Multiplication and Translocation of *Xylella fastidiosa* in Petioles and Stems of Grapevine Resistant, Tolerant, and Susceptible to Pierce's Disease

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ABSTRACT

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Petioles of *Vitis vinifera* 'French Colombard' (susceptible to Pierce's disease), and *V. rotundifolia* 'Carlos' (tolerant) and 'Noble' (resistant) were inoculated with virulent strain FC of *Xylella fastidiosa*. Populations of the bacterium increased rapidly in petioles of French Colombard but increased gradually in Carlos and Noble. Maximum populations were attained faster in French Colombard than in Carlos and Noble. *X. fastidiosa* rapidly colonized leaf veins of all cultivars. Stems of 5- and 7-wk-old plants were inoculated with strain FC or C of *X. fastidiosa*, and bacterial cfu/cm of stem 10, 20, and 50 cm above the inoculation point were determined over time. Both strains were translocated throughout French Colombard. The FC strain was reisolated at 50 cm in Carlos and 10 cm in Noble, 8 wk after 5-wk-old plants were inoculated. The C strain was not detected in Carlos or Noble. The FC strain was reisolated throughout Carlos and Noble, 8 wk after 7-wk-old plants were inoculated. The C strain was reisolated no higher than 10 cm above the inoculation point in Carlos and Noble.

Pierce's disease of grapevine is caused by the xylem-limited bacterium, *Xylella fastidiosa* Wells et al (11,20). The bacterium multiplies within the vascular system, resulting in plugging of the xylem vessels. Xylem vessels also become occluded with pectins, tyloses, and gums, which may be produced by the host in response to invasion by the bacterium (6,8,15,18).

As a result of plugging, and subsequent water stress (7), symptoms produced include marginal leaf necrosis, chlorosis, wilting of the fruit, and death of the plant. Bunch grapevines (*Vitis vinifera* L. and *V. labrusca* L.) are particularly susceptible to the bacterium. In contrast, muscadine grapevines (*V. rotundifolia* Michx.), which are native to the southeastern United States, may be tolerant or resistant, depending on the cultivar (2,13,15–17).

The multiplication and translocation of *X. fastidiosa* has been studied in bunch grapevines (14) as well as muscadine vines (12). Muscadine grapevines have become an important tool for studying host defense mechanisms because of their natural tolerance and resistance (15). To gain an understanding of the resistance mechanisms of muscadine vines to Pierce's disease, we determined the pattern of multiplication and translocation of *X. fastidiosa* in inoculated petioles and stems of resistant, tolerant, and susceptible grapevines. A preliminary study has been published (5).

MATERIALS AND METHODS

Inoculum. Strains FC and C of *X. fastidiosa* have been described (15). Inoculum was prepared from 4-day-old cultures of the FC strain and 5-day-old cultures of the C strain. The bacteria were suspended in succinate-citrate-phosphate (SCP) buffer (14) and the concentration adjusted to 8×10^7 cfu/ml ($A_{484nm} = 0.27$) with a spectrophotometer.

Petiole inoculation. Cultivars French Colombard, Carlos, and Noble were grown from rooted green cuttings in sand:soil:peat moss (1:1:1, v/v/v) in 25-cm-diameter clay pots. Carlos plants are considered tolerant to the disease because 25–50% of the leaves on an arm of a plant can be symptomatic with no effect on the yield (16). Noble plants do not express symptoms in the field, whereas French Colombard plants express symptoms readily. Plants were fertilized every 2 wk with a solution of Peter's 20-20-20 (Peter's Fertilizer Products, Fogelsville, PA) and once every 6 mo with Osmocote 19-6-12 (Sierra Chemical Co., Milpitas, CA). Temperatures in the greenhouse were maintained between 24 and 32 C. Plants were 6-mo-old when inoculated. The experiment was repeated with 2 1/2-mo-old plants.

A group of five petioles per plant, located 45–75 cm above the base of each vine, were inoculated with the FC strain. Ten microliters of bacterial suspension was transferred from a micropipette to each petiole at a point 6.5–7.0 cm from the junction of the petiole and leaf vein. A pin was used to inoculate each petiole. Control plants were inoculated with SCP buffer. Plants were arranged in a randomized complete block design. The experiment was a split plot with the cultivar-time combination as the whole plot and plant parts as the subplot. Each treatment was replicated four times in the first experiment and three times in the second experiment.

Inoculated petioles were removed from designated plants immediately after inoculation, and 2, 4, 8, and 12 wk later. A 2-cm-section of each petiole, located just above the inoculation point (toward the leaf), was removed, as was a 2-cm section of the base of the leaf vein. The sections were wrapped in moist paper towels, placed in plastic bags, and stored in the refrigerator until assayed. Sections were assayed within 16 hr of sampling.

Petiole and leaf vein sections were surface-sterilized in 1% sodium hypochlorite for 3 min and rinsed in four changes of sterile distilled water. Petiole sections from one plant were collectively ground in 5 ml of SCP buffer with a mortar and pestle. The suspension was filtered through four layers of cheesecloth into a sterile 15-ml glass centrifuge tube. An additional 5 ml of buffer was added to the mortar and the petioles were reground. The suspension was filtered through the cheesecloth into the same centrifuge tube and the filtrate centrifuged for 15 min at 4,000 *g*. The supernatant was discarded and the pellet resuspended in 2 ml of buffer. Serial dilutions in SCP buffer were completed and aliquots plated onto modified PD2 medium (3). Plates were incubated at 28 C for 11 days before colonies were counted.

Stem inoculation. Six- to 7-week-old plants (60–90 cm tall) that had been grown as described above were inoculated in the first experiment. The experiment was repeated with 5-wk-old plants (30–60 cm tall). All plants were trimmed so that one shoot of the desired height remained on each plant. Each shoot was inoculated in two places in the stem 2.5 cm above the base of the shoot. Twenty microliters of bacterial suspension or SCP buffer was placed at each inoculation point. A dissecting needle was used to prick the stem through the drop of inoculum. Plants were arranged in a randomized complete block design as described above. Treatments were replicated three times in each experiment.

Plants were sampled destructively immediately after inoculation, and 2, 4, and 8 wk later. Samples were taken immediately after inoculation of 5-wk-old plants only. Two-centimeter sections of stem centered on a point 10, 20, and 50 cm above the inoculation point were removed with razor blades or hand pruners. A 2-cm section of stem located just above the inoculation point was also removed from 5-wk-old plants immediately after inoculation to determine the efficiency of inoculation. Each stem section was surface-sterilized, cut into smaller pieces with a scalpel, and individually assayed as described above.

Movement of the bacterium into the leaves was monitored by making isolations onto PD2 medium by the technique described above. An ELISA kit (Agdia Inc., Mishawaka, IN) specific for xylem-limited bacteria was used to confirm the identity of colonies.

Data analysis. Data were transformed as log (n), where n = cfu/cm of petiole or leaf vein, or log ($n + 1$), where n = cfu/cm of stem, to stabilize variances. An analysis of variance was performed on all transformed data except those obtained immediately after inoculation. Data obtained from zero time samples were generated apart from the whole experiment and therefore were not included in the analysis. Means of the main effects and interactions were compared by Fisher's Protected Least Significant Difference test. Contrast statements were used to determine if multiplication of the bacterium after 2 wk was described by a linear or quadratic equation.

RESULTS

Petiole inoculation. Approximately 8×10^6 bacterial cells were inoculated into each petiole. Recovery of the bacterium immediately after inoculation was about 10 times greater in petioles of French Colombard than in Carlos and Noble, whereas populations were about 100 times greater in leaf veins of French Colombard than in Carlos and Noble (Fig. 1). Initial populations in the petioles of the muscadine cultivars were similar as were populations in the leaf veins.

Multiplication of *X. fastidiosa* in the petiole of the resistant muscadine cultivar Noble was described by a linear equation, whereas growth in the leaf vein was described by an equation consisting of linear and quadratic components (Fig. 1A). The bacterial population in the petiole and leaf vein reached a maximum after 8 wk, then stabilized. Multiplication of *X. fastidiosa* in the leaf vein was most rapid in the first few weeks.

Multiplication of *X. fastidiosa* in the petiole and leaf vein of the tolerant muscadine cultivar Carlos was described by a linear equation (Fig. 1B). The bacterial population in the petiole decreased, then increased gradually, whereas the population in the leaf vein increased over time. Populations of *X. fastidiosa* in Carlos and Noble were similar ($P = 0.05$) when averaged over time and plant parts.

When averaged over time and plant parts, populations of *X. fastidiosa* were higher in the susceptible cultivar French Colombard than in Carlos and Noble ($P = 0.05$). Growth of *X. fastidiosa* in the petiole and leaf vein of French Colombard was described by an equation consisting of linear and quadratic components. Populations decreased in petioles and veins then rapidly increased (Fig. 1C). Inoculated plants of French Colombard became defoliated by 12 wk, making samples unavailable. The bacterium was never recovered from control plants, the leaves of which remained healthy during the experiment.

Approximately 65% of inoculated French Colombard leaves exhibited general necrosis and chlorosis after 4 wk and 93% were necrotic after 8 wk. In some cases, the necrotic leaf was limp rather than dry and brittle, which is characteristic of marginal leaf burn.

About 5% of the inoculated leaves of Noble and Carlos exhibited slight marginal leaf burn after 4 wk. After 8 wk, 53 and 9% of inoculated leaves of Noble and Carlos, respectively, were symptomatic, whereas 50% of the leaves of both Noble and Carlos were symptomatic after 12 wk. The number of attached inoculated leaves tended to decrease over time as leaves dropped.

Stem inoculation. When averaged over time and plant parts, populations of the FC strain were significantly higher in French

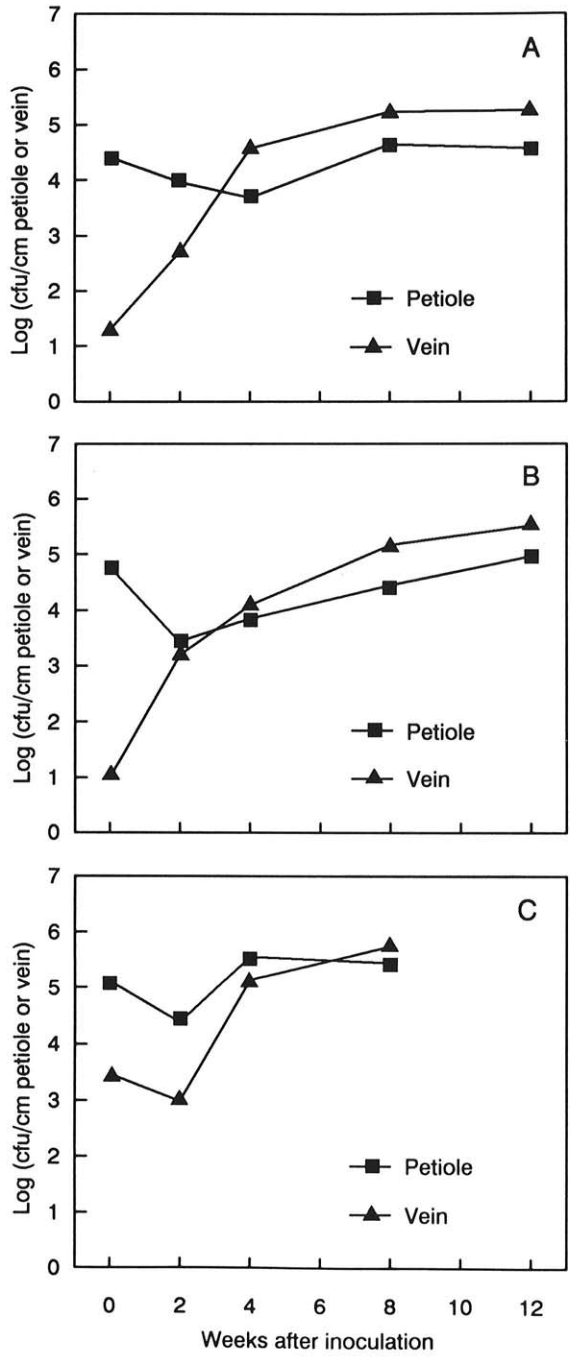

Fig. 1. Multiplication of the FC strain of *Xylella fastidiosa* in petioles and leaf veins of **A,** the resistant muscadine cultivar Noble; **B,** the tolerant muscadine cultivar Carlos; and **C,** the susceptible bunch grapevine French Colombard. Zero time samples were generated apart from the whole experiment.

Colombard than in Carlos and Noble, and similar ($P = 0.05$) between Carlos and Noble. Populations in French Colombard increased over time, whereas populations in the muscadine cultivars increased, then decreased (Fig. 2). The FC strain was detected 10 cm above the inoculation point in French Colombard after 2 wk, and in Carlos and Noble after 4 wk (Fig 2A). The patterns of multiplication at 20 cm were similar (Fig. 2B).

The FC strain was first isolated at 50 cm from all cultivars after 4 wk (Fig. 2C). The bacterium could not be reisolated at 50 cm in Noble after 8 wk. Multiplication of *X. fastidiosa* over time at 50 cm in Noble and French Colombard was described by an equation containing quadratic, and linear and quadratic components, respectively.

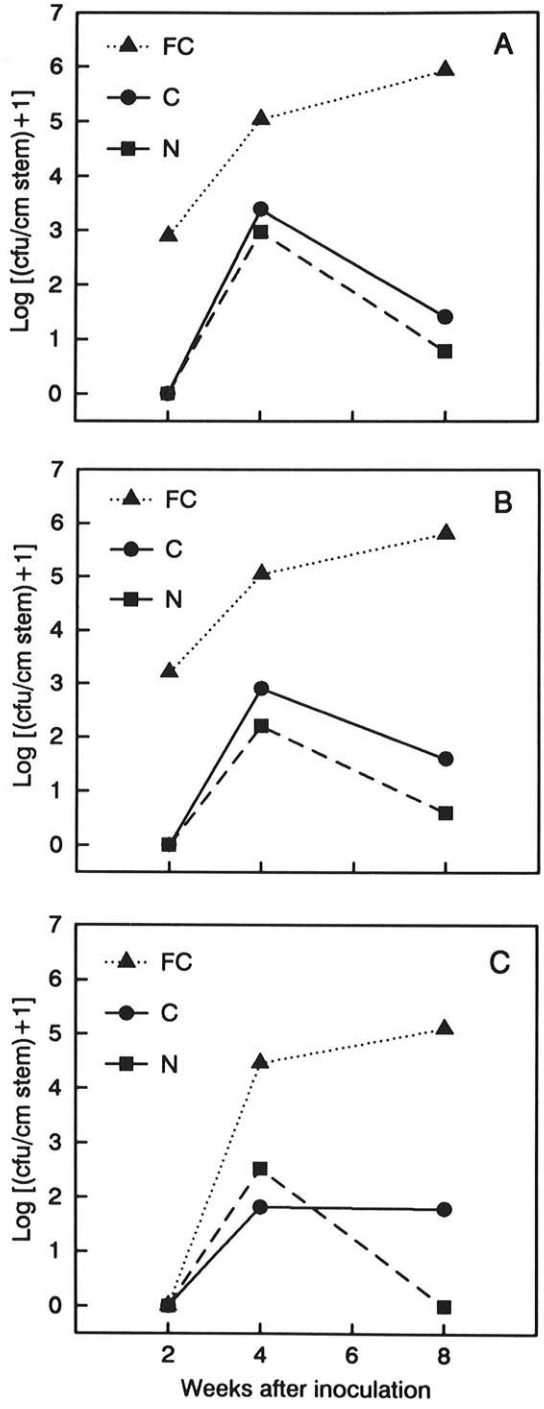

Fig. 2. Multiplication and translocation of the FC strain of *Xylella fastidiosa* in the susceptible cultivar French Colombard (FC), tolerant cultivar Carlos (C), and resistant cultivar Noble (N) **A,** 10 cm; **B,** 20 cm; and **C,** 50 cm above the point of inoculation in the stem. Test 1.

The pattern of multiplication of the C strain in French Colombard was similar to that of the FC strain, but populations were slightly lower. When averaged over time and plant parts, populations of the C strain were significantly higher in French Colombard than in Carlos and Noble, and similar ($P = 0.05$) between Carlos and Noble. The C strain was detected throughout French Colombard after 2 wk. Populations of 10^4 cfu/cm of stem were recovered throughout French Colombard after 8 wk. The C strain was recovered only at 10 cm in Carlos and Noble. Populations in Carlos were 10^1 cfu/cm of stem throughout the experiment. The bacterium was first detected at 10 cm in Noble after 8 wk.

The overall time × cultivar interaction was significant. The linear increase in the population of the FC and C strains over time in French Colombard was much greater than the change in populations in Carlos and Noble. When averaged over cultivar, time, and plant parts, populations of the FC strain were greater ($P = 0.05$) than populations of the C strain. The FC strain appears to be more aggressive in inoculated plants as well as in culture.

Approximately 3×10^6 bacterial cells of either strain were inoculated into each stem of 5-wk-old plants. Immediately after inoculation with the FC strain, 10^4 and 10^5 cfu/cm of stem were recovered from sections adjacent to the inoculation point in Carlos, and French Colombard and Noble, respectively. In the second experiment, populations of the FC strain in French Colombard were significantly higher than in Carlos and Noble when averaged over time and plant parts, whereas populations in Carlos were significantly higher than in Noble. The FC strain was detected at 10 cm in all cultivars immediately after inoculation (Fig. 3A). Populations at 10 cm increased in French Colombard over time. Populations in Carlos and Noble decreased before increasing. The bacterium was not detected above 10 cm in Noble.

The FC strain was reisolated at 20 cm in French Colombard and Carlos after 4 and 8 wk, respectively. Growth of the bacterium at 20 cm in French Colombard was described by a linear equation. When averaged over time and cultivar, populations of the FC strain at 50 cm were significantly lower than at 10 and 20 cm (Fig. 3C). Samples at 50 cm were not available immediately after inoculation, since the plants were not tall enough.

Immediately after inoculation with the C strain, 10^3 and 10^4 cfu/cm of stem were recovered from sections adjacent to the inoculation point in Carlos and Noble, and French Colombard, respectively. The bacterium was not recovered at 10 cm in French Colombard immediately after inoculation; however, it was detected at low levels (10^1 cfu/cm of stem) in Carlos and Noble. The C strain was not recovered at 20 cm immediately after inoculation from any cultivar and was not reisolated from Carlos or Noble thereafter. The pattern of multiplication of the C strain in French Colombard was similar to that illustrated for the FC strain.

Isolations from leaves of inoculated 5-wk-old plants revealed that the FC strain colonized the first one to two leaves, four to eight leaves, and seven to 15 leaves of French Colombard 2, 4, and 8 wk after inoculation, respectively. Leaves near the inoculation point as well as higher on the plant exhibited chlorosis or marginal leaf burn approximately 8 wk after inoculation. In the second experiment, the FC strain was not reisolated from petioles of Carlos plants, yet a few leaves exhibited symptoms after 8 wk. In contrast, several leaves from Carlos plants in the first experiment were colonized but asymptomatic. In the second experiment, the FC strain was reisolated from the first few leaves of Noble plants after 8 wk, but symptoms were not observed. The C and FC strains colonized French Colombard to a similar extent. The C strain was not recovered from Carlos leaves in either experiment but was detected in leaves from Noble from both experiments after 8 wk. Symptoms were never observed in Carlos or Noble inoculated with the C strain.

DISCUSSION

Symptom expression in response to infection by *X. fastidiosa* is influenced by time of bacterial accumulation and threshold

populations (10,13,14). Hopkins (13) reported that the most rapid increase in populations of *X. fastidiosa* in naturally infected petioles, leaf veins, and stems occurred 2 mo later in Carlos and Welder muscadine vines than in Schuyler bunch grapevines. Hopkins concluded that the delay in symptom development in the muscadine vines may have been due to the delay in accumulation of the bacterium. In addition, bacterial populations of 10^6 cfu/cm of leaf vein have been correlated with symptom expression in infected grapevines (10,14).

We observed similar effects of time of bacterial accumulation and populations on symptom expression in artificially inoculated susceptible, tolerant, and resistant grapevines. Symptom expression was more severe in petiole-inoculated French Colombard than in Carlos and Noble. Maximum populations were attained later in Noble and Carlos than in French Colombard. In addition, average populations of the FC strain were approximately 10 times greater in inoculated petioles of French Colombard than in Carlos and Noble, and 100–1,000 times greater in stems of French Colombard than in Carlos and Noble. Populations correlated with symptom expression were observed in leaf veins of French Colombard and Carlos 8 wk after petiole inoculation.

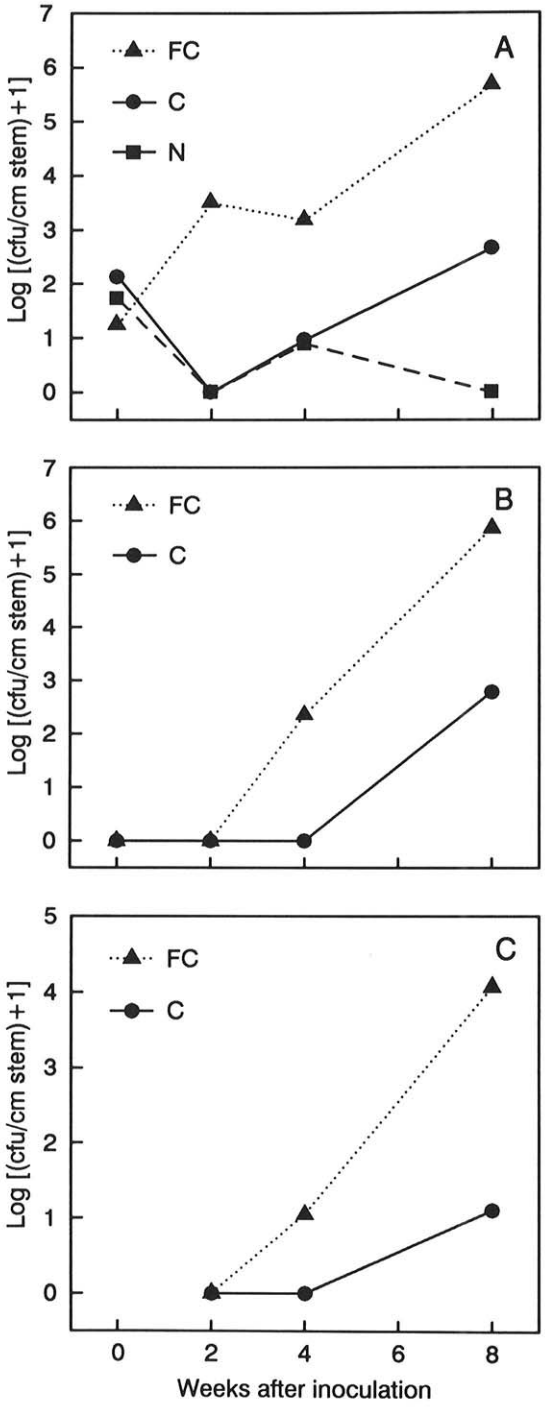

Fig. 3. Multiplication and translocation of the FC strain of *Xylella fastidiosa* in the susceptible cultivar French Colombard (FC), tolerant cultivar Carlos (C), and resistant cultivar Noble (N) **A,** 10 cm; **B,** 20 cm; and **C,** 50 cm above the point of inoculation in the stem. Data from zero time samples were generated apart from the whole experiment. Test 2.

Hopkins (10,14) indicated that a threshold of 10^6 cfu/cm of petiole was required before the bacterium was detected in the leaf vein of petiole-inoculated Carignane grapevines. As a result, the bacterium was detected in the leaf vein 2–4 wk later than in the petiole. This trend was not observed in our studies. *X. fastidiosa* was detected in leaf veins of all cultivars despite populations in the petiole. The difference between the two studies may be due to the distance between the inoculation point in the petiole and the sampling point in the leaf vein.

The apparent decrease in initial populations of *X. fastidiosa* in inoculated petioles has been reported (10,14). We also observed an apparent initial decrease in populations in inoculated stems of Carlos and Noble plants. The period between inoculation and increase in populations probably represents establishment of the bacterium in the host.

Structural barriers, including gums, tyloses, and pectins, have been proposed to prevent the spread of bacteria in muscadine vines and, therefore, prevent symptom expression (15,18). Though multiplication was inhibited in the petioles of Carlos and Noble, movement into the leaf vein was not. About 8×10^6 cells were inoculated into each of five petioles per plant. This inoculum density may have been too high, resulting in a breakdown in resistance in Carlos and Noble. In addition, this inoculum concentration may not be representative of what is introduced naturally. In support of this, we have noted that populations in the petioles of all cultivars inoculated with 10^1 cfu appeared to decrease over time (S. M. Fry, *unpublished*). Lower inoculum levels therefore may lead to the determination of a threshold population, which better differentiates susceptible and resistant grape cultivars based on multiplication studies.

The pattern of translocation of the FC and C strains in stems of French Colombard, Carlos, and Noble in the second experiment was comparable to that of the virulent, weakly virulent, and avirulent strains, respectively, in stem-inoculated Carignane grapevines (10). The virulent strain rapidly colonized the plant up to the 17th internode 8 wk after inoculation. The weakly virulent strain was translocated similarly, but after 5 wk remained around the 10th internode. The avirulent strain was recovered from the inoculated internode at 1 wk, but not thereafter. In our experiments, populations of the FC strain increased rapidly in the susceptible cultivar, and were detected at high levels at 50 cm (13th internode) 8 wk after inoculation. The bacterium was translocated similarly in the tolerant cultivar, but was detected at much lower levels. *X. fastidiosa* was not detected above 10 cm (fourth internode) in the resistant grapevine stems in the second experiment. Likewise, the C strain colonized the susceptible cultivar up to 50 cm but was recovered at 20 cm or not at all in the tolerant and resistant cultivars.

Populations of the FC strain in stems of Carlos and Noble were higher in the older plants used in the first experiment than in the younger plants inoculated in the second. In addition, the more virulent FC strain was delayed in its multiplication and translocation in the younger plants than in the older plants. The bacterium appears to multiply better in older inoculated grape tissues than in younger ones (9). The older plants may have a greater transpiration flow and are able to transport the bacterium further up the stem compared with younger plants.

In general, translocation of the bacterium into petioles was indicative of the relative position of the bacterium in the stem. Hopkins (10) observed that the position of the uppermost colonized leaf represented the location of the bacterium in stems of Carignane grapevines. Xylem vessels are larger in the stem, which would allow a stronger flow of nutrients up the plant as

opposed to flow directly into the leaf veins. The pattern of distribution in French Colombard appeared to be more sequential than in Carlos and Noble. Barriers in the stem may cause bacterial cells to be randomly diverted into leaves in Carlos and Noble. In French Colombard, however, populations were so high that bacterial cells colonized all leaves. In the greenhouse, symptomatic leaves on French Colombard and Carlos were either scattered on the vine or appeared to progress sequentially up the vine. Symptoms have been observed on Noble in the greenhouse but not in the field. Conditions in the greenhouse may favor breakdown of resistance mechanisms in Noble.

Inhibition of the multiplication and translocation of strains of *X. fastidiosa* in tolerant and resistant grape cultivars may be attributed to structural barriers (15,18), inhibitory chemicals or substances, or aging of the bacterium, which results in a loss of virulence (15). Differences in vascular anatomy also exist between *V. vinifera* and *V. rotundifolia* (21; S. M. Fry, *unpublished*). Several cases have been described where resistance is related to shorter and smaller vessels (1,4,19). The next step in characterizing the resistance mechanism of muscadine vines to Pierce's disease is to examine the role of vascular occlusions in detail.

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