds in inducing symptoms. In most cases, samples were then collected from three areas of the stem: within the wound, and at 5 mm above and 5 mm below the edge of the wound. Samples were processed according to one of the two following methods: (i) fresh samples were embedded in O.C.T. Compound (Miles Scientific, Slough, UK) at -20°C . Sections ($\sim 25\ \mu\text{m}$) were obtained with a Histostat cryomicrotome (Reichert, Vienna, Austria) and used with histochemical reagents (see below); or (ii) to study their fine anatomy, fresh samples were placed in 2% formaldehyde-2% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB) (pH 7.4), fixed overnight at 4°C , and then processed in a PELCO Microwave Pro oven (PELCO International, Redding, CA) according to the following schedule: 750 W, 0 W, and 750 W (20 s each), and 200 W, 0 W, and 200 W (40 s each) under vacuum (15 in of Hg), at a maximum temperature of 30°C . Buffer rinses were carried out three times in 0.1 M SCB for 45 s under the same vacuum at 300 W. Postfixation in 1% osmium tetroxide in 0.1 M SCB under the same vacuum was conducted at 100 W, 0 W, and 100 W for 3 min each, and rinsed as described above. After dehydration in ethanol gradients ranging from 30 to 100%, ethanol/propylene oxide (1:1, vol), and treatment with pure propylene oxide at 300 W for 45 s each, samples were infiltrated with JEMBED 812 epoxy (Canemco Inc., Lakefield, QC, Canada). A few samples were also embedded in hydrophilic acrylic resin Unicryl (British Biocell International, Cardiff, UK) to obtain better staining reactions. After the usual fixation and dehydration in graded series of ethanol, the samples were infiltrated with pure Unicryl twice for 1 h each time (Rath et al. 2014). After polymerization of both resins for at least 2 days at 60°C , sections ($\sim 1\ \mu\text{m}$) obtained with an Ultracut E microtome (Reichert-Jung, Vienna, Austria) were stained with epoxy tissue stain (ETS), a mixture of toluidine blue O and basic fuchsin (Canemco Inc., Lakefield, QC, Canada), and observed with a Polyvar light microscope (Reichert, Vienna, Austria). For transmission electron microscopy (TEM), ultrathin sections ($\sim 90\ \text{nm}$) obtained with the same microtome were contrasted with uranyl acetate followed by lead citrate (Reynolds 1963) and examined at Université Laval with a JEM-1230 (JEOL, Peabody, MA) transmission electron microscope operating at 80 kV. To help observe the lamellar structure of suberized layers in TEM, some sections were also contrasted with 1% OsO_4 , followed by a mixture of one volume of 2% uranyl acetate (UO_2Ac) and one volume of 1% potassium permanganate (KMnO_4), according to Wattendorf (1974).

Fluorescence microscopy, histochemical tests, and cytochemical tests. To detect lignin and suberin, sections obtained with the cryomicrotome were treated with phloroglucinol-HCl (Jensen 1962) and examined under either brightfield or UV-light excitation with a Polyvar microscope (see above), respectively (Biggs 1984). Phloroglucinol-HCl stains lignin, but in doing so, it also quenches its autofluorescence and thus helps better reveal the presence of suberin under UV illumination. The autofluorescence of suberin under UV, also at times detected with unstained sections, was obtained using a BP 330 to 380 exciter filter with a DS 420 separator mirror and a LP 418 barrier filter. Sudan black B was also used as additional evidence of the presence of suberin in host tissues (Jensen 1962). Blue light illumination was used to detect some phenols that frequently emit a yellow fluorescence under these conditions. Such blue illumination was produced using a BP 450

to 495 exciter filter combined with a DS 510 separator mirror and a LP 520 barrier filter.

To obtain images of fluorescence with better resolution and contrast, cryomicrotome and hand-made sections from unfixed material, some mounted on microscope slides according to Rath et al. (2014), and then at times also using JIM5 (see below) to localize pectin, were examined in confocal microscopy with an inverted IX83 microscope coupled to a Fluoview FV 1200 laser scanning unit (Olympus Corporation, Tokyo, Japan). This microscope, located at the LFC, is equipped with five lasers that can scan the material at seven wavelengths: four laser diodes producing 405, 559, 635, and 748 nm, and a multiargon laser that can provide 458, 488, and 515 nm. It also comprises a Z-stack acquisition system that can generate images in three dimensions.

In sections obtained with the cryomicrotome, condensed tannins and their catechin monomers were revealed using the vanillin-HCL test, as described in Valette et al. (1998). Neu's reagent (Neu 1956) was used to detect the presence of other phenolics, also as described in Valette et al. (1998). The flavonoids are then expected to take a shade of yellow to orange when excited under blue light illumination (Valette et al. 1998), while a yellow-green color would indicate the presence of caffeic acid derivatives (Andary et al. 1996).

Detection of pectin in TEM was carried out according to Rioux et al. (1995). Briefly, the monoclonal antibody JIM5 (provided by K. Roberts, John Innes Institute, Norwich, UK), which recognizes pectin with up to 50% esterification, was used with ultrathin sections mounted on nickel grids that were floated on a drop of PBS (0.01 M)-BSA (1%)-Tween 20 (0.05%) (pH 7.1) buffer for 10 min and thereafter incubated with the antibody for 2 h at 37°C . They were rinsed with the same buffer, floated on a drop of Tris-HCl (0.02 M)-BSA (1%)-Tween 20 (0.05%) (pH 7.2) buffer, and thereafter incubated with a goat anti-rat antibody (IgG) conjugated to colloidal gold (15 nm) diluted in the Tris-HCl-BSA Tween 20 buffer and then rinsed twice in water (5 min each time).

RESULTS

External symptoms. In 2012, no symptoms at all appeared on the seedlings at 42 dpi. After dormancy (290 dpi), external symptoms on the foliage (e.g., stunted and/or wilted leaves) were frequent in the inoculated seedlings and 46% of them were considered as dying or dead. Most of these seedlings, as well as asymptomatic ones, also had brown cankers and at times some black oozing liquid was seen around the inoculation point. *O. clavignenti-juglandacearum* was reisolated from 30 of the 48 inoculated seedlings. Substantial variation was observed concerning the length of bark necrosis, the length of internal lesions, and the rapidity of wound closure (*unpublished data*). However, the histopathological changes reported below were constantly observed in all seedlings under study.

Internal symptoms. Macro-histopathological changes related to compartmentalization. Before dormancy. In the four seedlings studied at 42 dpi, defense mechanisms appeared quite similar, whether or not they had been inoculated. The only variation observed was in relation to the closure of inoculation wounds. When the wounds were closed, the affected xylem was completely surrounded by tissues heavily impregnated with various substances (Fig. 1A to D), whereas the xylem that forms the largest part of the callus, the woundwood, was more intensely stained for lignin than the xylem extant at the time of inoculation (Fig. 1B). Under blue light illumination, the yellow fluorescence likely indicative of phenol compounds was generally intense in reaction zone (RZ) cells that seem to limit the tangential and inward spread of the invasion (Fig. 1C). These phenols were occasionally also observed in cells that formed a short tangential band at the edge of the fluorescing zone where the xylem was wounded during inoculation (Fig. 1C and F). Some isolated fluorescing ray cells were also noticeable

Fig. 1. Compartmentalization processes in asymptomatic seedlings before dormancy. **A to D**, Cross-sections within the wound of an inoculated SAD19. **A**, The dark invaded xylem appears surrounded by brown tissues (black arrow), and necrotic tissues (NTs) in the bark also appear compartmentalized by the necrophylactic periderm (NP, arrowhead). **B to D**, The same section stained with phloroglucinol-HCl and observed under three different illuminations. **B**, Under brightfield, the invaded xylem (asterisk) is entirely covered by a dark brown layer (white arrowhead). NTs are obvious outside the NP (white arrow). The woundwood (black arrowhead) consists of xylem cells more heavily stained for lignin than normal wood. Black compounds have accumulated in the cortex (blue arrowhead) and phloem (black arrows) and the gray/red arrowhead shows the limit of this accumulation around the affected tissues. **C**, Under blue illumination, the yellow autofluorescence of phenols is intense tangentially and internally (reaction zones) around the invaded xylem. This fluorescence extends slightly tangentially (white arrow) close the brown layer shown in **B**, and on its left side, a ray also emits the yellow autofluorescence (yellow arrow). A few woundwood cells also fluoresced (yellow arrowhead). **D**, Under UV, suberin of the NP left by the expanding callus (white arrow) is obvious (white arrow) over the invaded xylem, in the NP and in the exophylactic periderm (yellow arrow). The yellow arrowhead indicates a small injury in the exophylactic periderm delimited by a NP. The suberized tube on the left is in continuity with the NP. **E and F**, The same section, from an inoculated SAD11, examined under **E**, UV and **F**, blue light. **E**, The NP of the expanding callus leaves a layer of suberized cells (white arrow) over the invaded xylem. The NP is continuous with the exophylactic periderm (white arrowhead) and its thinnest part (yellow arrow) is seen where it reaches the vascular cambium. Bark tissues (B, cortex and phloem) and woundwood (WW) in the expanding callus. **F**, The yellow autofluorescence of phenols is obvious in the reaction zone, and its lateral portion (arrow) is in continuity with the limit of the NP (arrowhead). **G**, In this control (SAD14) with an open inoculation wound, the yellow autofluorescence internally compartmentalizes the affected xylem and is continuous with the slightly autofluorescent NP (arrowhead) at the cambium level (arrow). ST = suberized tubes.

occasionally in this area (Fig. 1C). In the xylem directly exposed to air following wounding, the affected xylem took a natural dark appearance (Fig. 1A) and only the cells surrounding laterally and internally this invaded xylem showed yellow autofluorescence under blue light (Fig. 1C). The invaded xylem was externally covered by suberized tissues (Fig. 1D) that derived from the necrophylactic periderm (NP) that unfolds over the damaged xylem during wound closure when the callus develops and moves toward the center of the inoculation point (Fig. 1E). When the wounds were still at least partially open, an RZ was formed internally to the affected xylem tissues that also appeared to be mainly composed of cells containing phenols, and this was observed in inoculated seedlings (Fig. 1F) and controls as well (Fig. 1G). This barrier rich in phenols was then always in continuity with the NP cells formed in the bark at the point where the suberized cells reached the cambial zone (Fig. 1E to G). In the most resistant butternut scion that was inoculated in Ontario (S. Greifenhagen, OMNRF, *personal communication*), such autofluorescence was again detected all around the tissues that appeared invaded by the pathogen (Supplementary Fig. S1), while in other scions with canker symptoms such autofluorescence was also present but less intense and always scattered in a few cells within or surrounding the discolored xylem.

After dormancy. In all controls and in the only inoculated seedling that was still apparently healthy at the time of sampling and that presented intact compartmentalized tissues, it was possible to observe compartmentalization reactions similar to those found in seedlings before dormancy. In those seedlings, suberin was easily detectable in the NP, using either Sudan black B or suberin autofluorescence (Fig. 2A), or in the suberized layer included within the xylem that covered the affected area after wound closure (Fig. 2B and C). However, phenols in the reactive xylem generally displayed a yellow autofluorescence that was less intense under blue light than before dormancy, and/or in areas more limited in size, in both controls and inoculated seedlings, even when the latter had well compartmentalized the invaded xylem (Fig. 2D).

In addition to having walls that seemed more lignified (Figs. 1B and 2H), the first differentiated cells in woundwood stained differently with the dyes used in our study (Figs. 1B and 2E to G), or emitted autofluorescence that was slightly different than elsewhere in the woundwood (Fig. 2C and D). In addition, these cells deviated from their usual radial course (Fig. 2E and H), and some of them close to the suberized layer were even perpendicular to their normal orientation (Fig. 2E). In comparison with normal xylem, vessels were rare and smaller in woundwood (Fig. 2E). Some of the other xylem elements were parenchyma cells that appeared at times quite numerous. Shortly after wound closure, the vascular cambium gradually resumed its normal production of xylem elements (Fig. 2C and D).

With the vanillin test, there was a positive reaction with suberized cells of the periderms, such as the ones formed over the damaged xylem (Fig. 2F), as well as with the cells close to those suberized cells which had a natural dark coloration in Figure 2E. The red staining with vanillin also corresponded in some areas to the yellow autofluorescence revealed under blue light, particularly in some occluded vessels and in ray cells surrounding the invaded xylem (see similar sections from a SAD11 seedling under blue illumination (Fig. 2D) and the red staining with vanillin (Fig. 2F)). Some xylem cells of the woundwood also appeared red, but then only close to the edge of the suberized layer (Fig. 2F). A typical reaction was also observed within or around the bark included in the xylem (Fig. 2F), or in healthy bark tissues contiguous to the NP (results not shown). With Neu's reagent, an emission of yellow-green fluorescence indicative of caffeic acid derivatives was at times observed around bark tissues left in the xylem by the callus as well as a yellow fluorescence that indicated the presence of flavonoids in adjacent woundwood (Fig. 2G). Woundwood was

generally observed even in seedlings that were dead or dying, but then the callus rarely succeeded in completely closing the inoculation wound (Fig. 2H). In such seedlings, the necrosis was usually obvious in the xylem and in the bark that often extended beyond the NP (Fig. 2H), where the host attempted to limit its progression by apparently forming suberized cells, some of the latter being similar to the suberized tubes shown in previous figures (Figs. 1A to D and 2A).

Most inoculated seedlings displayed obvious external symptoms such as stunted and wilted leaves (Fig. 3A) and, as shown in Figure 2H, frequently had open inoculation wounds surrounded by cankered tissues (Fig. 3A and B). With the exception of one seedling, those that were apparently healthy following inoculation, for instance a SAD11 seedling that showed a big callus having closed the inoculation wound (Fig. 3C and D), showed extensive internal necrotic tissues when examined in stereomicroscopy that went beyond the first compartmentalization barriers (Fig. 3E). In such seedlings, or when they were killed or dying, the lesions were usually more prominent in the bark than in the xylem, and this was more obvious in samples collected at 1.5 cm above the inoculation wound (Fig. 3E). Phenols initially present around the invaded xylem zone, such as those reported before dormancy, were still detectable under blue light illumination, but again the autofluorescence seemed to have faded (Fig. 3F). Suberized tissues that helped close the inoculation wound as well as the NP were still easily detectable (Fig. 3G). Bordering the initial NP were other suberized structures that were more numerous in inoculated seedlings (Figs. 1A to D, 2A, and 3G) than in controls, indicating that this area was also injured and that the tree had tried to restrict the development of the damaged tissues. These suberized tissues often appeared to form longitudinal tubes or canals in conventional fluorescence microscopy (Figs. 1D, 2A, and 3G), but when several continuous sections were examined and merged in confocal microscopy, it was clear that at least some of them were inclined in relation to the longitudinal axis (Fig. 3H). These tubes were usually closely associated with the NP and, at times, were clearly in continuity with them in some places (Figs. 1D and 3H). Confocal microscopy observations also revealed that the NP could be very thin or even indicated discontinuity in some places (Fig. 3H). In another SAD11 seedling that was still apparently healthy, except for the presence of a black liquid slightly oozing from the bark around the inoculation point, cross-sections taken at the inoculation wound revealed that the invaded xylem was still well compartmentalized and that the callus was apparently healthy (Fig. 3I). However, the bark and a part of the xylem close to the cambium displayed the conspicuous presence of dark necrosis contiguous to both sides of the callus, while a few small black lesions were at times also visible in the xylem (Fig. 3I). In all inoculated seedlings that were killed by the pathogen, the cross-sections were entirely necrotic.

Fine histopathological changes. Unless specified otherwise in the Figure legends, most of the following observations were obtained from samples collected after dormancy. In confocal microscopy, phenolic deposition corresponding to the yellow fluorescence in previous figures was clearly distinguishable in parenchyma cells, vessel occlusions, and cell walls (Fig. 4A). As indicated in Figure 1F, few ray cells seem to have accumulated such phenolics in the fluorescing zone (Fig. 4A), although at times phenols intensely stained with toluidine blue O using thin resin sections were seen in some ray and axial parenchyma cells bordering the affected xylem (Fig. 4B). As previously mentioned in relation to Figure 1E to G, those reacting cells were continuous with, or in the vicinity of, the NP cells that reached the cambium (Fig. 4B). Elsewhere in the invaded xylem, fungal cells were frequently noticeable, particularly in xylem elements that were close to the suberized cells left by the callus (Fig. 4C), whereas they were absent in woundwood. In samples embedded in the epoxy resin, it was clear that the suberized walls were difficult to

impregnate as gaps representing artifacts were often seen right in the middle of such suberized layers (Fig. 4C).

Although at times vessels with an internal layer that was clearly lamellar (Fig. 4D) were observed in brightfield, it was impossible, for instance using UV illumination, to clearly reveal the presence of suberin in such layers. In TEM, the lamellar structure was not apparent, or at least not as obvious as that very occasionally seen by the senior author in the nonhost *Prunus pensylvanica* inoculated with the Dutch elm disease pathogen (Supplementary Fig. S2). Elsewhere in the invaded xylem, gels (at times called gums in the literature; Fig. 4E to G) and tyloses (Figs. 4G and 5A to C) were found in most vessels, but these occluding structures

were more abundant in the vicinity or in the area where the yellow autofluorescence was distinguishable under blue light and then they seemed to contain at least some phenolics detected in confocal microscopy (Fig. 4A) or in brightfield using toluidine blue O (Figs. 4B and E to G and 5A and B). It was frequent to observe in the parenchyma bordering such occluded vessels, whether in ray or axial cells, an internal wall layer that was generally thicker than elsewhere in the cell and also frequently stained a shade of pink when using ETS, but only so in the part facing the vessel side (Figs. 4F and G and 5A to C). At times, only the part of this wall layer in front of the pit membranes showed such thickenings (Fig. 4D and F). When gels were seen, the portion

Fig. 2. Samples collected from seedlings after dormancy. **A**, The necrophylactic periderm (NP) is evident under UV (white arrow) in this asymptomatic MBGHYB. The ventral part (yellow arrowhead) is thinner than the expanding portion (white arrow) of the NP. Woundwood rays are obliquely oriented (white arrowhead). Yellow arrow = Suberized tube. **B to F**, An asymptomatic inoculated JOL1 having compartmentalized the invaded xylem and completely closed the inoculation wound. **B**, The black invaded xylem at the inoculation point (left) appears well compartmentalized. The wound is closed, but its center is still slightly sunken (arrowhead). The arrow points to some bark included where the NPs of the callus have merged (see also **C**). The asymptomatic section on the right was taken at 1.5 cm above the inoculation point. **C and D**, The same unstained section examined under **C**, UV and **D**, blue light. **C**, The suberized layer covering the dark invaded xylem is obvious and the point where both NPs merge is indicated (white arrow). Just outside the suberized layer, some dark bark tissues that were present in the callus at the time of fusion of the NPs are noticeable. Lignin autofluorescence is evident everywhere in the woundwood, but takes on a brighter appearance (black arrows) close to the compartmentalized tissues. **D**, The yellow autofluorescence of phenols is visible (arrow) but has faded compared with that observed before dormancy (Fig. 1C, E, and F). **E**, After treatment with phloroglucinol-HCl, the lignin is stained and woundwood elements such as vessels are small and some rays are horizontally oriented (yellow arrow). The white arrowhead points to the suberized layer and just externally to it, white cortical cells that appear free of lignin monomers can be seen. Some xylem cells that have a natural brown color (white arrow, see also the white arrowhead in Fig. 1B) seem slightly stained with phloroglucinol-HCl, while others at the tangential edge of woundwood take a bright red color (yellow arrowhead). **F and G**, Cryomicrotome sections from the same sample. **F**, The positive red reaction with vanillin-HCl is particularly obvious in the suberized layer (black arrowhead), in the rays and vessels of the internal reaction zone (RZ, white arrowhead) compartmentalizing the affected xylem and in the zone corresponding to the included bark (black arrow). At both tangential limits of the suberized layer, the staining is also noticeable in some woundwood cells (gray/blue arrowheads) and in RZs (white arrows). **G**, After reacting to Neu's reagent, the area just above the suberized layer displays a green fluorescence (black arrow) under blue illumination and a yellow one (white arrows) in the woundwood close to this layer. **H**, A cryomicrotome section stained with phloroglucinol-HCl from a dead inoculated SAD14 showing the NP (black arrowhead) and the oblique orientation of rays in woundwood (white arrowhead). This arrowhead also indicates that the staining for lignin is more intense in this area than elsewhere in the xylem. The inoculation wound is partly closed and the lesion extends beyond the NP in the bark (white arrow). Black necrotic xylem elements are also discernible (black arrow).

adjacent to the parenchyma was at times stained differently, taking on a pinky hue resembling that present in the internal wall layers of parenchyma cells facing the occluding vessels (Fig. 4F and G), suggesting possible secretion of material from the parenchyma cells into the vessels. These plugs were regularly infused with phenolics, as indicated by their blue color after staining with toluidine blue O (Fig. 4E to G). In addition in these occluding structures, we did not distinguish any layer similar to a wall, for instance over the aperture of bordered pits (Fig. 4F), while with tyloses, a dark wall was generally easily discernable in brightfield contiguous to the vessel wall (Fig. 4G), occasionally covering pit apertures (Fig. 5A and B). Tyloses often appeared collapsed, at times with broken walls, while others were completely empty (Fig. 5C).

In only a few cases, suberin was revealed in tylosis walls under UV light (Fig. 5D). As expected in confocal microscopy, the walls of the rare suberized tyloses were more easily discernible (Fig. 5E) than in conventional fluorescence microscopy. In TEM, the internal suberized wall layer of tyloses generally had a characteristic whitish appearance (Fig. 5F). At high magnification, the typical lamellar structure of suberin was at times distinguishable, especially when such a layer appeared to be in formation and examined concomitant with using OsO_4 - UO_2Ac - KMnO_4 to improve the contrast of the constituents of this polymer (Fig. 5G). Suberized layers then displayed lamellae presenting a contrast that varied from dark gray to white.

The colloidal-gold labeling with JIM5 allowed the detection of pectic material associated with the occluding structures in vessels.

Fig. 3. Seedlings examined after dormancy. **A and B**, A dying inoculated ODA1 with stunted and wilted leaves (**A**, arrow), with an inoculation wound still open and surrounded by a brown canker that appears sunken (**B**, arrow). **C and D**, The inoculation wound is closed. **C**, Front view of the callus in which the center appears as a furrow similar to that shown in Figure 2B. **D**, Side view of the callus that is clearly swollen. The bark appears brown next to the occluded wound (arrow). **E**, On the left, at the inoculation point, necrotic tissues have developed beyond the original compartmentalized xylem (white arrow) and necrophylactic periderm (NP; arrowhead). A new NP at the edge of the lesion is obvious in the bark (black arrow; see also **G**). On the right, the necrosis is conspicuous at 1.5 cm above the inoculation point, particularly in the bark. **F and G**, A sample similar to that on the left in **E**, but stained with phloroglucinol-HCl and examined under **F**, blue light and **G**, UV. **F**, Phenols surrounding the original infected xylem are still discernible (black arrow), but the yellow autofluorescence is less intense than before dormancy. The white arrow shows a new but weak yellow autofluorescence at the edge of the necrotic xylem. **G**, The suberin is evident over the original invaded xylem (yellow arrowhead), in the suberized tubes (white arrowheads) bordering the old NP (yellow arrow) as well as in a NP recently formed at the limit of the necrosis (white arrow) and continuous with the new band of phenols in the xylem (white arrow in **F**). **H**, A micrograph taken in confocal microscopy (a z-stack, 102 μm thick, 12 sections) with the laser 405 nm showing suberin autofluorescence. An apparent discontinuity is indicated (yellow arrow) in the NP. The suberized tubes (arrows) appear closely associated with the NP and one in particular in this image in three dimensions is clearly inclined (arrowhead). EP = exophylactic periderm. **I**, This cross-section of the stem of an inoculated SAD11 that did not express any foliage symptoms shows a complete closure of the inoculation wound (between the white arrows) and also reveals that the colonization occurred mainly in the bark beyond the old NP (white arrows). New NPs are present (white arrowheads) at the limit of the necrosis in the bark. The invaded xylem still appears well compartmentalized (black arrowhead). Black arrows show isolated necrotic spots in the xylem.

Fig. 4. **A**, Phenol autofluorescence surrounding the invaded xylem revealed with the laser 405 nm in confocal microscopy. The material is the same as that of Figure 1E and F. The fluorescence is visible within vessel occlusions (yellow arrowheads), in some ray parenchyma cells (gray/red arrowheads), and in cell walls (yellow arrow). Most ray cells of the reacting zone do not show any fluorescence (white arrowheads). The white arrow points to the junction of the necrophylactic periderm (NP, not shown) and the fluorescing zone. **B to D, F, and G**, Epoxy sections. **E**, Unicryl section. **B**, An inoculated SAD19 before dormancy. The NP (gray/red arrow) is thinner where it reaches the cambium (yellow arrow). In the lateral reaction zone, toluidine blue O has stained the content of a few xylem cells (black arrow) in front of the yellow arrow, in several axial and ray parenchyma cells internally in the invaded xylem where occluded vessels also contain such phenolics (arrowheads), and in most cortical cells (asterisk) contiguous to the NP. **C**, An inoculated JOL1. Several fungal cells (arrowheads) are shown in the invaded xylem. The gray/red arrowhead indicates some suberized cells covering the invaded xylem next to which a gap is evident. Broken vessel walls are also distinguishable (black arrows). **D**, An inoculated SAD19 before dormancy. A layer coating the vessel wall with a lamellar appearance (arrow). The internal protective layer of axial parenchyma cells is thicker (arrowheads) when facing the vessel, but this is obvious only in front of the pits in the cell at the top of the figure. **E**, An inoculated SAD11 after dormancy. Several gels stained with toluidine blue O occlude the vessels of the reaction zone at the tangential limit of the invaded xylem. The cytoplasm of most ray cells appears normal (arrows), where many starch grains are distinguishable. **F**, An inoculated SAD1 after dormancy. Four vessels occluded by gels that stain blue with toluidine O but also pink (yellow arrows) in some areas next to parenchyma cells. In those cells, internal wall layers are thicker and pink (black arrows) when facing occluded vessels and at time thicker just in front of the pits in a ray cell (asterisk). Bordered pits are not covered by a clear layer in such vessels (white arrow). **G**, An inoculated FRE105 after dormancy. Some gels (white asterisks) and a ray cell (gray/red arrow) are stained with toluidine blue O. Small arrows indicate fungal cells. The internal wall layer of several parenchyma cells is thicker and pinkish (large black arrows) when facing occluded vessels. A gel stained pink is seen (yellow arrow) next to a parenchyma cell with similar coloration of its internal layer. The black asterisk shows a tylosis with a black wall coating the vessel.

Fig. 5. Vessels occluded by tyloses. All inoculated seedlings examined after dormancy. **A to C**, Epoxy sections from a SAD11. **D**, Cryomicrotome section from a SAD1. **E**, Unicyrl sections from a SAD11. **F and G**, Epoxy section from a SAD1. **A**, The wall of a budding tylosis (white arrow) is continuous with the thick internal pink layer of the mother ray cell (yellow arrow). There is another tylosis in the vessel with a wall darkly stained, particularly in front of parenchyma cells, that covers bordered pit apertures in some places (arrowhead). **B**, A tylosis with dark-stained content and wall (arrow), the latter particularly thick next to an axial parenchyma cell with a noticeable pink internal layer (arrowhead) facing the vessel. **C**, Four tyloses appearing empty (asterisks) are shown in a vessel while wall remnants of another tylosis (black arrowheads) are seen in another vessel. Several parenchyma cells have internal wall layers that are thick and pinkish (yellow arrowheads) when facing occluded vessels. **D**, A section stained with phloroglucinol-HCl and examined under UV in conventional fluorescence microscopy reveals two tyloses with suberized walls (arrowheads). **E**, Confocal view of a section scanned with the laser 405 nm revealing suberin in the walls of two tyloses (white arrowheads) and in the internal wall layers of parenchyma cells in front of these vessels (yellow arrowheads). **F and G**, TEM examination. **F**, This vessel is completely blocked by a tylosis. The internal suberized tylosis wall layer is whitish (white arrow) whereas its primary wall appears darker (black arrow). P = parenchyma cell. **G**, After treatment with $\text{OsO}_4\text{-UO}_2\text{Ac-KMnO}_4$, this fully expanded tylosis shows light and dark lamellae in its nascent suberized wall layer (arrow). TW = tylosis wall. VSW = vessel secondary wall. * external wall layer of the tylosis similar to a middle lamella.

In gels, labeling in TEM was often positive in protective layers of parenchyma cells (Fig. 6A and B) and in adjacent vessels (Fig. 6B). Retraction of the plasmalemma seemed obvious in most parenchyma cells that were apparently involved in gel formation (Fig. 6A and B). In expanding tyloses that only had a primary wall (Fig. 6C), pectic material was revealed with JIM5 in vessels and gold particles were also present over the protective layer of adjacent parenchyma cells (Fig. 6D). Background labeling was scarce to absent, for instance over the secondary walls, while abundant gold particles always occurred over compound middle lamellae and pit membranes (Fig. 6A to D).

At higher magnification in conventional fluorescence microscopy, suberized tubes, similar to those already shown (Figs. 1A to D, 2A, and 3G), were at times clearly connected to the NP (Fig. 7A). The presence of fibers was noticeable within such canals, usually in or near the center (Fig. 7B). Cells that displayed a yellow autofluorescence within such suberized tubes under blue illumination (Fig. 1C) also took on a blue color in the presence of toluidine blue O (Fig. 7B). This blue color, indicative of phenols, was also obvious in suberized cells (Fig. 7B). In confocal microscopy using the laser 405, we obtained better resolution images of the autofluorescence emitted from suberin than that caught by the

Fig. 6. After dormancy, transmission electron microscopy (TEM) observations of gels (**A and B**, an inoculated SAD1) and tyloses (**C and D**, an inoculated SAD11) occluding vessels, and immunogold labeling for pectin with JIM5. Note the near absence of background labeling over secondary wall layers. **A**, The protective layer of this ray cell, the pit membranes (asterisks) and the middle lamella are covered with gold particles whereas the labeling is variable within the vessel. The arrow shows a lighter part of the labeled protective layer that may correspond to the retraction of the plasmalemma. **B**, The labeling is intense over the protective layer (arrow) of a parenchyma cell, the pit membrane (arrowhead), the middle lamella and within the vessel as well (asterisk). **C**, A vessel, next to a parenchyma cell, completely clogged by a tylosis. The labeling is positive over some material in the vessel (asterisk) external to the tylosis primary wall (white arrow) as well as over the junction between xylary cells (black arrows). **D**, A closer view of the labeling in the box of **C** over the protective layer (asterisk), the middle lamella (black arrow) and the material external (arrowhead) to the tylosis primary wall (white arrow). VSW = vessel secondary wall.

conventional fluorescence microscope (compare Fig. 7A with Fig. 7C and F). Within the canals, the cells that reacted with toluidine blue O (Fig. 7B) or autofluoresced when excited by the blue light (Fig. 1C), and the fibers as well, emitted some light when scanned

with the laser 405, and even a weak one when using the laser 488 (Fig. 7D). All cortical cells showed a uniform autofluorescence when the laser 635 was used while that of all cells of suberized tubes was almost nil (Fig. 7E). When the images obtained with these

Fig. 7. Suberized tubes (ST) connected to the necrophylactic periderm, or in its vicinity. Samples collected before (**B**) or after dormancy (**A** and **C** to **F**). **A**, A cryomicrotome section from a control JOL1. The autofluorescence of suberized cells in conventional microscopy appears slightly blurred. The arrow marks the connection of an ST with the NP. **B**, An epoxy section from an inoculated SAD19. The toluidine blue O has produced a light blue color within most suberized cells, but the most external ones show a dark blue content (gray/red arrow). Some parenchyma cells in these two ST have also reacted with this dye (white arrows) and groups of fibers are shown in the center of these ST. **C** to **F**, Confocal micrographs showing fine and clear details of the fluorescence of a cryomicrotome section from an inoculated MBGHB scanned with wavelengths **C**, 405, **D**, 488, and **E**, 635 nm. In **F**, the three previous micrographs are merged. ST suberized cells connected to the NP in **C** and **F** (white arrows). In the latter figures, fibers and parenchyma cells are noticeable in STs and are also slightly autofluorescent in **D**. Cortical cells emit a red autofluorescence under 635 nm while the emission of ST cells is nil (**E**), giving a nice contrast to all cells when the images are merged (**F**). Z-stack of nine continuous sections, each 1.38 µm thick. CO = cortex. NP = necrophylactic periderm. White arrowheads = fibers. Yellow arrows = parenchyma cells.

wavelengths were merged, it was then possible to see better details of all the cells, whether suberized or not, in part because of the nice background provided by the cortical cells (Fig. 7F).

Assays in confocal microscopy to detect the pathogen in these canals by using the lectin wheat germ agglutinin (WGA) and by merging images were not conclusive (data not shown), even though at times, in scanning electron microscopy we observed one or two structures quite similar to hyphae (results not shown), but then only in 2 tubes out of around 10 that were examined. Most of the time in TEM, as for other suberized cells (Fig. 4C), such suberized tubes appeared badly infiltrated by the resin and big holes were frequent within the ultrathin sections.

DISCUSSION

In the present investigation, we presented, for the first time, an in-depth microscopic description of butternut defense mechanisms, essentially related to compartmentalization of tissue damage, following inoculation with *O. clavignenti-juglandacearum*, the butternut canker pathogen. The concept of compartmentalization, in the broad sense, has been extensively cited as an efficient defense mechanism in trees reacting to mechanical injuries or attacks by various pathogens. Excellent reviews have been published on this topic, most of the time when the xylem is involved (Blanchette 1992; Pearce 1996; Shigo 1984; Smith 2006), but also when defensive tissues such as NPs occur in the bark in response to various injuries (Biggs 1992a, b; Pearce 1996). It is worth noting that compartmentalization boundaries also form in tree roots in response to wounding or disease (Tippett and Shigo 1981), and such defensive tissues have even been described in herbaceous plants resisting to fungal pathogens (Baayen et al. 1996).

Even though our study was not intended at first to follow the fine time-course histopathological changes that take place after inoculation, it was obvious that compartmentalization barriers, particularly those in lateral and internal positions often called RZs produced by living cells extant at the time of inoculation (Shigo 1984), seem to play an important role in limiting the colonization of *O. clavignenti-juglandacearum*. Before dormancy, such compartmentalization tissues were observed in two control and two inoculated seedlings, whether or not they had closed the inoculation wound. At that moment, all seedlings were apparently healthy, to the extent that we thought the inoculation method had failed to allow pathogen invasion of host tissues. After dormancy, these defensive tissues were still intact in all controls and in one of the JOL1 seedlings inoculated with *O. clavignenti-juglandacearum* (Fig. 2B to D), from which *O. clavignenti-juglandacearum* was successfully reisolated. As these reactions were also discernible in all the other inoculated seedlings (Fig. 3E to G), this clearly indicates that they always formed during the first weeks after inoculation when the seedlings were actively growing.

As previously mentioned, after dormancy, only 1 seedling out of 48 was symptomless and had internal compartmentalization tissues that were not breached by the pathogen. Another inoculated SAD1 seedling seemed resistant, but now it appears most likely that two tags were mixed up during inoculation since that seedling was tagged number 15, while the SAD1 number 14 control, immediately adjacent to number 15 in the greenhouse, showed obvious signs of necrosis similar to the inoculated seedlings. Unfortunately, we were not able to reisolate the pathogen from either seedling, which could have confirmed that number 14 had indeed been inoculated by mistake. In many PDA Petri dishes used for reisolating the pathogen, contamination with other fungi was common and clearly negatively impacted the growth of *O. clavignenti-juglandacearum*, thus making the morphological identification of the pathogen difficult at times. It has been suggested by others that *O. clavignenti-juglandacearum* may eventually be outcompeted by secondary fungi in old cankers, making isolations difficult (Brosi 2010). Consistent with such observations, and although

O. clavignenti-juglandacearum was regularly detected in cankers using a sensitive qPCR method recently developed at the LFC (Tanguay et al. 2018), comparisons of cycle threshold values in the latter study also showed that *O. clavignenti-juglandacearum* DNA was more easily detected in recently infected epicormic shoots than in cankers, suggesting that intact *O. clavignenti-juglandacearum* propagules were less numerous in cankers than in such shoots.

As reported for other canker-causing fungi (Enebak and Blanchette 1989; Weiland and Stanosz 2007), new NPs may form (Fig. 3E to G and I) when the original ones fail to contain the invasion. Several inoculated seedlings, such as the SAD11 seedling in this figure, had asymptomatic foliage, although the bark occasionally became necrotic around inoculation wounds (Fig. 3D). Although it is difficult to predict if this seedling (Fig. 3E) would have remained healthy throughout the second growing season after inoculation, it is most likely that the infection would have continued to progress after a second period of dormancy and that the seedling would have then succumbed after being entirely girdled. In forest pathology textbooks, it is written that perennial cankers such as those caused by *Nectria*-like pathogens colonize trees more easily during the dormant or near-dormant seasons (Boyce 1961), apparently by penetrating through periderm barriers (Manion 1991). Artificial inoculations with such pathogens have also shown numerous times that when the host is less active, its susceptibility seems to reach a peak. For instance, *Neonectria ditissima* induced longer lesions around various wounds in apple trees when inoculated either in fall or winter than at the onset of the growing season (Alves and Nunes 2017). Similarly with butternut in response to *O. clavignenti-juglandacearum*, it has been demonstrated that the grafting scions from putatively resistant butternuts were more vulnerable when the inoculations were carried out in fall than during the active growing season (Ostry and Moore 2008). Consistent with this vulnerability, we have reported in a recent survey that open and dominant butternuts, likely growing in favorable conditions, show less dieback symptoms associated with butternut canker than intermediate or suppressed individuals (Sambaraju et al. 2018). According to some of our observations (Figs. 2H and 3I), during dormancy, it is likely that the pathogen first breaches the defensive tissues of the bark before further colonizing the host instead of attacking xylem tissues that compartmentalize the discolored wood. In the present study, discontinuities in NPs observed in confocal microscopy suggest that the pathogen could cross these defensive tissues through such interruptions in suberized layers, as also suggested for other canker pathogens (Biggs 1986; Weiland and Stanosz 2007). Another possibility is that *O. clavignenti-juglandacearum* can circumvent the NP by colonizing its ventral region, where suberized cells are the last differentiated and thus constitute a layer that is usually thinner than the rest of the NP (Figs. 1E and 2A), a point of weakness that appears to be also exploited by several canker pathogens (Biggs 1986, 1992b; Enebak and Blanchette 1989; Weiland and Stanosz 2007).

As the compartmentalization processes were quite similar in both control and inoculated seedlings, though they were more obvious and extended farther away from the inoculation point in the latter, these reactions are considered nonspecific and their induction may primarily occur to prevent the drying and aeration of the host tissues adjacent to cells that were affected by exposition to the atmosphere after wounding (Pearce 1996). The reduction in water content and concomitant higher concentrations of O₂ in xylem tissues caused by wounding would generally be a prerequisite for infection by various pathogens (see review by Boddy 1992). Incidentally, it is interesting to note that water levels, revealed by nuclear magnetic resonance imaging, in xylem RZs of *Acer pseudoplatanus* limiting the invasion of *Ustulina deusta* were 2.5 to 3 times higher than in adjacent healthy sapwood, whereas in a compatible interaction with *Chondrostereum purpureum*, no accumulation of water occurred in these zones (Pearce et al. 1994). This increase in water

content would represent a passive defense mechanism that could act together with the active accumulation of phenols in RZs, which was also revealed in *A. pseudoplatanus* to hinder *U. deusta* invasion (Pearce et al. 1994).

Accumulation of phenols has often been reported at lesion margins in living xylem of several tree species that apparently restricted pathogen colonization (Pearce et al. 1994; Shain 1971; Wong and Preece 1978; Yamada 1992) and it has been briefly reported by Nair (1999) to explain the resistance of hybrids and black walnut after artificial inoculations with *O. clavignenti-juglandacearum*. However, to our knowledge, the presence of phenols, notably at the limit of necrotic xylem that reach the cambium and always in continuity with NPs (Figs. 1E to G and 4A and B), has never been demonstrated so conclusively before. It is also interesting to underline that such deposition of phenols was revealed under blue light in the most resistant scion provided and tested by the OMNRF. In confocal microscopy, the phenols were shown to accumulate in the cytoplasm of parenchyma cells, in cell walls and vessel occlusions, seemingly reinforcing the cells involved in compartmentalization. The staining obtained with toluidine blue O in the present study also supports the phenolic nature of these compounds. We have reported many times the presence of such phenols, using either the detection of a yellow autofluorescence under blue light illumination and/or using this stain or additional ones, in other hardwood species, for instance in *Ulmus americana* (Et-Touil et al. 2005) and *Populus balsamifera* (Rioux and Ouellette 1991) inoculated with the Dutch elm disease pathogen. In the present study, a large part of the phenols that display a yellow autofluorescence under blue light appears to mostly consist of catechins to condensed tannins (see similar sections under blue light [Fig. 2D] and the red staining with the vanillin-HCL test [Fig. 2F]). As suggested in other studies (Geiger et al. 1989; Shain 1971), some of these phenols, as well as those of a flavonoid nature detected with Neu's reagent, may have been modified by different enzymes when exposed to oxygen before wound closure, and some of them may then give the natural dark brown color associated with wood discoloration (Fig. 1A). Woundwood cells that formed afterward also seemed to be composed of phenolics different from those occurring elsewhere in the normal xylem (Fig. 2F and G). In addition, the intense staining obtained with phloroglucinol-HCl in woundwood (Fig. 1B) suggests that lignin more intensely impregnates these cell walls or that its chemical polyphenolic nature is different from that of the wood extant at the time of inoculation. The unusual orientation, composition and size of xylem elements in woundwood have also been frequently reported in other studies (Eyles et al. 2003; Rioux and Ouellette 1991; Smith 1980; Smith and Sutherland 1999). As shown in Figure 1B and elsewhere (Armstrong et al. 1981; Smith and Sutherland 1999), radial growth associated with wound closure is usually greater in woundwood than in unaltered wood.

Axial xylem parenchyma cells in butternut are described as apotracheal-diffuse to uniseriate banded, as well as paratracheal to vasicentric at times (Panshin and de Zeeuw 1980), this being visible in some of our sections (Figs. 4 and 5). These abundant parenchyma cells allow the accumulation of phenols that appear continuous internally and tangentially around the affected xylem and seems to efficiently restrict pathogen invasion. The different functions of xylem parenchyma cells in tree defense have been reviewed elsewhere (Morris et al. 2016).

In their study of 65 mature woody species, Bonsen and Kučera (1990) reported that only one type of vessel occlusion, either tyloses or gels (=gums), occurs in dysfunctional xylem at the species level, whereas at the genus level, both types can be recorded in vessels, but then in a very sporadic fashion. While tylosis formation was reported in different mature *Juglans* trees in aging xylem or in response to injury (Armstrong et al. 1981; Bonsen and Kučera 1990; McElrone et al. 2010; Panshin and de Zeeuw 1980), Nair (1999) was able to show their presence, together with a few gels, in

butternut and black walnut twigs inoculated with *O. clavignenti-juglandacearum*. Tyloses were detected in the present study, but they were more difficult to observe in seedlings than anticipated, probably because gels also plugged the vessels and that most examined samples were collected at 290 dpi. Tyloses were present in the core of affected xylem, but they were more abundant in tissues surrounding the injured area, where they had generally accumulated phenols. Only some of the tyloses were suberized in the present work and, in general, suberin deposition did not appear to be prominent in the xylem. The ultrastructure of tylosis walls in butternut was quite similar to that described for other tree species, particularly *Populus balsamifera* (Rioux et al. 1995), and the lamellae of suberized layers were made evident using OsO₄-UO₂Ac-KMnO₄ (Wattendorff 1974), as we have also reported for other defense mechanisms, notably in elm callus reacting to inoculation with *Ophiostoma novo-ulmi* (Aoun et al. 2009). The suberized layers observed over the affected xylem at the inoculation point always originated from NPs that unfolded over the injured xylem, so they should not be equated to wall 4 of the CODIT model, which is laid down by the vascular cambium (Shigo 1984). In studies of wounded *Juglans* species, such suberized layers seem to have gone unnoticed (e.g., Figure 1D in Armstrong and McGinnes 1979).

Immunogold labeling revealed that some pectic material was secreted in butternut vessels by expanding tyloses. In another study (Rioux et al. 1998), it was shown that such pectin production helped tyloses to completely block vessel elements, in areas such as where the vessel secondary wall presents acute angles difficult to reach by the tylosis primary wall. This pectin obviously might also help glue the tylosis wall to whatever structures encountered during its development, even to other tyloses, and this is reminiscent of the role played by middle lamellae (see the asterisk in Fig. 5G), as discussed elsewhere (Bonsen and Kučera 1990; De Micco et al. 2016). In Rioux et al. (1995, 1998), tylosis primary wall was described as an extension of the so-called protective layer of parenchyma cells. Besides, when gels are formed instead of tyloses, for instance in *Prunus pensylvanica* (Rioux et al. 1998), JIM5 labeling also showed that pectin transits from this layer before being secreted into vessels via the pits. We have seen a few cases that clearly suggest that gels occlude butternut vessel elements, and although we cannot exclude the possibility that such material might have been produced by tyloses present above or below such material in cross-sections, our immunolabeling demonstrates that pectin can be directly secreted by parenchyma cells to block adjacent vessels. As mentioned in Bonsen and Kučera (1990), the protective layer is generally thicker in the part along the vessel side. Actually, in the present study, this layer was regularly thicker when formed adjacent to apparently dysfunctional occluded vessels (Figs. 4D, F, and G, 5A to C, and 6A and B) than when formed adjacent to unaffected ones, and when gels were noted in brightfield, the staining of this layer was often similar to that found in nearby vessels (Fig. 4F and G).

The main findings reported by Bonsen and Kučera (1990) are that a maximal vessel diameter (MVD) of less than 80 µm and the smallest diameter of apertures of vessel-parenchyma pit pairs, called the minimum pit aperture diameters (MPD), of less than 3 µm nearly always favor the production of gels (=gums), whereas over these values, the tree reacts by forming tyloses. According to these authors, large vessels would be hard to plug with gums while a pit aperture smaller than 3 µm would not easily permit the passage of the double tylosis primary walls. In our seedlings, vessel diameters were rarely over 50 µm and, obviously, tyloses still occurred in some of them, so this rule does not seem to hold with such young xylem tissues. In mature wood, butternut vessels can reach 300 µm and those clearly blocked with tyloses in Panshin and de Zeeuw (1980) varied from 240 to 300 µm in diameter. However, our preliminary measures of pit membrane apertures support the findings by Bonsen and Kucera. With cross-sections, even though we can only underestimate such apertures, the latter being ideally observed in radial planes with sections at least 30 µm thick, pits that were

involved in pectin secretion (Fig. 6A and B) had a diameter of around 2.5 μm or less while those observed in light microscopy during tylosis formation (Fig. 5A) had a diameter of at least 4 μm . We measured at random, and with some difficulty with thin sections, 10 pit pairs in light microscopy using radial sections, and the average MPD was 3.18 μm (SD = 0.48). Obviously, as it is well known that nearly all wood properties greatly vary between juvenile and mature wood (Panshin and de Zeeuw 1980), it is most likely that the MPD of such pit pairs would have been much larger if the material studied had come from the last growth rings of mature butternut trunks. It is also worth mentioning that in *Juglans*, *Salix* and *Corylus* genera, trees appear to have evolved from narrow pit apertures allowing gel production to large vessels with MPD over 3 μm that were then occluded by tyloses (Bonsen and Kučera 1990). This suggests that the occlusion of vessels by gels remains a possibility when MPD and MVD are less than 3 and 80 μm , respectively, as in some parenchyma-vessel combinations in butternut seedlings. Gums were briefly described in butternut vessels by Nair (1999), but then their presence seemed linked to susceptibility, since they were not observed in the more resistant individuals (e.g., hybrids and black walnut) and their origin was associated with by-products of wall degradation by the pathogen.

In the damaged bark analyzed in our study, NPs were observed in all the seedlings examined. The formation of such periderms has been reported as a key response to delay or stop the advance of various canker pathogens (Biggs 1992b). Although the accumulation of phenols in the healthy tissues was also noticed contiguous to the NP (Fig. 4B), those cells appeared to be readily invaded when the NP failed to stop pathogen advance. High concentrations of constitutive juglone in the bark have been reported as a potential characteristic for selecting resistant butternuts (Moore et al. 2015). Although we did not investigate if juglone was produced in greater amounts in the bark following wounding or inoculation, preliminary assays were carried out to assess whether this phenol might be related to the yellow autofluorescence revealed in the xylem following wounding or inoculation. Two wood samples from unwounded seedlings and two from inoculated seedlings showing this fluorescence were analyzed in ultra-performance liquid chromatography–tandem mass spectrometer, as employed by Moore et al. (2015), at Université Laval by the chemist Pascal Dubé (Institut sur la nutrition et les aliments fonctionnels, *unpublished data*), and no link was established between the concentration of juglone and such fluorescence. Actually, juglone was present in much higher amounts in a control, i.e., 436.4 μg per gram of xylem, than in the other samples where the concentration varied from 9.4 to 14.5 $\mu\text{g/g}$. However, this might be the first time that juglone is detected in the wood of a *Juglans* species.

The presence of suberized tubes in the bark around inoculation wounds was unexpected. As they were more numerous after inoculation than after wounding alone, at first we thought that such longitudinal periderm canals could represent a route exploited by the pathogen to invade deeper into the bark. Our tests with the lectin WGA, having an affinity for chitin, to detect the presence of *O. clavignenti-juglandacearum* in these tubes have not supported this hypothesis so far (*data not shown*). It is to be noted that when this lectin was conjugated to a fluorescent dye such as Alexa, it was clear that the fiber walls in such tubes were fluorescent, making the identification of fungal cells, if any, quite difficult. The presence of *N*-acetyl-glucosamine residues, monomers of chitin, has been reported at times in the secondary walls of many plant species (Benhamou and Asselin 1989). As it is apparently the case with most compartmentalization processes or other defense reactions in sapwood, the main factor triggering the formation of such suberized canals appears to be the presence of air, mainly transported through the fibers present within the tubes, as reported following wounding of the stem of different woody species (Lev-Yadum and Aloni 1991). These suberized tubes were even described in intact stems of

Vitis vinifera where such canals appear at some point to extend among peridermal cells exposed to the atmosphere that might have induced their formation (Aloni and Peterson 1991). The formation of NPs, reminiscent of these suberized tubes, around phloem fibers has also been reported in other histopathological studies (Weiland and Stanosz 2007; Wisniewski et al. 1984).

One of the main goals of our project, which started in 2010, was to identify potentially resistant butternuts that could be used in programs aimed at conserving and restoring viable butternut populations. To screen their resistance, seedlings were artificially wounded and inoculated with PDA plugs colonized by *O. clavignenti-juglandacearum*. We used such large plugs, relative to the diameter of the stem, because they represent massive amounts of inoculum, so if some expression of resistance were to occur following inoculation, the tested seedlings should be considered resistant with a good level of confidence. Others have even reported that such plugs appear to be too much loaded with inoculum, to the point that it is impossible to discriminate between susceptible and resistant butternuts (Crystal and Jacobs 2012). Similar plugs, although smaller in size, were successfully used to identify experimental hosts of *O. clavignenti-juglandacearum* (Ostry and Moore 2007). The latter authors also used material from canker-free butternuts growing in nature and those individuals were intensely colonized by *O. clavignenti-juglandacearum* following artificial inoculation. Our results clearly indicate that it is possible to disclose the presence of defense mechanisms in butternut using a similar method of inoculation. Compartmentalization barriers appear important for limiting *O. clavignenti-juglandacearum* colonization and when the seedlings are killed, these barriers had been regularly breached. The restriction of pathogen invasion by compartmentalization processes occurring in the xylem (Beier et al. 2017) or in the bark (Weiland and Stanosz 2007) in trees has even been suggested as a key parameter for screening individuals potentially resistant to some diseases. Finally, when the material tested appears to be resistant to butternut canker after at least one period of dormancy, we strongly suggest to vegetatively propagate this material and thereafter cut open the individual to assess whether the compartmentalization processes were efficient in limiting pathogen development.

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