

Response of Resistant, Tolerant, and Susceptible Grapevine Tissues to Invasion by the Pierce's Disease Bacterium, *Xylella fastidiosa*

S. M. Fry and R. D. Milholland

Graduate research assistant and professor, respectively, Department of Plant Pathology, North Carolina State University, Raleigh 27695-7616.

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ABSTRACT

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Microtechniques were used to quantify occlusion of leaf veins of grapevines inoculated with *Xylella fastidiosa*. Bacteria and pectins were responsible for most occlusions in xylem vessels of inoculated *Vitis vinifera* 'French Colombard' (susceptible to Pierce's disease) and *V. rotundifolia* 'Carlos' (tolerant) and 'Noble' (resistant). The average percentage of vessels in leaf veins colonized by the bacterium and occluded by pectins was greatest in French Colombard. Occlusion by tyloses occurred to a similar

extent among all cultivars. The average percentage of vessels occluded by gums and tannins was greatest in Noble, and similar between French Colombard and Carlos. The majority of pectins, bacteria, and tyloses occluded leaf vein vessels partially. In general, fewer occlusions were observed in buffer-inoculated (control) plants than in pathogen-inoculated plants.

Pierce's disease of the grapevine is caused by the xylem-limited bacterium, *Xylella fastidiosa* (19,37). Marginal leaf burn, chlorosis, and death of the plant are characteristic symptoms of this vascular disease. Bunch grapevines (*Vitis vinifera* L. and *V. labrusca* L.) are especially susceptible to the bacterium. Muscadine grapevines (*V. rotundifolia* Michx.), however, may be tolerant or resistant to the bacterium, depending on the cultivar (10,20,22,25,27).

Studies on the mechanisms of resistance of muscadine grapevines to Pierce's disease indicate that tyloses and gums occur more frequently in naturally infected muscadine grapevines than in bunch grapevines (28). It has been postulated that the bacteria become trapped in these substances, thereby preventing systemic movement of the pathogen and further multiplication (28). Vascular plugs consist of pectins, gums, and/or tannins (23). Though vascular plugging may prevent the spread of *X. fastidiosa*, blockage of the xylem also causes water stress and subsequent necrosis and chlorosis (16).

Previous studies (13,14,21) indicate that the multiplication and translocation of *X. fastidiosa* is inhibited in petioles and stems of resistant and tolerant muscadine grapevines. The objective of this study was to characterize the mechanism of resistance in muscadine grapevines by using light microscopy to determine the development of occlusions in xylem vessels of grapevines artificially inoculated with *X. fastidiosa*.

MATERIALS AND METHODS

Grape cultivars French Colombard, Carlos, and Noble were grown and inoculated in the petiole as previously described (14). Each treatment was replicated four times. One-centimeter sections of petiole and leaf vein adjacent to samples taken after 2, 4, 8, and 12 wk for multiplication studies (14) were removed and fixed in Formalin-2-propanol-propionic acid (24). The samples were dehydrated in an isopropyl alcohol series, and infiltrated and embedded in Paraplast Plus (Monoject Scientific, St. Louis, MO). To soften the material, trimmed paraplast blocks were

soaked at 4 C for 3-7 days in a solution of Dreft detergent and glycerin (1). Samples were sectioned on a rotary microtome at 12 μ m.

One leaf vein from each of three replicate plants per cultivar inoculated with either the FC strain or SCP buffer was observed from samples taken 4 and 8 wk after inoculation. Leaf veins were observed to avoid misinterpretation of formation of occlusions in the petiole due to injury from the inoculation procedure. Sixteen slides per paraffin block were prepared, each containing 14 serial sections. To visualize bacteria and tyloses the first, fifth, ninth, and 13th slides were stained with Harris hematoxylin and counterstained with Orange G (24). A modified version of the iron adsorption method (32) was used to detect pectins in the second, sixth, 10th, and 14th slides. The third, seventh, 11th, and 15th slides were stained with phloroglucinol (32) for gums. A modified version of the ferric sulfate reaction (24) was used to stain tannins in the fourth, eighth, 12th, and 16th slides. All xylem vessels in approximately 10 transverse-sections per slide were counted and the percentage of vessels containing bacteria, tyloses, pectins, gums, or tannins determined. Approximately 2,000-5,000 vessel transverse-sections per leaf vein were observed. Each occlusion was recorded as totally or partially plugging the vessel.

An analysis of variance was performed for each type of occlusion. An analysis appropriate for a randomized complete block design was used because data from one position on the plants was taken. Means of the main effects and interactions were compared by Fisher's protected least significant difference test.

RESULTS

Bacteria, tyloses, and pectins were the main substances occluding xylem vessels in petioles inoculated with the FC strain of *X. fastidiosa*. Bacteria stained purple and were free in the lumen of the xylem vessel as well as appressed to the xylem vessel cell wall in all cultivars. Bacteria were also embedded in a substance that stained positive for pectins.

The percentage of vessels containing the bacterium increased approximately four-, three-, and onefold between 4 and 8 wk in French Colombard, Carlos, and Noble, respectively (Table 1). When averaged over time, the percentage of vessels occluded by

bacteria was significantly higher in French Colombard than in Carlos. Colonization of Noble was similar ($P = 0.05$) to that in French Colombard and Carlos. There was a similar ($P = 0.05$) increase in bacterial colonization of all cultivars over time. Bacteria were not observed in buffer-inoculated (control) plants.

The percentage of vessels with tyloses increased about 5-, 21-, and 7-fold between 4 and 8 wk in pathogen-inoculated French Colombard, Carlos, and Noble, respectively (Table 1). In general, tylose production in control plants tended to decrease or remain about the same. When averaged over time, the percentage of vessels containing tyloses was similar ($P = 0.05$) among cultivars inoculated with the pathogen or buffer. The increase in production of tyloses over time was also similar ($P = 0.05$) among cultivars inoculated with the pathogen or buffer. Pathogen- and buffer-inoculated plants produced similar amounts of tyloses. There was, however, a greater ($P = 0.05$) increase in the presence of tyloses in pathogen-inoculated plants between 4 and 8 wk than in control plants.

The most abundant occlusion of host origin was pectic substances. Pectins stained blue and were appressed to the xylem vessel wall and/or free in the lumen of the vessel in French Colombard. Pectins were mainly appressed to the xylem vessel wall in Carlos and Noble. When a high percentage of a plug consisted of bacteria, the plug appeared blue-green to yellow-green when stained for pectins.

The percentage of vessels occluded by pectins increased about three-, five-, and one-fold in pathogen-inoculated French Colombard, Carlos, and Noble, respectively (Table 1). In contrast, pectin production remained almost unchanged over time in control plants. When averaged over time, the percentage of vessels occluded by pectins was significantly higher in pathogen-inoculated French Colombard than in Carlos and Noble, whereas Carlos and Noble produced similar ($P = 0.05$) amounts of pectin. Pectin production was similar ($P = 0.05$) among buffer-inoculated cultivars. The increase in pectin production over time was similar ($P = 0.05$) among pathogen- and buffer-inoculated plants. Pathogen-inoculated plants produced significantly greater

amounts of pectins than control plants. The increase in the presence of pectins over time was greater ($P = 0.05$) in pathogen-inoculated plants than in control plants.

The percentage of vessels with gums increased slightly in pathogen-inoculated French Colombard and Carlos; however, there was an 82-fold increase in Noble between 4 and 8 wk (Table 1). Gum production remained almost unchanged over time in control plants. When averaged over time, the percentage of vessels with gums was significantly higher in pathogen-inoculated Noble than in Carlos and French Colombard, whereas French Colombard and Carlos produced similar ($P = 0.05$) amounts of gum. Gum production was similar ($P = 0.05$) among buffer-inoculated cultivars. A significant cultivar \times time interaction was detected among pathogen- and buffer-inoculated cultivars. Pathogen-inoculated plants produced significantly greater amounts of gums than control plants, and the increase in the presence of gums over time was greater in pathogen-inoculated plants than in control plants.

Few vessels were occluded by tannins in pathogen- and buffer-inoculated cultivars (Table 1). When averaged over time, the percentage of vessels with tannins was significantly greater in pathogen-inoculated Noble than in French Colombard and Carlos, whereas French Colombard and Carlos produced similar ($P = 0.05$) amounts of tannins. Tannin production was significantly greater in buffer-inoculated Noble than Carlos, whereas French Colombard produced amounts similar ($P = 0.05$) to Noble and Carlos. A significant cultivar \times time interaction was detected among pathogen- and buffer-inoculated cultivars. Pathogen-inoculated and control plants produced similar amounts of tannin. The production of tannins decreased significantly in pathogen-inoculated plants and increased significantly in control plants over time.

The majority of occlusions by bacteria, tyloses, and pectins in pathogen- and buffer-inoculated plants were partial (Table 2). The presence of total or partial occlusions in control plants was generally less than that in pathogen-inoculated plants. The percentage of vessels completely or partially occluded by tyloses

TABLE 1. The percentage of observed vessels containing bacteria and occlusions in leaf veins of the susceptible cultivar French Colombard, tolerant cultivar Carlos, and resistant cultivar Noble inoculated with *Xylella fastidiosa* strain FC or buffer (control)^a

Cultivar	Time after inoculation (wk)	Bacteria ^b	Pectins		Tyloses		Gums		Tannins	
			Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
French Colombard	4	3.8	7.9	0.4	0.4	3.5	0.1	0.2	0.1	0.2
	8	16.3	21.9	0.6	2.1	0.4	0.1	0.0	0.0	0.7
Carlos	4	1.5	1.0	0.1	0.3	0.7	0.0	0.0	0.1	0.1
	8	4.4	5.4	0.2	5.7	0.4	0.4	0.1	0.1	0.0
Noble	4	5.4	6.3	0.9	0.7	0.5	0.0	0.0	1.4	0.1
	8	6.6	7.8	1.1	4.7	1.8	3.3	0.0	0.0	1.2
LSD ^c		1.4	0.2	0.3	0.6

^aData presented were averaged over three leaf veins (~40 sections/leaf vein; ~2,000–5,000 vessel transverse-sections/leaf vein).

^bData from bacterial pathogen- and buffer-inoculated plants (control) are presented. Bacteria were not observed in control plants.

^cLSD ($P = 0.05$) presented is for comparing two cultivars within a time or two times within a cultivar.

TABLE 2. The percentage of the observed vessels partially or totally occluded by bacteria, tyloses, or pectins in leaf veins of the susceptible cultivar French Colombard, tolerant cultivar Carlos, and resistant cultivar Noble inoculated with *Xylella fastidiosa* strain FC or buffer (control)^a

Cultivar	Time after inoculation (wk)	Bacteria ^b		Pectins				Tyloses			
				Partial		Complete		Partial		Complete	
		Partial	Complete	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
French Colombard	4	3.8	0.1	7.5	0.4	0.2	0.0	0.3	3.5	0.1	0.1
	8	14.4	1.8	20.4	0.6	1.5	0.0	1.7	0.3	0.4	0.0
Carlos	4	1.5	0.1	1.0	0.1	0.0	0.0	0.1	0.4	0.1	0.3
	8	3.6	0.8	5.1	0.2	0.2	0.0	2.6	0.3	3.1	0.2
Noble	4	4.3	1.1	6.1	0.9	0.2	0.0	0.5	0.3	0.2	0.3
	8	5.9	0.6	7.4	1.1	0.4	0.0	3.1	1.0	1.6	0.8
LSD ^c	

^aData presented were averaged over three leaf veins (~40 section/leaf vein; ~2,000–5,000 vessel transverse-sections/leaf vein).

^bData from bacterial pathogen- and buffer-inoculated (control) plants are presented. Bacteria were not observed in control plants.

^cLSD ($P = 0.05$) presented is for comparing two cultivars within a time or two times within a cultivar.

and pectins was similar ($P = 0.05$) among buffer-inoculated cultivars. Partial occlusion by bacteria was greater in French Colombard than Carlos, however, was similar ($P = 0.05$) between French Colombard and Noble. Total occlusion by bacteria and tyloses was similar ($P = 0.05$) among all cultivars. Total and partial occlusion of leaf vein vessels by pectins was greatest ($P = 0.05$) in French Colombard.

DISCUSSION

Histological observations indicated that populations of *X. fastidiosa* in leaf veins of artificially inoculated French Colombard are greater than in Carlos and Noble after 8 wk, even though multiplication studies (14) indicated that all cultivars harbor similar numbers of the bacterium after 8 wk. Rapid colonization of naturally infected bunch grapevines and not muscadine grapevines has been observed (21). The discrepancy between the multiplication and histological studies may be due to the few samples that were observed microscopically. Other explanations may be that a substance surrounded the bacterium in Carlos and Noble, thus preventing staining of the bacterium, or that the large amount of pectin associated with the bacterium in French Colombard decreased the sensitivity of the dilution technique.

The susceptible cultivar French Colombard apparently cannot localize the bacteria with tyloses and gums and prevent their systematic movement up the plant. Gums, which originate from the cell wall or middle lamella (2,9,35), and tyloses occurred infrequently in leaf vein sections. The rare occurrence of tyloses and gums has also been observed in naturally infected susceptible bunch grapevines (28). Leaf veins of French Colombard were, however, predominantly occluded by pectins. Individual transverse-sections usually contained several vessels occluded by plugs that stained positive for both bacteria and pectin. Pectins, which originate from middle lamellae, perforation plates, end walls, and pit membranes (9,30,35), partially occluded xylem vessels. Hopkins (18) found that *X. fastidiosa* did not produce pectolytic, proteolytic, or cellulolytic enzymes in culture and, therefore, the bacterium appears unable to degrade cell wall constituents. However, *X. fastidiosa* may produce these enzymes in the host.

The presence of many partial occlusions throughout the vascular system is characteristic of a susceptible reaction to a vascular infection (3,4,6). For example, Beckman (6) proposed that a susceptible banana cultivar infected with *Fusarium oxysporum* f. sp. *cubense* was able to initiate production of gels and tyloses. The gels and tyloses, however, were weak or delayed in development, allowing fungal spores to be swept up in the xylem where the localization process was initiated again. As the pathogen became systemic, many "pockets of resistance" became established in the vascular system. As a result, overproduction of tyloses and gels caused water stress and symptom development. Overproduction of occlusions is also responsible for symptom development in other pathosystems (31,34). The same susceptible reaction appears to be operative in *X. fastidiosa*-infected French Colombard. Our study indicates that pectins may have been overproduced throughout the vascular system in an attempt to localize the pathogen. The rapidity and magnitude with which the bacteria and pectins occlude leaf veins of the susceptible cultivar enhances marginal leaf necrosis or death of the plant. Hopkins and Thompson (21) indicated that bacterial populations in naturally infected susceptible Schuyler grapevines increased rapidly in May through June, such that high populations existed in the plants throughout the summer allowing a long period of water stress. We suggest that pectins also contribute largely to water stress and, therefore, symptom development.

Previous studies (28) have correlated the presence of tyloses and gums to resistance of muscadine grapevines to *X. fastidiosa*. Using transmission electron microscopy, Mollenhauer and Hopkins (28) found that tyloses and gums predominated in vessel transverse-sections of petioles and leaf veins of naturally infected tolerant muscadine grapevines, but not in a susceptible bunch grapevine. We also observed tyloses more frequently in artificially

inoculated muscadine grapevines than a bunch grapevine; however, gums were infrequently observed. Mollenhauer and Hopkins observed a much higher percentage of vessels with tyloses (27%) and gums (35%) than we observed in Carlos or Noble 8 wk after inoculation. Esau (12) also found that gums occurred infrequently in major veins of *X. fastidiosa*-infected grapevines. One reason for the discrepancy may be that, because Mollenhauer and Hopkins did not use histochemical tests, pectins were inadvertently categorized as gums. Gels or plugs may consist of pectins, hemicellulose, carbohydrate, and/or traces of protein, depending on the pathosystem (9,15,23,28,35). Histochemical tests, therefore, are necessary for characterizing the components of vascular plugs. Other reasons for the discrepancy between our results and those of Mollenhauer and Hopkins are that the resistance of the naturally infected vines was apparently breaking down, and the vines were probably infected longer than our vines.

Successful localization of a vascular pathogen by a resistant cultivar is characterized by rapid and complete occlusion of the infected area (3,4,6). For example, Beckman and Halmos (6) proposed that a banana cultivar resistant to *Fusarium oxysporum* f. sp. *cubense* was able to completely occlude vessel elements above the infection site with gel, tyloses, and phenols 5 days after infection. We found that tyloses and pectins were produced in moderate amounts in leaf vein vessels of artificially inoculated Carlos and Noble. Though rarely observed, greater amounts of gums were produced in Noble than in Carlos and French Colombard. The appearance of pectins early in the infection process may have aided localization of the pathogen in Noble. Tyloses, on the other hand, appeared to develop later in both muscadine cultivars. Individual leaf vein transverse-sections in Noble usually contained few occlusions, whereas other transverse-sections were almost totally occluded. Some vessels were occluded by a combination of tyloses, pectins, and/or gums. The localization process in muscadine grapevines may occur over a longer period of time. Inhibition of bacterial colonization, however, has been observed. Hopkins and Thompson (21) indicated that bacterial populations in naturally infected muscadine grapevines increased gradually during the summer, reaching a maximum in the fall. Populations then declined due to cooler weather. If low bacterial populations and moderate amounts of vascular occlusions occur over time in muscadine grapevines, as we have found, plants may survive to produce new growth or may produce new xylem to compensate for blocked vessels.

Tannins are polyhydroxyphenols that occur in many plants and may form cross-links with proteins (17,29). Esau (12) observed tannin-containing xylem parenchyma cells, and tannin-containing and tannin-free tyloses in xylem vessels of *X. fastidiosa*-infected grapevines. In our studies, tannins were scarce, randomly distributed, and sometimes associated with tyloses.

Phenols have been reported to occur in specialized cells of many plants (7,26,36). Beckman et al (8) reported that phenols melanized structural barriers in infected banana roots to create a durable plug, resistant to degradation, between healthy and diseased tissue. The presence of the phenols appears to regulate the localization response of the host. The localization response may be delayed if the release of phenolics is too slow, and may be shut down if released too fast. The presence of tannins in Noble after 4 wk may play a role in initiating a localization response. Once initiated, tannin production is decreased as observed after 8 wk. It is not known, however, if the amount of tannin observed in our study is sufficient to elicit such responses. In addition, the amount of tannin produced by pathogen- and buffer-inoculated appeared to be similar.

The localization response is a widespread mechanism of resistance of plants to vascular infections (3-6,11,33). Comparison of occlusions produced by *X. fastidiosa*- and buffer-inoculated grapevines indicates that pectins, tyloses, and gums were produced in response to the presence of the pathogen and not the buffer. We have found that the overproduction of pectins by the susceptible cultivar may actually contribute to symptom development. Mollenhauer and Hopkins (28) proposed that the localization response was operative in *X. fastidiosa*-infected muscadine grape-

vines. It is unclear, however, from our studies whether the low percentage of vessels occluded with gums, tyloses, pectins, and tannins, and their time of appearance is enough to provide tolerance or resistance to muscadine grapevines. Perhaps, the localization response acts in combination with the rapid aging of *X. fastidiosa* (23) in muscadine grapevines to provide resistance.

Previous studies with the susceptible bunch grapevine, French Colombard, inoculated with *X. fastidiosa*, showed that most bacteria in the tracheary elements had smooth cell walls and were completely encapsulated by an electron-dense substance (23). This encapsulation of rapidly aging bacteria by an electron-dense substance apparently plays an important role in the incompatibility of this host-pathogen interaction. Results of transmission electron microscopy studies we conducted during the past 3 yr showed that rippled cell-walled bacteria predominated in the susceptible cultivar, whereas a combination of rippled and smooth cell-walled bacteria were present in the tolerant cultivar, and mainly smooth cell-walled bacteria colonized the resistant cultivar 8 wk after inoculation. The smooth cell-walled bacteria were encapsulated in an electron-dense substance. Based on our histochemical tests, this material surrounding the aging bacteria consisted primarily of pectins and some gums (S. M. Fry, *unpublished*).

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