Phytopathologia Mediterranea (2013) 52, 2, 313–323

RESEARCH PAPER

Histopathological study of response of *Vitis vinifera* cv. Cabernet Sauvignon to bark and wood injury with and without inoculation by *Phaeomoniella chlamydospora*

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Summary. *Phaeomoniella chlamydospora* (*Pch*) is one of the main causal agents of tracheomycosis in grapevine. We characterize how this fungus affects the response of *Vitis vinifera* cv. Cabernet Sauvignon to bark and xylem-tissue wounding after six weeks post-treatment. A histological investigation shows that, in xylem tissue, cell-wall modifications in response to wounding are related to suberin deposits rather than to lignin-induced wall thickening. The xylem response does not appear to be disturbed by *Pch* infection. Therefore, cell-wall modification strongly inhibits the development of wound-closure tissue (WCT) but does not prevent the differentiation of the necrophylactic periderm. Hyphae localization in tissue surrounding the wound or inoculation sites indicates that *Pch* colonizes all cell types, such as vascular tissues, paratracheal parenchyma cells, fibers and rays. The results also suggest that efficient compartmentalization separating fascicular xylem portions is assured by thick suberized cell walls bordering the ray parenchyma.

Key words: Grapevine vine-trunk disease, *Phaeomoniella chlamydospora*, CODIT, compartmentalization, bark healing.

Introduction

Grapevine trunk diseases constitute real threats to the worldwide grape and wine industry. Of the numerous grapevine wood pathogens identified to date, *Phaeomoniella chlamydospora (Pch)* is one of the most commonly isolated and also one of the most virulent (Mugnai *et al*., 1999; Eskalen and Gubler, 2001; Marchi *et al*., 2001; Laveau *et al*., 2009; Luque *et al*., 2009; Kuntzmann *et al*., 2010). It is ascomycetous fungus responsible for tracheomycosis in grapevine (Surico, 2009). Infection can lead to different symptoms depending on the level of infection and the age of the vine plant (Surico, 2009). In young vines, *Pch* infection can lead to young-vine decline also called Petri disease. Decline caused by *Pch* in young vines is associated with dark exudates (named black goo) running out of xylem vessels. These black spots appear as wood streaking in longitudinal sections. In young and old vines alike, *Pch* is isolated from vine wood with tiger-striped leaf symptoms associated with the esca syndrome (Mugnai *et al*., 1999; Marchi *et al*., 2001). Vines with esca disease can exhibit a wide variety of symptoms in both trunks and arms, such as hard central brown discoloration, hard V-shaped discoloration, black spot (mostly organized in a concentric manner), or white soft rot (Mugnai *et al*., 1999; Luque *et al*., 2009; Kuntzmann *et al*., 2010). These various symptoms can be explained by the combination of fungal pathogens usually found in the wood of mature vines. These pathogens include the ascomycota *Eutypa lata, Phaeoacremonium* spp.,

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ISSN (print): 0031-9465
ISSN (online): 1593-2095

Botryosphaeriaceae spp., and other basidiomycetous fungi (e.g., *Fomitiporia mediterranea*) (Mugnai *et al*., 1999; Luque *et al*., 2009; Kuntzmann *et al*., 2010). From among this variety of symptoms, *Pch* is isolated mainly from black spots and central brown discolorations (Mugnai *et al*., 1999; Luque *et al*., 2009; Kuntzmann *et al*., 2010). In addition, studies based on the characterization of wood microbiota as well as pathogenicity tests indicate that *Pch* may produce sectorial necrosis and canker (Laveau *et al*., 2009; Kuntzmann *et al*., 2010).

Pathogens infecting plants must overcome the defense mechanisms developed by their host. These defense mechanisms can be pre-existing (e.g., physical or chemical barriers such as the cuticle or constitutive antifungal compounds, respectively) or induced. In woody tissues, compartmentalization of pathogens involves anatomical modifications at the junction of healthy and infected tissues, which constitutes a delayed defense mechanism. In pre-existing woody tissues, an area called the reaction zone (RZ) (where the active response of the host occurs) appears after the injury (Pearce, 1996). Another boundary, called the barrier zone (BZ), forms at the time of injury in the plane of the vascular cambium. According to Shigo and the model of compartmentalization of decay in trees (CODIT), four different boundaries can be distinguished in injured tree wood (Shigo and Marx, 1977). The least-effective barrier is the occlusion of xylem vessels by tyloses and gummosis. The next-more effective barrier consists of the last layers of cells produced by the vascular cambium in the annual rings. A still more effective barrier is the rays, but this does not provide a continuous barrier because rays only extend over a limited longitudinal portion of perennial tissues. In the CODIT model, these first three barriers are considered part of the RZ. The fourth barrier constitutes the BZ and seems to be the most impervious and durable barrier with regards to limiting the spread of pathogens. The BZ effectively protects the youngest wood tissue and the vascular cambium (Pearce, 1996). In the RZ and BZ, cell walls may thicken because of lignin or suberin and phenolic compounds may accumulate in cell lumen (Pearce, 1996). It is also known that the ways in which wood responds to injury are likely to vary between woody-plant species (Pearce, 1996; Deflorio *et al*., 2009). The RZ and BZ do not form in a specific manner because they can be produced in response to both infection and wound (Pearce, 1996).

Another fundamental aspect of wound response is the compartmentalization process that occurs in the bark and the development of WCT. After wounding, a ligno-suberized layer of cells usually develops at the periphery on the phloem tissue (Biggs, 1984; Biggs *et al*., 1984). This ligno-suberized layer seems necessary for the regeneration of a new periderm, also called the necrophylactic periderm (NPd). The initiation of WCT begins by the proliferation of a mass of undifferentiated cells (callus) that originates in the vascular cambium. When the callus grows and covers the wound, a new vascular cambium and a new periderm regenerate near the vascular-cambium surface, and the callus gradually becomes differentiated (Armstrong *et al*., 1981). The healing process is considered complete when the continuity of the vascular cambium is restored.

The anatomical features of wood and tissue where suberized cell walls form in response to injury vary from one tree species to another (Biggs, 1987). Even if grapevine is a woody plant, it cannot be considered a tree. Vines are woody plants adapted to climbing existing natural supports to reach the canopy. Therefore, they allocate fewer resources than they otherwise would to building a self-supporting structure and so have a much smaller radial-growth rate than ordinary trees (Carlquist, 1985). The anatomy of cane evolved to efficiently transport water through a plant with a higher leaf/xylem surface ratio than trees (Carlquist, 1985). Today, the compartmentalization process in the woody tissue of vines remains poorly understood.

Characterization of the biochemical activity of enzyme secreted by *Pch* indicates that this fungus poorly metabolizes lignified cell walls (Del Rio *et al*., 2004; Valtaud *et al*., 2009). However, toxins secreted by *Pch*, especially the polypeptide fraction, strongly affect physiological cell processes, which leads to reduced plant responses, cell death, and tissue necrosis (Abou-Mansour *et al*., 2004; Luini *et al*., 2010). In the field of grapevine trunk diseases, compartmentalization of pathogens and pathogen routes in the woody tissue is poorly understood. A genetically modified strain of *Pch* that constitutively expresses DsRed fluorescent protein seems mainly restricted to the lumen of the xylem vessels and, to a lesser extent, to intracellular spaces in cells around the vessels (Mutawila *et al*., 2011). In the same study, other fungi expressing this kind of fluorescent reporter were not detected in the woody tissue, although they could

be isolated by microbiological methods. It thus appears that the fluorescent-reporter approach can lead to a less-demonstrative visualization of pathogens because of the loss of the fluorescence signal in fungal hyphae or because of a high autofluorescence background in woody tissue. The location of fungal hyphae (obtained by transmission electron microscopy) indicates that *Pch* may colonize the intracellular space of the xylem fibers (Valtaud *et al*., 2009).

The objective of the current study is to characterize, at the histological level, the effects of *Pch* inoculation on the response to injury of bark and xylem in grapevine. To achieve this goal, we first characterized the response to injury and then characterized the response in inoculated plants. In addition, after quenching the autofluorescence of the woody tissue, we located the fungal hyphae at the tissue level through an epifluorescent strategy involving Calcofluor White stain.

Materials and methods

Plant material

Fifteen canes of the *Vitis vinifera* cv. Cabernet Sauvignon clone were harvested and cut into 10-cm-average lengths to obtain cuttings with a single internode and with homogenous size and diameter. Cuttings were incubated in a PDA medium at 26°C for 16 h days and 8 h nights in plastic boxes containing humid sterile glass wool until buds broke and the first roots appeared. Plants were potted in a sterile substrate consisting of universal peat, river sand, and perlite (1:1:1). Throughout the entire experiment, plants were grown in a Mylar box chamber for 16 h days and 8 h nights.

Fungal material and preparation of conidia solution

Phaeomoniella chlamydospora isolate CBS 239.74 was grown in a PDA medium at 26°C in the dark. A margin of two-week-old culture (a cube averaging $5 \times 5 \times 5$ mm^3) was harvested with a sterile scalpel and introduced into 1 mL of sterile 10 mM phosphate buffer (pH = 7). The solution was gently mixed, following which a plug of solid medium was removed from the solution. The solution was centrifuged at 1000 rpm and the excess water was removed. Conidia were washed with a sterile 10 mM phosphate buffer, centrifuged, and gently suspended by pipetting into a 500 µL phosphate buffer solution. Conidia were counted with a Malassez counting cell and the solution molarity was adjusted to obtain 1000 conidia in a 50 µL drop.

Plant treatment

A total of 18 plants were used in this study. Three replications were performed, with each replicate consisting of three control and three inoculated plants. One-month-old bark cuttings were surface cleaned with a 70% ethanol solution and the young trunk was wounded with a 3-mm-diameter cooled sterile drill 40 mm under the center off the upper node. The drill wound was perpendicular to the longitudinal axis of the bud. Plants were lain down and inoculated with 50 µL of fresh conidia solution (1000 conidia) or 50 µL of 10 mM phosphate buffer (control). When the drop of solution was totally absorbed by the plant tissue, the wound was covered with parafilm. Plants were grown as described above and were analyzed six weeks after treatment.

Histological methods

A stem segment averaging 30 mm in length was removed using a razor blade. At the center of each stem was a wound or inoculation site. The stems were dehydrated by exposure at 4°C to ethanol solutions (25, 50, 80%; three times 30 min). Samples were conserved in the 80% ethanol solution at 4°C for a maximum of 2 weeks. For histological preparation, samples were rehydrated by exposure to ethanol-water solutions (50, 25%, water) and fixed on aluminum plates with cyanocrylate glue. Samples were cut into 30- and 200-µm-thick sections with a vibratome Leica VT 100S equipped with a sapphire knife. We characterized the plant's reaction by optical microscopy and epifluorescence of the 30 *µm* sections.

Samples were stained with safranine O-Astra Blue (SfrO-AB) to differentiate cellulosic lignified cell walls (stained in red) from nonlignified cell walls (stained in blue) (Srebotnik and Messner, 1994). The lignin and suberin zones in the woody tissue were investigated by using phloroglucinol-HCl (Phl-HCl) (Biggs, 1987). Phl-HCl reacts specifically with lignin and, according to Biggs, also quenches lignin self-fluorescence, which makes it possible to detect suberin by illuminating the sample with ultraviolet

(UV) light. The 200-µm-thick sections were used for fungal localization. These sections were bleached in sodium hypochlorite (2.5% active chlorine) until tissue coloration was weak or extinct (approximately 5 min), and then rinsed three times in deionized water for 5 min each time. To stain fungal hyphae, sections were covered for 2 min with a drop of Calcofluor White M2R, then briefly rinsed in water and mounted with water between slide and cover slip.

Epifluorescence analysis of the sections was done with a Leica DM IRBE microscope equipped with a 100 W HBO mercury-vapor lamp. To both analyze the plant response and localize the fungus in plant tissue, we used a di amido phenyl indol (DAPI) filter (excitation at 340–380 nm, emission is at 425 nm). A Leica DM6000 equipped with a confocal head (AOBS TCS SP2) was used for confocal imaging. To distinguish fungal hyphae, micrographs were acquired for two emission and two excitation wavelengths (excitation at 405 nm, emission at 420–480 nm; shown in blue) from cell-wall signals (excitation at 488 nm, emission at 520–600 nm; shown in red).

Results

Characterization of response to injury in wood of vine cuttings

Six weeks after treatment, longitudinal sections revealed a thin layer on the xylem tissue surrounding the wound (Figures 1A and 1B). Some control plants developed short brown discolorations in the xylem tissue up to the wound (Figure 1B). All nine control plants developed WCT that entirely covered the wound (data not shown).

In the cross sections, the histological structure of WCT is distinguishable from other tissue (Figure 1C). A thick layer of phellem developed at the abaxial surface of the WCT formed from each margin of the wound (Figure 1C). More specifically, the newly formed bark was composed of a periderm layer and a differentiated phloem (Figure 1C). The Vascular cambium separated the phloem from the newly formed xylem (Figure 1C), which was mainly composed of fibers and ray parenchyma and had only a limited number of vessels of small diameter. From the base to the end of the WCT, both bark and xylem tissues were progressively less organized. At the adaxial face of the WCT, the periderm extended from the base of the wood wound to form a continuous structure with the periderm of the abaxial face (Figure 1C). Under the periderm, only a mass of connective tissue composed of undifferentiated cells was observed. These cells reacted to the Phl-HCl test in purple, indicating that they harbored a thin wall that can be weakly lignified with Guaiacyl monomers (Figure 1D).

In the injured xylem, a RZ appears in the tissue around the wound. This RZ is characterized by a more intense coloration of the cell wall by safranine O (Figure 1C) and is restricted at the NPd-xylem junction. The RZ was not distinguishable using the Phl-HCl reaction, which indicates that the lignin content may not have changed or may have undergone only limited change (Figure 1D). After Phl-HCl treatment, UV illumination of the RZ resulted in emission of residual autofluorescence, which indicates that the staining variations resulting from SfrO-AB treatment may have resulted from suberin deposits (Figure 1E). Suberin deposits occur in fibers, large pluriseriate rays, mature tyloses, and paratracheal parenchyma (Figure 1E). No deposits were observed in the necrotic brown areas in the rays. The strongest remaining autofluorescence signal comes from periderms and from cells bordering the rays and fibers at the periphery of the RZ. Although cells displaying this signal were usually organized in one- to two-seriate continuous layers at ray borders, some gaps consisting of a group of two to four cells also appeared along the ray boundary (Figure 1I). In the transversal plane crossing the middle of the wound, tyloses led to vessel occlusions. The tyloses formed mostly in vessels surrounding the zone where reacting fibers and rays were detected, but not within this zone itself (Figure 1E). Some vessel occlusions in the RZ also appeared up to the wound, but with no obvious organized distribution.

Coming out of the NPd-xylem junction, two boundaries appear that are parallel to the plane formed by the vascular cambium (Figure 1FI). The cells of these two layers displayed walls stained deep red with SfrO-AB (Figure 1FG). The wounds made for the experiment led to the first layer (which was at the end of the growth ring of the previous year) and the second layer, both of which were then matched with the BZ (Figure 1G). The growth-ring boundary is formed by a band of one to four flattened thick-walled cells. Their walls react strongly to Phl-HCl staining, producing a deep-purple coloration that contrasts with the woody surrounding

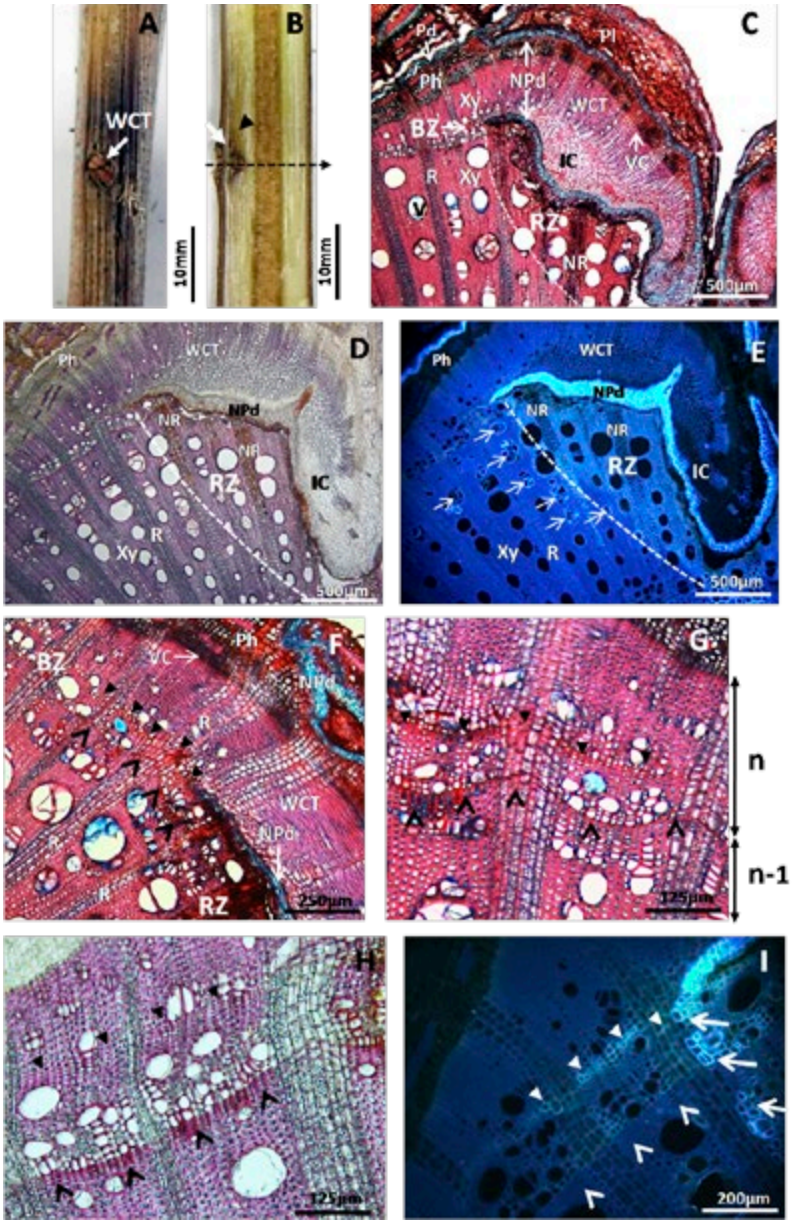

Figure 1. *Vitis vinifera* cv. Cabernet Sauvignon response to injury six weeks after wounding. (A, B) Photographs of phenotype observed in control plant from (A) outer view and (B) in longitudinal section. (A) WCT indicates wound closure tissue. (B) White arrow indicates WCT, black arrow head indicates the brown wood occurring up to the wound in control plants, and dotted arrow indicates the section shown in panels (C–I). (C–E) Large-field micrographs (X5) of (C) injured tissue stained with SfrO-AB, (D) Phl-HCl observed with bright field, and (E) under UV illumination. Dotted lines indicate boundaries of the RZ. (E) Arrows indicate xylem vessels occluded with tyloses. (F–I) Closer view of (F, G) junction between necrophylactic periderm (NPd) and xylem tissue under SfrO-AB staining, (H) Phl-HCl observed with bright field, and (I) under UV illumination. V-shaped arrow heads indicate barrier at the boundary between tissue formed a year prior to this experiment [n-1 in panel (G)] and tissue formed during experiment [n in panel (G)]. Arrow head indicates the barrier formed in response to injury. (I) Arrows indicate the layer of cell exhibiting a strong primary autofluorescence at the border of rays encircling the RZ. The notation IC stands for undifferentiated cells, NR is for necrotic ray, NPd is for necrophylactic periderm, Pd is for periderm, Pl is for phellem, Ph is for phloem, BZ is for barrier zone, RZ is for reaction zone, V is for vessels, Vc is for vascular cambium, WCT is for wound closure tissue, and Xy is for xylem.

tissue (Figure 1H). Under UV illumination, no residual autofluorescence originated from the cell walls in this layer, which indicates an absence of suberin deposits (Figure 1I). Weak residual autofluorescence also came from a thin layer of one to three thin-walled cells composed of primary cells that formed on the annual rings. In the layer consecutive to the drill wound, flat cells were also observed immediately centripetal of the vascular-cambium position at the time of wound (Figure 1H). These cells either do not react to Phl-HCl or do so only in a weak and discontinuous manner. Ultraviolet illumination reveals that the walls of these cells constitute a continuous suberized barrier in fibers and rays (Figure 1I).

Six weeks after wounding, several anatomical changes occurred in response to injury, including the development of WCT, the deposition of suberin in fibers and ray cells in the RZ, and the occlusion by tyloses of vessels surrounding the RZ.

Effect of *Pch* infection on responses of bark and wood injury

Plants inoculated with *Pch* developed black streaking (about 5 mm in length) and brown discoloration in the xylem up and down the wound (Figure 2A). Macroscopically, no WCT was noticed for all the nine plants inoculated with *Pch* (Figure 2A).Nevertheless, NPd was seen at the wound margin (Figures 2B–2D). This NPd separates the living phloem from the dead necrotic phloem (Figure 2C). Under this periderm, WCT consisted of a smaller number of undifferentiated cells. More precisely, the new xylem and phloem tissue formed after the wounding indicates that the vascular cambium remained active at the margin of the wound. Howeversignificantly less xylem tissues are formed (n = 9, $P = 0.002$) than in the control plants (Figures 1C and 2B; data not shown). In woody tissue, the RZ appeared the same as that in control plants, both in terms of constitution and

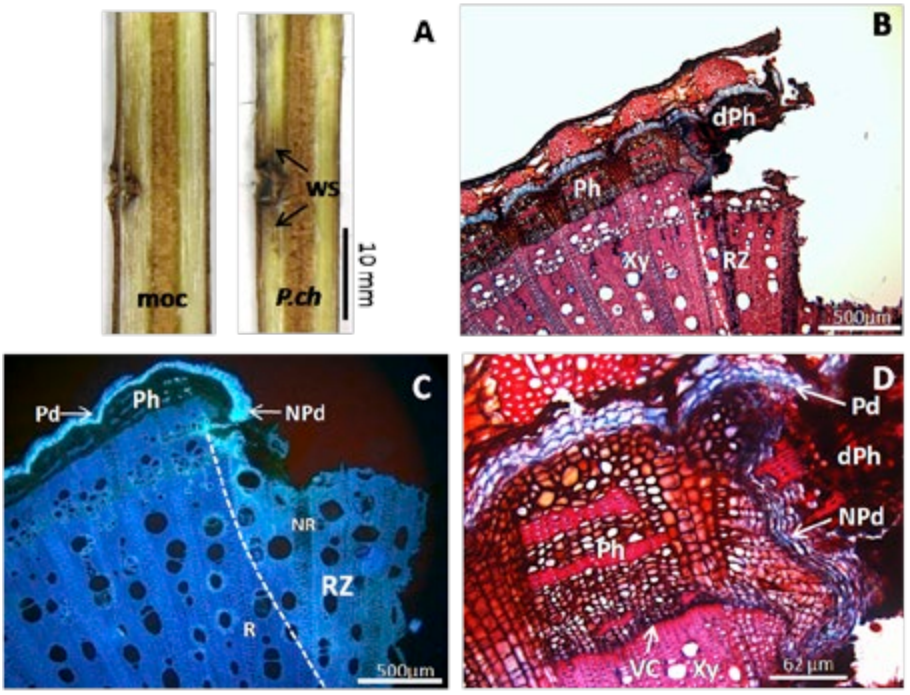

Figure 2. Effect of *Pch* on *Vitis vinifera* cv. Cabernet Sauvignon response to wounding. (A) Photographs of macroscopic phenotype observed in moc (control) and *Pch*-inoculated plants. WS indicates wood streaking in xylem on each side of the wound. (B, C) Large-field micrographs of (B) injured tissue stained with SfrO-AB and (C) Phl-HCl observed under UV illumination. (D) Closer view of bark structure at the border of the wound. The notation dPh stands for dead phloem, NR is for necrotic ray, NPd is for necrophylactic periderm, Pd is for periderm, Ph is for phloem, RZ is for reaction zone, V is for vessels, and Xy is for xylem.

of extension (Figures 1C, 1E, 2B, 2D). In addition, at the RZ periphery, the occlusion of vessels was not affected (Figures 3A–3D).

Thus, *Pch* infection affects the response to injury mainly by inhibiting wood-wound development. Any change in the response of the pre-existing woody tissue is either limited or cannot be distinguished using our approach.

Localization of fungal hyphae in the woody tissue

To localize fungal hyphae in woody samples, the autofluorescence background of woody tissue was quenched and samples were stained with Calcofluor White M2R afterwards (Figures 3 and 4). This approach allows *Pch* propagules in grapevine wood to be clearly detected under UV illumination (Figures 3 and 4). In control plants, we detected on rare occasions the hypha structure of unidentified endophyte fungi in the cell lumina of xylem fibers and vessels. These endophytic fungi were always restricted to a limited area containing a loose network of hyphae (Figures 3A and 3B).

An extended cluster of dense hypha network appeared in the xylem tissue up to the inoculation site of samples from plants inoculated with *Pch*, (Figures 3C–3E). Hypha structures in this cluster were always located in cell lumina and were never detected in cell walls. The overall hypha network seems to be restricted to an area that matches the RZ (Figure 3C). In infected tissue, hypha structures develop in fibers, paratracheal parenchyma cells, rays, and xylem vessels both open and occluded by tyloses (Figures 3D–3F and 4D). Fungal hyphae were also observed in piths, in the surrounding xylem tissue, and in the dead phloem tissue (data not shown). We also found infected vessels in the neighborhood of infected fibers but separated from healthy xylem tissue (Figure 3D).

At the junction of NPd and pre-existing xylem tissue (i.e., xylem tissue that existed before the wound), the dense network of hyphae was limited by the layer of cells bordering the ray (Figures 1I and 4A–4C). These cells had thickened suberized walls and appeared to form a periderm-like continuity in the ray (Figures 1I and 4A–4B). We found no fungal structure in these modified ray cells, as was the case for NPd and newly formed xylem tissue (Figures 4A–4C).

Discussion

In this study, anatomical changes appearing after injury in the xylem of *Vitis vinifera* consist mainly of suberin rather than lignin deposits at the cell-wall level. A feature of grapevine wood is the absence of apotracheal parenchyma cells in the fascicular portion of xylem. In this case, the conjunctive tissue between vessels mainly consists of fibers, even if tracheids rarely appear (Schoch *et al*., 2004). This feature may help to increase stem stiffness by requiring only a minimum amount of conjunctive tissue to avoid damaging the fascicular portion under twisting (Carlquist, 1985). In angiosperm tree species, postinjury modifications in the xylem mainly occur in apotracheal parenchyma cells and in rays but are absent from fiber, which, in tree wood, are nonliving cells (Biggs, 1987; Pearce, 2000). This response can lead to the deposit of lignin or suberin in tree wood. However, in grapevine wood, it seems that fibers react by modifying their wall, thereby playing the role in the compartmentalization process of the apotracheal parenchyma cells of tree wood. This feature may also confer to grapevine wood the ability to build continuous suberized layers such as walls 2, 3, and 4 of the CODIT model. Deposition of suberin may help responsive tissues to conserve its mechanical properties, thereby preventing stems from cracking at the wound (or healing) site.

The vessel occlusions observed in our experiments were mainly consisted in tyloses. Woody-plant species that respond to wounding by depositing suberin tend to occlude their vessels with tyloses rather than gels (Pearce, 1996). In grapevine wood, the type of occlusion also depends on the season (Sun *et al*., 2008). Because this species occludes its vessels with tyloses in summer and gel in winter, our results may be attributed to the temperate conditions of our trials. Suberin deposits also occur in tyloses. Although this phenomenon is known in woody species (Rioux *et al*., 1995), it has never been reported before in grapevine wood. It is interesting that only a very small number of vessel occlusions appear in portions of the xylem where fibers and rays react. In contrast, vessels fully occluded with tyloses appear in the RZ periphery and are organized all around the RZ, which corresponds strongly with wall 3 of the CODIT model (Figures 1E and 3A). Although the injury response in wood might restrict microbe invasion, it must first maintain the water status of the plant by limiting water loss through the wound.

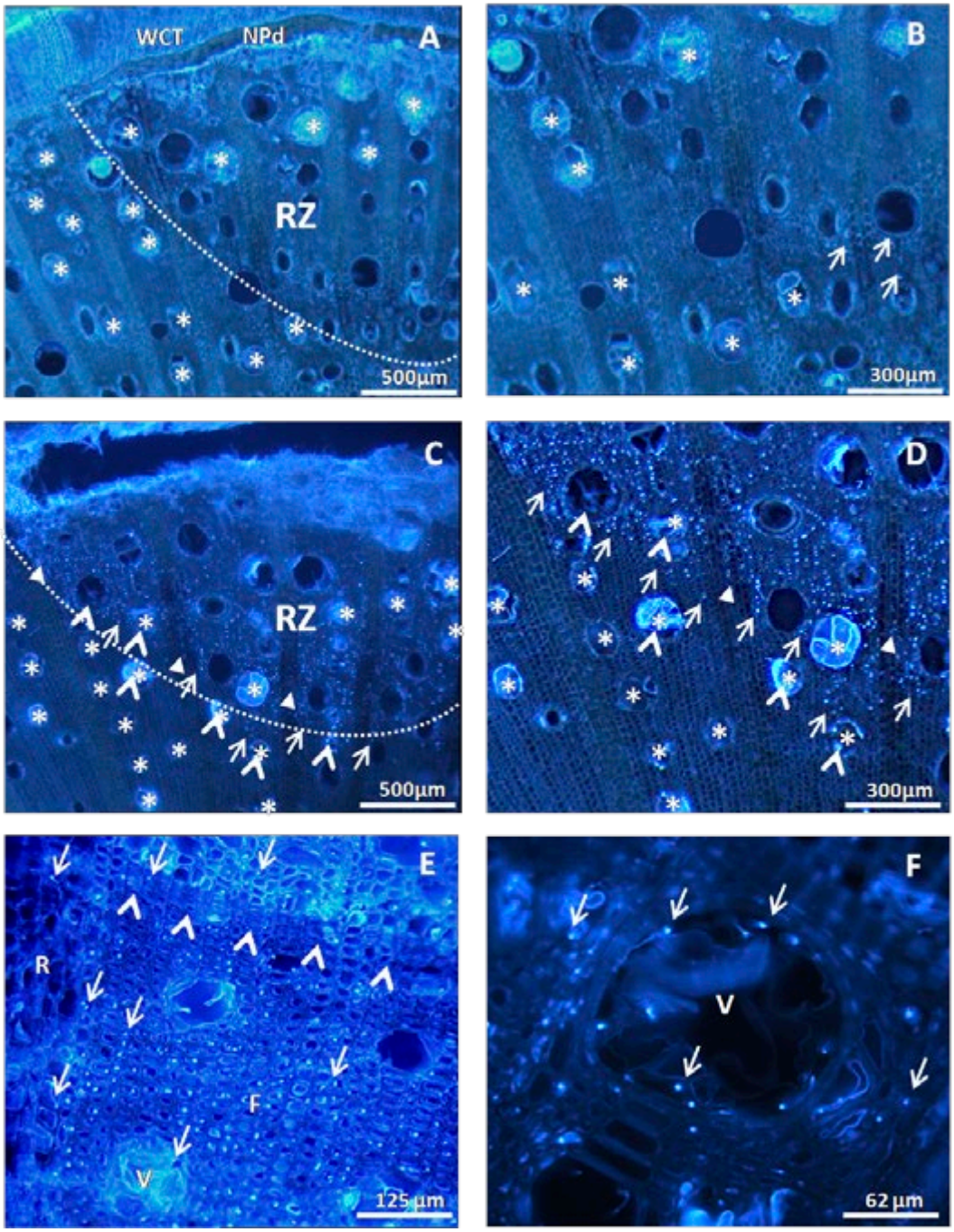

Figure 3. Localization of *Pch* hypha in xylem. (A–F) Epifluorescence micrographs of sections stained with Calcofluor White M2R (UV illumination). (A–D) Cross sections of (A, B) wound of control plant and (C, D) of plant inoculated with *Pch*. Black stars indicate vessels fully occluded with tyloses, arrows indicate fungal hyphae in fibers, arrow heads indicate fungal hyphae in rays, V-shaped arrow heads indicate vessels where fungal hyphae occurs. (A, C) RZ boundaries are indicated by dotted lines. (E) Micrograph showing the dense network of hyphae in infected xylem. Arrow heads indicate the layer of cells delimiting the growth ring, and arrows indicate different xylem-cell types where hyphae are seen. (F) Close view of fungal hyphae (arrows) in xylem vessels occluded with tyloses. The notation F stands for fibers, NPd is for necrophylactic periderm, R is for ray, RZ is for reaction zone, V is for vessel; and WCT is for wound closure tissue.

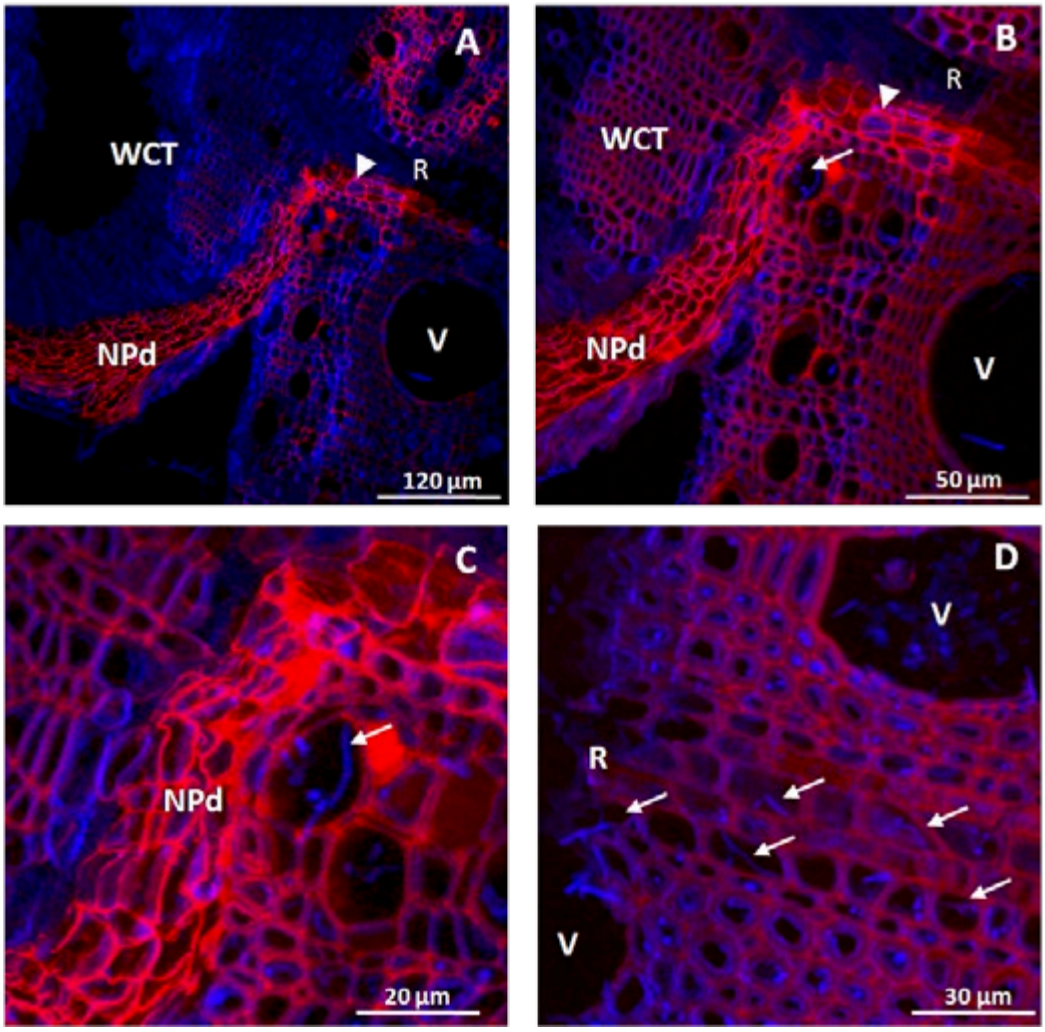

Figure 4. (A–C) Localization of *Pch* hyphae at junction of necrophylactic periderm and xylem tissue. Layout pictures of confocal micrographs obtained with filter Aand filter B. Arrows indicate fungal hyphae near the junction, and arrow head indicates cell layer bordering the ray. (D) Close view of infected xylem. Arrows indicate hyphae progressing in a ray. Note the continuity of hypha in the vessel close to the ray in the bottom-right corner. The notation NPd stands for necrophylactic periderm, R is for ray, V is for vessel, and WCT is for wound closure tissue.

To efficiently prevent water loss around the injured area, plasmodesma closure may accompany the development of the suberized layers in fibers. This process may thus prevent symplastic transport of nutrients and limit the flow of carbon sources required for the synthesis of a significant amount of occluding material in the large vessels of grapevine wood. In this context, the massive water intake around injured tissue must be suppressed by occluding the vessels exiting the RZ.

This study shows that inoculation with 1000 conidia of *Pch* is sufficient to stop callus growth but not tissue differentiation, as for the NPd. The colonized-plug method used in others studies (Laveau *et al*., 2009; Luque *et al*., 2009) may lead to over infection of tissues and enhanced fungus aggressivity. The infection method used in the present study allows the compartmentalization process to be initiated in the xylem and bark. Several phytotoxic compounds have been purified from medium-cultured *Pch*

(Abou-Mansour *et al*., 2004; Bruno and Sparapano, 2007; Luini *et al*., 2010). *In vitro* tests indicate that these toxins reduce callus growth, alter grapevine-cell physiology, and inhibit induced defense mechanisms (Abou-Mansour *et al*., 2004; Luini *et al*., 2010). We find no *Pch* hypha in the newly formed tissue or across the rays limiting the infected xylem portion (Figures 4A–4C). In this context, the thick suberized layers observed here (NPd and cells bordering the rays at the RZ periphery) may provide impervious barriers that efficiently prevent the spread of *Pch* in the newly formed xylem tissue and the adjacent portion of the fascicular xylem. It has often been observed that the layer bordering rays may not provide a continuous suberized barrier along the rays. Thus, we hypothesize that the gaps observed along rays may contribute to a failure in *Pch* compartmentalization by wall 3 of the CODIT model. Inhibition of callus growth and reduction of cambial activity then imply that toxins may circulate (most probably through the symplastic pathway) from infected to surrounding healthy tissue. Assuming that toxins produced by *Pch* are strong necrosis inducers and that these toxins act far from their production site, it appears that *Pch* might behave as a necrotroph pathogen in grapevine wood.

The response to *Pch* infection of occluding xylem vessels with tyloses and gel is documented (Del Rio *et al*., 2001; Mutawila *et al*., 2011). An *in vivo* study showed that *Pch* is able to degrade pectin and use it as a sole source of carbon (Marchi *et al*., 2001). Because pectin polymers are one of the main components of tyloses and gels in occluded vessels, it is not surprising that vessel occlusions (CODIT model wall 1) do not provide an efficient mechanism to restrict the spread of decay. This statement is supported by the fact that networks of *Pch* propagules appear in occluded vessels. *Pch* may spread more quickly in plants through vessels than by other xylem cells, but it may not be restricted strictly to vessels. The colonization of other xylem areas surrounding the vessels (i.e., fibers, tracheids, and rays) could be a second step of the infection process, allowing the slow spread of *Pch* from the infected growth ring to cells of the healthy outer ring, and finally to the vascular cambium. As found here, the cells bordering rays may be one of the most strategic zones in the compartmentalization process in grapevine wood, especially in mature grapevine wood, where large vessels are mostly in contact with surrounding rays (Schoch *et al*., 2004). The black spots from which *Pch* can be isolated are mainly organized in a concentric manner and often in a line that follows portions of a growth-ring-like boundary (CODIT model wall 4) (Mugnai *et al*., 1999; Luque *et al*., 2009). We found that the BZ (CODIT model wall 4), which developed six weeks after injury, consisted only of a thin layer of one to two suberized cells, which contrasts with the thick lignified layer found in tree species (Pearce, 1996). Fully understanding how native cell layers (and eventually modified cell layers) restrict the centrifugal spread of *Pch* in fascicular xylem portions and in rays requires further investigations.

The goals of this study were to characterize the reactions of grapevine woody stem to bark and wood injury and to determine how these responses are affected by *Pch* infection. This approach provides a simple and convenient method to more deeply understand plant-pathogens interactions in the field of grapevine trunk disease. Fully understanding how native (limits of annual rings: CODIT model wall 2) and modified cell layers (BZ: CODIT model wall 4) restrict the centrifugal spread of *Pch* in fascicular xylem portions and in rays requires further investigations.

Acknowledgments

This research was supported by Loire Viti Vini Distribution (LVVD, Z.A du Landreau, 49610 Mozé sur Louet, France), and "Agence Nationale de la Recherche et de la Technologie". We would like to thank Dr Reichheld for providing histological expertise.

Literature cited

Abou-Mansour E., E. Couché and R. Tabacchi, 2004. Do fungal naphthalenones have a role in the development of Esca symptoms? *Phytopathologia Mediterranea* 43, 75–82.

Armstrong E., A.L. Shigo, D.T. Funk, E.A. McGinnes and D.E. Smith, 1981. A macroscopic and microscopic study of compartmentalization and wound closure after mechanical wounding of black Walnut trees. *Wood and Fiber Science* 13, 275–291.

Biggs A.R., 1984. Boundary-zone formation in peach bark in response to wound and *Cytospora leucostoma* infection. *Canadian Journal of Botany* 62, 2814–2821.

Biggs A.R., 1987. Occurrence and location of suberin in wound reaction zones in xylem of 17 tree species. *Phytopathology* 77, 718–725.

Biggs A.R., W. Merrill and D.D. Davis, 1984. Discussion: re-

sponse of bark tissues to injury and infection. *Canadian Journal of Forest Research* 14, 351–356.

Bruno G. and L. Sparapano, 2007. Effects of three Esca-associated fungi on *Vitis vinifera* L.: I. Characterization of secondary metabolites in culture media and host responses to the pathogens in calli. *Physiological and Molecular Plant Pathology* 69, 209–223.

Carlquist S., 1985. Observations on functional wood histology of vines and lianas, vessel dimorphism, tracheids, vasicentric tracheids, narrow vessels and parenchyma. *Aliso* 11, 139–157.

Deflorio G., F. Erwin, S. Fink and F.W.M.R. Schwarze, 2009. Host responses in the xylem of trees after inoculation with six wood-decay fungi differing in invasiveness. *Botany* 87, 26–35.

Del Rio J.A., P. Gomez, A. Baidez, M.D. Fuster, A. Ortuno and V. Frias, 2004. Phenolic compounds have a role in the defence mechanism protecting grapevine against the fungi involved in Petri disease. *Phytopathologia Mediterranea* 43, 87–94.

Del Rio J. A., A. Gonzalez, M.D. Fuster, J.M. Botia, P. Gomez, V. Frias and M. Ortuno, 2001. Tylose formation and changes in phenolic compounds of grape roots infected with *Phaeomoniella chlamydospora* and *Phaeoacremonium* species. *Phytophatologia Mediterranea* 40, S394–S399.

Eskalen A. and W.D. Gubler, 2001. Association of spores of *Phaeomoniella chlamydospora*, *Phaeoacremonium inflatipes*, and *Pm. aleophilum* with grapevine cordons in California. *Phytopathologia Mediterranea* 40, S429–S432.

Kuntzmann P., S. Villaume, P. Larignon and C. Bertsch, 2010. Esca, BDA and Eutypiosis: foliar symptoms, trunk lesions and fungi observed in diseased vinestocks in two vineyards in Alsace. *Vitis* 49, 71–76.

Laveau C., A. Letouze, G. Louvet, S. Bastien and L. Guerin-Dubrana, 2009. Differential aggressiveness of fungi implicated in Esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea* 48, 32–46.

Luini E., P. Fleurat-Lessard, L. Rousseau, G. Roblin and J.M. Berjeaud, 2010. Inhibitory effects of polypeptides secreted by the grapevine pathogens *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* on plant cell activities. *Physiological and Molecular Plant Pathology* 74, 403–411.

Luque J., S. Martos, A. Aroca, R. Raposo and F. Garcia-Figueres, 2009. Symptoms and fungi associated with declining mature Grapevine plants in northeast Spain. *Journal of Plant Pathology* 91, 381–390.

Marchi G., I. Pascoe and J. Edwards, 2001. Young Esca in Australia. *Phytopathologia Mediterranea* 40, S303–S310.

Marchi G., S. Roberti, R. D'Ovidio, L. Mugnai and G. Surico, 2001. Pectic enzymes production by *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 40, S407–S416.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Disease* 83, 404–418.

Mutawila C., P. Fourie, F. Halleen and L. Mostert, 2011. Histopathology study of the growth of *Trichoderma harzianum*, *Phaeomoniella chlamydospora* and *Eutypa lata* on grapevine pruning wounds. *Phytophatologia Mediterranea* 50, S46–S60.

Pearce R.B., 1996. Antimicrobial Defences in the Wood of Living Trees. *New Phytologist* 132, 203–233.

Pearce R.B., 2000. Decay development and its restriction in trees. *Journal of Arboriculture* 26, 1–11.

Rioux D., H. Chamberland, M. Simard and G.B. Ouellette, 1995. Suberized tyloses in trees: An ultrastructural and cytochemical study. *Planta* 196, 125–140.

Schoch W., I. Heller, F.H. Schweingruber and F. Kienast, 2004. Wood anatomy of central European Species. Online version http://www.woodanatomy.ch/

Shigo A.L. and H.G. Marx, 1977. Compartmentalization of Decay in Trees. *Agriculture information bulletins* 405, 73 pp.

Srebotnik E. and K. Messner, 1994. A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi. *Applied and Environmental Microbiology* 60, 1383–1386.

Sun Q., T.L. Rost and M.A. Matthews, 2008. Wound-induced vascular occlusions in *Vitis vinifera* (*Vitaceae*): tyloses in semmer and gels in winter. *American Journal of Botany* 95, 1498–1505.

Surico G., 2009. Towards a redefinition of the diseases within the Esca complex of grapevine. *Phytopathologia Mediterranea* 48, 5–10.

Valtaud C., P. Larignon, G. Roblin and P. Fleurat-Lessard, 2009. Developmental and ultrastructural features of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in relation to xylem degradation in Esca disease of the grapevine. *Journal of Plant Pathology* 91, 37–51.

Accepted for publication: May 14, 2013