

Association of *Xylella fastidiosa* with Yield Loss and Altered Fruit Quality in a Naturally Infected Rabbit-eye Blueberry Orchard

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Abstract. *Xylella fastidiosa* Wells et al. causes disease in a number of plants in the southeastern United States, including southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids), but little was known concerning its potential impact in rabbiteye blueberry (*Vaccinium virgatum* Aiton syn. *Vaccinium ashei* Reade). In a naturally infected orchard in Louisiana, mean yields of *X. fastidiosa*-positive plants were 55% and 62% less than those of *X. fastidiosa*-negative plants in 2013 and 2014, respectively. Average berry weight was also lower in *X. fastidiosa*-positive plants. Within 3 years of testing positive for *X. fastidiosa*, four of nine *X. fastidiosa*-positive plants appeared dead. However, plants that were *X. fastidiosa*-negative in 2013 remained so until 2015, indicating that the bacterium did not spread rapidly in this established orchard during this time. Other factors, including soil chemistry variables, *Phytophthora cinnamomi*, ring nematode, and ringspot symptoms, were also investigated to determine if one of these might predispose plants to infection with *X. fastidiosa* or be partly responsible for observed yield loss. In most cases, interactions were not found, but associations with soil Cu and Zn suggest a need for further research on whether these elements predispose rabbiteye blueberry to *X. fastidiosa* infection and thereby contribute to yield losses. Researchers, extension workers, and growers should be aware of *X. fastidiosa* as a potential yield- and survival-impacting factor in rabbiteye blueberry.

Diseases incited by *X. fastidiosa* are especially prevalent in the southeastern United States (Hopkins and Purcell, 2002). Proposed reasons for the bacterium's success in this region include a long growing season and warm nights (Hopkins and Purcell, 2002). The feeding habits of the glassy-winged

sharpshooter [*Homalodisca vitripennis* (Germar)], a vector of *X. fastidiosa* that is common in the southeastern United States, likely contribute as well (Hopkins and Purcell, 2002). Several *X. fastidiosa*-caused diseases found in the Southeast include Pierce's disease, phony peach disease, pecan bacterial leaf scorch, and oleander leaf scorch (Hopkins and Purcell, 2002; Sanderlin and Heyderich-Alger, 2000). Since 2004, researchers in Georgia and Florida have confirmed *X. fastidiosa* as the cause of a leaf scorch disease of southern highbush blueberries (*Vaccinium corymbosum* interspecific hybrids) (Chang et al., 2009; Harmon and Hopkins, 2009). Inoculation of rabbiteye blueberry (*V. virgatum* syn. *V. ashei*) plants with *X. fastidiosa* resulted in no detected infection in 'Premier' and in local colonization of two of six 'Powderblue' plants. In infected 'Powderblue' plants, symptoms did not progress past the inoculated stem within a 14-month period (Chang et al., 2009). However, *X. fastidiosa* has been detected in samples from rabbiteye blueberry plants at farms in Louisiana that have experienced noticeable plant decline and death (Ferguson, 2016; R. Sanderlin,

personal communication). Rabbit-eye blueberry comprises the majority of the ≈3000 acres of blueberry plantings in Louisiana and Mississippi (E. Stafne, personal communication; USDA-NASS, 2014).

Bacterial leaf scorch symptoms described on southern highbush plants include marginal leaf necrosis, thin twigs on new growth, leaf drop, stem yellowing, and plant death (Brannen et al., 2016). In FL 86-19 southern highbush plants, symptoms progressed from marginal necrosis to yellow stems and leaf loss, on both inoculated and noninoculated branches (Chang et al., 2009). Defoliation combined with yellowed stems is characteristic of blueberry plants with bacterial leaf scorch, and these symptoms help differentiate between this disease and other biotic and abiotic causes of plant decline (Brannen et al., 2016). Death of southern highbush blueberry plants typically occurs a year or more after plants first show symptoms (Brannen et al., 2016).

Rabbiteye blueberry plant symptoms noted in Louisiana and suspected of being associated with *X. fastidiosa* include plant death, shoot dieback, marginal necrosis, leaf reddening, and extensive defoliation combined with yellow stems (D. Ferrin and R. Sanderlin, personal communication; M.H. Ferguson, unpublished data), similar to symptoms described in Georgia on southern highbush blueberry (Brannen et al., 2016).

There are other factors that may cause decline in rabbiteye blueberry plants. *Phytophthora cinnamomi* Rands incites root rot in blueberry, which can result in yellowing of leaves and leaf loss in rabbiteye cultivars (Milholland, 1995). Ring nematode (*Mesocriconema ornatum*) has been observed in association with plants symptomatic for blueberry replant disease, although experiments did not reveal significant differences in rabbiteye blueberry plant growth after inoculation with them (Jagdale et al., 2013). Several viruses cause ringspot or ring blotch symptoms on blueberry plants (Martin et al., 2012). One of these, blueberry red ringspot virus, has been detected in rabbiteye blueberry (Polashock et al., 2009). It is important to consider such alternative causes of symptoms when assessing the possible impact of *X. fastidiosa* in rabbiteye blueberry.

In some crops, such as susceptible cultivars of *Vitis vinifera* L. grape, death of plants infected with *X. fastidiosa* takes place quickly enough that the proportion of yield reduction that individual infected plants experience, while they remain alive, is likely of minor interest (Hopkins and Purcell, 2002). Southern highbush blueberry plants with bacterial leaf scorch can also die soon after symptoms are observed (Brannen et al., 2016). However, in other crops, such as peach and citrus, infected plants can decline slowly with prolonged reduced yield and altered fruit quality (Gonçalves et al., 2011; Hartung et al., 1994; Hutchins, 1933).

There are crops for which yield loss in the presence and absence of *X. fastidiosa* has been compared. In almond [*Prunus dulcis*

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(Mill.) D.A. Webb] in California, symptomatic/infected trees of one cultivar had estimated yields 37% or 40% lower than those of trees lacking symptoms of almond leaf scorch disease (Sisterson et al., 2008, 2012). For a second cultivar, average estimated yields of symptomatic/infected trees were 17% or 19% lower than those of asymptomatic trees (Sisterson et al., 2008, 2012). In 13 of a total of 18-year orchard-cultivar combinations, the estimated yields of symptomatic trees were significantly lower than those of asymptomatic trees (Sisterson et al., 2008, 2012).

In pecan [*Carya illinoensis* (Wangenh.) K. Koch] in Louisiana, kernel weight was significantly lower (13% to 18%) in nuts from *X. fastidiosa*-infected than noninfected trees each year for 3 years, and total nut weight was significantly lower in 2 years and marginally lower in a third year (Sanderlin and Heyderich-Alger, 2003). In Brazil, sweet orange (*Citrus* sp.) plants inoculated with *X. fastidiosa* before planting yielded \approx 20% less than ones that were not inoculated before planting, over an 8-year period (Gonçalves et al., 2011).

How quickly *X. fastidiosa* spreads among plants in an orchard in which infected plants are found is another factor pertinent to management decisions. The rate of spread of *X. fastidiosa* within orchards would affect the economics of removing infected plants (Sisterson et al., 2008). In one pecan study, none of 10 originally noninfected plants of a susceptible cultivar Cape Fear become infected during a 3-year study period (Sanderlin and Heyderich-Alger, 2003). In an orchard with younger pecan trees, the infection incidence in the same susceptible cultivar increased from 5% to 64% over a period of 6 years, and rates of infection in less susceptible cultivars increased from 0 to 2.6 and 10.5% during the same period (Sanderlin et al., 2009). However, an analysis of spread in the latter orchard did not provide conclusive evidence that locations of infected trees impacted the locations of future infections (Li et al., 2011; Sanderlin et al., 2009). Sisterson et al. (2012) found similar results in almond, contributing to a conclusion that it may not necessarily be beneficial to remove infected plants from orchards that will be replanted soon. However, in an area of California where the glassy-winged sharpshooter was present, Park et al. (2006) found that grapevines infected with Pierce's disease were frequently close to dead vines and/or locations from which older vines had been removed.

Even in crops in which *X. fastidiosa* infection does not result in rapid plant death, death may occur at a higher rate in infected than in noninfected plants. In almond, 9% of trees tested and confirmed to be *X. fastidiosa*-positive in the first years of 6- to 7-year surveys of two orchards were found to be dead in the final years of the surveys, and a higher proportion would likely have been dead if trees had not been removed (Sisterson et al., 2012). Only 1% of initially asymptomatic trees were dead at the ends of the respective surveys (Sisterson et al., 2012).

Objectives of this study included determining whether there was an association between *X. fastidiosa* infection and reduced yield and/or fruit quality in rabbiteye blueberry and to what extent *X. fastidiosa* spread within a rabbiteye orchard. Soil chemistry variables, *P. cinnamomi*, ring nematode, and ringspot symptom status were also investigated to determine if they might be related to infection with *X. fastidiosa*.

Materials and Methods

Selection of plants for yield comparison and detection of X. fastidiosa in yield study plants. Because *X. fastidiosa* is a slow-growing organism, and it was not known whether an infection could be artificially established or how long it would take for an infection to become established and result in yield loss, this study used naturally infected plants. Fifteen blocks of three plants were selected from within rows of 'Tifblue' rabbiteye blueberry plants at a commercial farm in East Feliciana Parish, LA, where *X. fastidiosa* had been detected. Within each block, the three plants were separated by a plant, a place where a plant was formerly located, or both. Plants in each block were roughly uniform in size at the beginning of the study. Five plants were removed from the study because of the determination, based on foliar appearance, time of bloom, or both, that they were a cultivar other than 'Tifblue', leaving a total of 40 plants in the study. The orchard was originally planted in the late 1970s or early 1980s, making the orchard \approx 30 to 35 years old at the time of harvest. The orchard was \approx 1 ha in size, unirrigated, managed using organic practices, and harvested by hand. The soil was a Tangi silt loam, a fine-silty mixed semiactive thermic Typic Fragiudult (USDA-NRCS, 2014, 2016).

In an initial effort to determine which study plants were infected, real-time polymerase chain reaction (PCR) was used to test stem sap for *X. fastidiosa*. One cane was cut from the base of each plant in Feb. 2013. After returning from the field, fresh cuts were made at the base of each stem and roughly 15–20 cm from the location of the first cut, using pruning loppers wiped between cuts with 70% ethanol (EtOH), based on a protocol described by Holland et al. (2014). A vise was used to squeeze sap from the resulting stem piece, and sap was pipetted into autoclaved microcentrifuge tubes. Sap was held for 2 d at \approx 4 °C and then at -70 °C until DNA was extracted from 50 μ L of sap, or less, if 50 μ L was not available, using a PowerPlant Pro DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA).

Real-time PCR was performed using Applied Biosystems Power SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA) and EFTu_3 forward and reverse primers (forward: 5'-TGAGGTG-GAAATTGTTGGCATT-3'; reverse: 5'-AG-CCTGACCTTGATCCAATAA-3'; Holland et al., 2014). An ABI Prism 7000 Sequence Detection System (Applied Biosystems,

Foster City, CA) thermocycler and the following program was used: 10 min at 95 °C; 40 cycles of 10 s at 95 °C and 1 min at 60 °C; and a dissociation step of 15 s at 95 °C, 20 s at 60 °C, and 15 s at 95 °C (Holland et al., 2014). One well (technical replicate) per plant was tested. Positive samples had cycle threshold (C_t) values of 35 or less and melting temperatures between 77.2 and 78.3 °C. Until a positive sample from blueberry was detected, an extract of *X. fastidiosa* from grape (A-08; Melanson et al., 2012) was used as a positive control.

Leaf samples were then taken from plants in late July or early Aug. 2013. Several leafy shoots of roughly 15 cm in length were cut from various locations on each plant. The samples were held at \approx 5.6 °C until shortly before petioles and lower midribs were cut from leaves using a scalpel blade, which was flamed between samples. A commercial enzyme-linked immunosorbent assay (ELISA) kit using the double antibody sandwich method with polyclonal antibodies (Agdia, Inc., Elkhart, IN) was used to detect *X. fastidiosa* in 0.3–0.5 g of petiole and midrib tissue of the leaves. The samples were tested in duplicate. Absorbance was measured at 630 nm on a Microplate Autoreader EL311 (BioTek Instruments, Inc., Winooski, VT). An absorbance of 0.100 or higher, relative to a buffer control and accompanied by color change, was used as the criterion for a positive sample, similar to the threshold (0.100 at 620 nm) used by Chang et al. (2009).

Additional foliar samples (5–6 shoots/plant) were taken from all but two plants, which lacked foliage, in September of 2013. After excision with a flamed scalpel blade, petioles and lower midribs were held at -20 °C until they were ground in liquid nitrogen using mortars and pestles. DNA was extracted from 0.04 to 0.05 g of tissue using the PowerPlant Pro DNA Isolation Kit. Real-time PCR was performed as described earlier for detection of *X. fastidiosa* in sap. The samples were tested in duplicate (two technical replicates).

Annual sampling of the 40 plants in the yield study was repeated in Aug. 2014 and Sept. 2015, to determine if the infection incidence had changed, in comparison with that found in 2013. Each time, five to seven leafy shoots were collected. After returning from the field, the samples were held at \approx 5.6 °C until shortly before petioles and lower midribs were excised. Some petioles and midribs from each plant were used for testing by ELISA, as previously described, whereas others were held at -20 °C for DNA extraction. Tissue for DNA extraction was ground in liquid nitrogen. DNA was extracted from 0.05 to 0.06 g of tissue using a cetyltrimethylammonium bromide (CTAB) protocol based on that of Li et al. (2008). DNA was dissolved in sterile nuclease-free water and held at -70 °C. Real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), along with the previously described

EFTu_3 primers. Positive samples still had C_t values of 35 or less, but because a different master mix was used, the range for melting temperatures of positive samples was between 81.2 and 82.4 °C. The samples were retested if there was a conflict between wells or if a sample tested positive but the amplification curve appeared atypical.

Yield and average berry weight. Fruit was harvested by hand once per week between 13 June and 5 July in 2013 and between 12 June and 10 July in 2014. Each week's yield was weighed, and weekly yields were summed for each sample, to get total yield per plant for each season. At the time of each harvest, a subsample of berries was set aside for collection of average berry weight data and stored at ≈ 5.6 °C. Twenty-five berries, or fewer if 25 berries were not available, were weighed, and mean berry weight was recorded for each plant after each harvest date. The overall mean of averages from each harvest date was used as the average berry weight for the season, for each plant. If there were dates when a plant had fewer than 25 berries, the contribution of that week's average to the seasonal average was reduced proportionally so that the weights from those dates did not disproportionately affect the seasonal average.

Soluble solids and titratable acidity. During one week of harvest in June 2014, a subsample of berries from each plant was set aside for measurements of soluble solids concentration (SSC) and titratable acidity (TA). The berries were held at -70 °C until the day of sample preparation. To prepare samples for measurements, berries were thawed, homogenized in a blender, and centrifuged in three to four 50-mL tubes per sample at 484 g for 15 min. The supernatant was filtered through a double layer of cheesecloth and centrifuged a second time at 830 g for 15 min, in 15 mL tubes.

Ten milliliters (or less, if 10 mL was not available) of the resulting juice was used for TA analysis, and the rest was held at ≈ 4 °C, or at -20 °C for longer periods, for SSC measurement. SSC was measured using a Bellingham and Stanley RFM 80 Refractometer (Kent, UK). To measure TA, 0.1 N NaOH was added while stirring the blueberry juice, until the pH measured \geq pH 8.1. The volume of 0.1 N NaOH used to raise the pH to ≥ 8.1 was measured. This volume was multiplied by 0.1 (to account for the normality of the NaOH solution) and 0.059 [to account for the formula weight and number of dissociable hydrogen ions of a succinic acid molecule (Ehlenfeldt et al., 1994), as well as a conversion from milliliters to liters] and divided by the volume of juice used (in milliliters), to get a TA value. Calculations were based on the properties of succinic acid because Ehlenfeldt et al. (1994) found this acid to exist at the highest concentration among measured acids (including citric, malic, and quinic, along with succinic) in fruit from rabbiteye cultivars in general and from 'Tifblue', specifically.

Soil sampling. Six soil cores were taken under the canopy of each bush, to a depth of

≈ 15 to 20 cm, in Nov. 2013. Samples for each plant were mixed and placed in a plastic bag, which was then sealed. The bags were stored at 14 °C. Soil was analyzed for plant nutrient (Ca, Cu, K, Mg, P, S, and Zn) and Na content, along with pH, by the Louisiana State University Agricultural Center Soil Testing and Plant Analysis Laboratory.

Nematode populations. A 100-mL portion of the soil collected in Nov. 2013 was assayed for ring nematodes. Nematodes were extracted using the semiautomatic elutriation and sugar centrifugation method (Byrd et al., 1976; Jenkins, 1964). Using 100 mL of soil, the nematodes were trapped in a 400-mesh sieve (38- μ m openings). Sieve contents were stored in water at ≈ 4 °C for at least 4 h and then added with tap water to a ≈ 40 mL centrifuge tube. The contents were centrifuged for 5 min at ≈ 350 g. The supernatant was poured off, and a solution containing 454 g·L⁻¹ of sucrose was added to the tubes and stirred to mix with the contents. This mixture was centrifuged for 1 min at ≈ 350 g and then poured through a 400-mesh sieve. The contents were washed in tap water and stored at ≈ 4 °C until nematodes were identified and counted by D. Xavier-Mis at the Louisiana State University Agricultural Center Nematode Advisory Service. The counts were converted into populations of nematodes per 500 cm³.

Phytophthora assay. In Jan. 2015, four soil cores were collected from within 1 m of the base of each plant that had been part of the yield study, to depths of ≈ 15 to 20 cm. The samples were placed in plastic bags, which were sealed and stored at ≈ 5.6 °C, then set out at room temperature for 2 or 6 d before the first and second rounds of baiting, respectively. Each time, using a protocol based on that in Ferguson and Jeffers (1999), ≈ 100 cm³ of soil from around each plant was placed in a 13 cm \times 9 cm \times 6 cm food storage container, and 200 mL of distilled, deionized water was poured over it. Eight disks cut from *Camellia japonica* L. leaves using a single-hole punch were floated on top of the water in each container. Leaves had been dipped in 70% EtOH and allowed to dry, and the hole punch used to cut out disks had been dipped in 70% EtOH and flamed.

Three days after each round of baiting began, baits were removed from the containers, using sterilized forceps that were dipped in 70% EtOH and flamed between samples. For each sample, five disks, from among those that had not sunk, were placed on a clean paper towel and blotted dry (Ferguson and Jeffers, 1999). Forceps were used to insert the five disks into a selective medium. PARPH (pimaricin-ampicillin-rifamycin-pentachloronitrobenzohymexazol) medium was used after the first round of baiting, whereas pimaricin was left out the second time, so that the medium was effectively ARPH (Ferguson and Jeffers, 1999; Jeffers and Martin, 1986). Antibiotic concentrations of the media were those in Ferguson and Jeffers (1999), but cornmeal agar was used and V8 was omitted, as in Jeffers and Martin (1986).

Thirteen to 14 d after inserting leaf disks into PARPH after the first round of baiting, medium from new plates of PARPH was overlaid on the original plates. There was no overlay step for plates from the second round of baiting. One to three pieces of agar (≈ 0.2 cm²) containing hyphae were transferred to PARPH or ARPH media from the overlaid plates (from first round) ≈ 6.5 weeks after overlay or from original plates (from second round) ≈ 2 weeks after insertion of disks. Eleven days after the previous transfers, one to three pieces of agar (≈ 0.2 cm²) containing hyphae were transferred to clarified V8 agar (cV8A-10%) (Jeffers, 2007).

Between 13 and 15 d after transfer to cV8A-10%, hyphae from ≈ 2 cm² were scraped from the plates and placed in 1.5 mL microcentrifuge tubes with 500 μ L of 10 mM Tris-HCl (pH 7.5), based on the method of Kong et al. (2003). The tubes were placed in boiling water for 20 min and then centrifuged for 3 min. The supernatant (200 μ L) was transferred to new microcentrifuge tubes, and these were stored at -20 °C.

PCR using Lpv2 (forward: 5'-ACTGGGTC-GACAACGACTGCTTG-3'; reverse: 5'-GTCC-AAACCGACTCTTGCTCGATG-3') and Lpv3 primers (forward: 5'-GTGCAGACTGTC-GATGTG-3'; reverse: 5'-GAACCACAA-CAGGCACGT-3') was performed following the protocols outlined in Kong et al. (2003). Because DNA concentrations in the extracts were low, the volume of template in the PCR reaction was successively increased from 2 to 4 to 8 to 16.9 μ L, if no product was found for a given sample at a lower concentration. Because a positive result with both primers is necessary to confirm a sample as *P. cinnamomi*, the samples were run with Lpv3 primers at increased template concentrations only if positive results were obtained with the Lpv2 primers.

Ringspots. Leaves of yield study plants were observed in Aug. 2014 and Sept. 2015 for the presence of ringspots, to determine if a ringspot-causing virus was likely to be present.

Data analysis. Data were analyzed using SAS software (SAS 9.4; SAS Institute Inc., Cary, NC). Analyses of variance (ANOVAs) were performed using PROC MIXED and the Kenward-Roger df method. Repeated measures analyses were used when 2 years of data were analyzed together. When only 1 year of data was analyzed at one time, a likelihood ratio chi-square test was done in Microsoft Excel (Microsoft Corp., Redmond, WA) using -2 Res Log Likelihood values and the df of the covariance structures, to determine whether a better fit resulted from using combined or separate variances by plant infection status. If there was not a significant difference in the fits, the simpler model was used. Likewise, when 2 years of data were analyzed together, likelihood ratio chi-square tests were done to determine if a better fit resulted from using combined or separate variances by plant infection status or by year and if an autoregressive covariance structure resulted in a better fit than the default covariance

structure. In all cases, models with non-normal residuals distributions, according to a Shapiro–Wilk test ($\alpha = 0.05$), were not used. Ranked rather than raw data were used for SSC analysis, as residuals were non-normal when raw data were used. Once a satisfactory model for yield was selected, ANOVAs were also performed with *P. cinnamomi* status, 2014 ringspot status, ring nematode population, and soil chemistry variables (Ca, Cu, K, Mg, P, S, Zn, Na, or pH), individually, as covariables.

To determine whether the presence of *P. cinnamomi*, ring nematodes, or a ringspot-causing virus possibly predisposed plants to colonization with *X. fastidiosa*, logistic analyses were performed using PROC GLIMMIX, to account for the arrangement of plants in blocks. A binary distribution was specified, and the Kenward–Roger df method was used. It was also considered desirable to determine if any soil chemistry variables (nutrient or Na concentrations, or pH) might be predisposing plants to *X. fastidiosa* colonization, but because there were nine soil chemistry variables, these were subjected to a principal components analysis using PROC FACTOR in SAS. Once the top three factors were determined, the factor scores were also used as independent variables in a logistic analysis of infection incidence. For all ANOVAs and logistic analyses, reported *P* values are based on partial (type III) sums of squares.

Results

Only one plant of 40 in the commercial orchard in East Feliciana Parish was positive for *X. fastidiosa* based on sap from stems collected in Feb. 2013. However, nine plants, including the one that previously tested positive, tested positive by ELISA of petiole/midrib samples collected in late July and early August of 2013. None of these tested positive by real-time PCR when petiole/midrib tissue was collected in Sept. 2013, and DNA was extracted using the PowerPlant Pro DNA Isolation Kit; however, all but one of these nine plants later tested positive by real-time PCR in 2014 and/or 2015, when the CTAB extraction method was used to extract DNA from petiole/midrib tissue.

In Aug. 2014, eight plants still had a sufficient number of leaves for testing by both ELISA and real-time PCR, and samples from five of these plants tested positive by ELISA, while samples from six tested positive by real-time PCR. In 2015, outcomes of ELISA and real-time PCR tests were the same, with leaves from four of a total of six undefoliated plants testing positive by both methods. Plants that tested negative for *X. fastidiosa* by ELISA in 2013 continued to test negative by both ELISA and real-time PCR in 2014 and 2015.

By Apr. 2016, no foliage was present on four of the nine plants from the yield study in which *X. fastidiosa* had been detected. The yield study plants that consistently tested negative for *X. fastidiosa* had new growth at this time. Besides death of the aboveground

parts of plants, symptoms noted in association with infected plants included foliar reddening and chlorosis and, less commonly, marginal necrosis (Fig. 1). However, the incidence of foliar symptoms was not subjected to statistical analysis, and obvious symptoms were not always observed.

The nine plants that tested positive for *X. fastidiosa* yielded significantly less than the 31 that did not, with mean yields of *X. fastidiosa*-positive plants being 55% and 62% less than *X. fastidiosa*-negative plants in 2013 and 2014, respectively (Table 1). There was a significant ($P = 0.01$) infection status by year interaction with respect to yield, but observation of mean values for each year revealed that the trends were the same in both years (i.e., positive plants yielded less than half of what negative plants did), so yield data from both years were analyzed together. The difference between average berry weights was also significant (Table 1).

In berries harvested during 1 week in 2014, the SSC was higher in fruit from plants in which *X. fastidiosa* had been detected than in fruit from plants in which it had not been detected (Table 2). No significant difference in TA was found (Table 2).

Phytophthora cinnamomi was detected in soil from around 56% of *X. fastidiosa*-positive plants and 23% of *X. fastidiosa*-negative plants. The logistic regression analysis to check for a possible predisposing effect of *P. cinnamomi* on *X. fastidiosa* infection was not significant ($P = 0.138$). Ringspots were observed on 33% of *X. fastidiosa*-positive plants and 65% of *X. fastidiosa*-negative plants in Aug. 2014. The *P* value (0.194) resulting from logistic regression did not suggest an association between ringspot symptoms and *X. fastidiosa* infection status. Ringspots were not observed in Sept. 2015.

Mean population estimates of ring nematodes per 500 cm³ soil were 572 and 419 for *X. fastidiosa*-positive and negative plants,

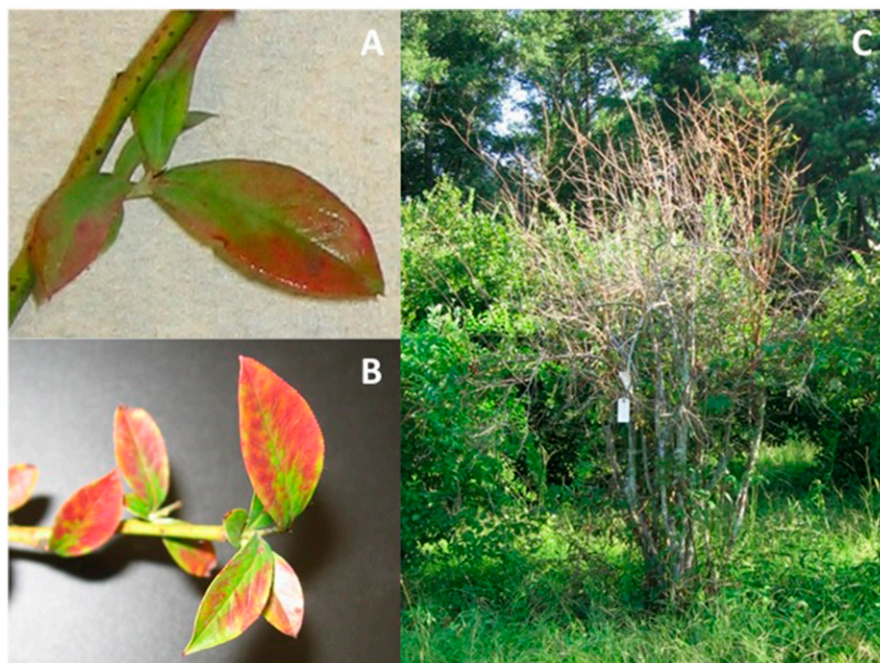


Fig. 1. Symptoms associated with *Xylella fastidiosa*-positive plants include chlorosis and reddening of foliage (A and B) and plant death (C).

Table 1. Yield of ripe blueberries per plant and average berry weight of ‘Tifblue’ rabbiteye blueberry plants in which *Xylella fastidiosa* was and was not detected.

<i>X. fastidiosa</i> status ^a	Yr	Yield/plant (kg)	Avg berry wt (g)
Positive ^y	2013	4.0 ± 1.4 ^x	1.2 ± 0.08
	2014	6.3 ± 2.3	1.3 ± 0.07
Negative ^w	2013	8.9 ± 0.6	1.4 ± 0.01
	2014	16.7 ± 0.9	1.4 ± 0.01
<i>P</i> values			
<i>X. fastidiosa</i> status (Xf)		<0.0001	0.0507
Year		0.0002	0.2531
Xf × year		0.0124	0.0933

^aBased on enzyme-linked immunosorbent assays of leaf petioles/midribs in July or August of 2013.

^y*N* = 9 plants in both years for yield/plant and in 2013 for average berry weight. *N* = 6 plants in 2014 for average berry weight.

^xMean ± SE.

^w*N* = 31 plants in both years, for both variables.

respectively. Results of logistic regression analysis ($P = 0.316$) did not suggest that ring nematodes might predispose plants to infection by *X. fastidiosa*. The P value for the “effect” of *X. fastidiosa* status on yield remained highly significant ($P < 0.0001$) when *P. cinnamomi* status, ringspot status, ring nematode population estimate, or any of the soil chemistry variables were included as covariables.

A principal components analysis revealed three factors that accounted for 74% of the variation in the soil chemistry data. Factor 1 accounted for 31% of the variation in the soil chemistry data, and the most highly correlated variables were Ca, Mg, and K. Factor 2 accounted for 29% of the variation, with P, Cu, and Zn being most highly correlated with it. Factor 3, with which S, Na, and pH were most highly correlated, accounted for 15% of the variation. The Factor 2 score was the only one that resulted in a significant P value ($P = 0.040$) in a logistic analysis, and the variables most highly correlated with this factor were examined individually. Means and P values for the logistic analyses using these variables are shown in Table 3. Because pH was not one of the variables most highly correlated with Factor 2, results of logistic regression using pH as an independent variable are not shown. However, mean pH values for soil around *X. fastidiosa*-positive and negative plants were pH 4.76 and pH 4.83, which are near the middle of the range recommended for blueberries (Puls, 1999).

Discussion

Results suggest that *X. fastidiosa* infection may cause a substantial decline in productivity of ‘Tifblue’ rabbiteye blueberry plants but that spread is limited. ELISA tests in July or Aug. 2013 indicated that nine of 40 plants were infected. *X. fastidiosa* was later

Table 2. Soluble solids concentrations and titratable acidity of berries harvested during 1 week of 2014 from ‘Tifblue’ rabbiteye blueberry plants in which *Xylella fastidiosa* was and was not detected.

	Soluble solids (%)	Titratable acidity (%)
<i>X. fastidiosa</i> -positive ^a	11.5 ± 0.5 ^a	0.57 ± 0.02
<i>X. fastidiosa</i> -negative ^b	10.1 ± 0.1	0.57 ± 0.005
P value	0.0217 ^w	0.8324

^a $N = 6$ plants.

^bMean ± SE.

^c $N = 31$ plants.

^wRanks of soluble solid values were analyzed because of a lack of normality.

Table 3. Concentrations of nutrients in soil around plants in which *Xylella fastidiosa* was ($N = 9$) and was not ($N = 31$) detected.

	<i>X. fastidiosa</i> -positive	<i>X. fastidiosa</i> -negative	P value ^a
P (ppm)	30.8	24.1	0.1250
Cu (ppm)	1.62	1.35	0.0479
Zn (ppm)	1.63	1.41	0.0850

^a P values are from logistic analyses performed to determine if the concentration of this nutrient might be related to infection by *X. fastidiosa*.

detected by real-time PCR in all but one of these. The initial ELISA result from the single plant in which infection was not later detected may have reflected an infection that did not spread systemically in the plant. Six of the nine *Xylella*-positive plants were located in blocks with at least one other positive plant, seeming to suggest that spread between plants may be important. This is consistent with the findings of Park et al. (2006) in grape, in an area in which the glassy-winged sharpshooter was present but contrasts with results of Sisterson et al. (2012) in almond and Li et al. (2011) in pecan.

The fact that none of the 31 yield study plants that initially tested as *X. fastidiosa*-negative tested positive in 2014 or 2015 suggests that spread between plants, or spread followed by colonization to a degree that allows detection, is not rapid. It has been suggested that, in such a situation, using noninfected planting material may limit the number of infections in an orchard (Sanderlin and Heyderich-Alger 2003). However, it is possible that the infection incidence would increase more quickly in a younger orchard, and it is likely that new infections would eventually be detected if plants were monitored for a longer period. In a pecan orchard that was ≈25 years old when data collection was initiated, Sanderlin and Heyderich-Alger (2003) did not detect new infections in 10 initially *X. fastidiosa*-negative pecan trees during the course of a three year study. However, in another pecan orchard in which trees were only 14 years old when the study began, infection incidence in trees of the same susceptible cultivar increased from 5% to 64% over a 6-year period (Sanderlin et al., 2009). Future research should investigate whether spread occurs at a different rate in young rabbiteye orchards than was observed in the old orchard surveyed in this study.

Four of the nine *X. fastidiosa*-positive plants appeared dead when survival was assessed in Apr. 2016. Because positive plants had been sampled more extensively after the final yield study harvest, as part of a seasonal detection study (Ferguson, 2016), they differed in this respect from *X. fastidiosa*-negative plants by this time. However, based on plant vigor, it appeared that all but one of the 31 *X. fastidiosa*-negative plants would still have had living aboveground growth at this time, had they been sampled in the same way.

Results clearly indicate that the detectable presence of *X. fastidiosa* is associated with

yield loss in ‘Tifblue’ rabbiteye blueberry. Yield reductions of *X. fastidiosa*-positive plants were 55% in 2013 and 62% in 2014, compared with negative plants, and were greater than losses or estimated losses reported in almond, pecan, or citrus (Gonçalves et al., 2011; Sanderlin and Heyderich-Alger 2003; Sisterson et al., 2008, 2012). To fully assess the importance of *X. fastidiosa* in rabbiteye blueberry, it would be helpful to have data from other naturally infected orchards.

Average berry weight was lower in infected plants, as well. Only six of the nine *X. fastidiosa*-positive plants had fruit in the second season, and those that did were plants that appeared to be in earlier stages of decline. The difference in average berry weight might have been greater if more *X. fastidiosa*-positive plants had produced fruit in the second year. The greater SSC observed in a limited sample of fruit from infected plants is consistent with what might be expected in plants with reduced water uptake because of plugging of the xylem. Also, studies involving other fruit crops have sometimes shown that greater SSCs were associated with reduced crop loads (Berkey et al., 2011; Einhorn et al., 2011; Serra et al., 2016). Researchers should be aware of a possible effect of *X. fastidiosa* in case unexplained variations in soluble solids are observed, for example, in a cultivar or breeding line evaluation of a plant species in which *X. fastidiosa* can be a chronic issue (P. Perkins-Veczie, personal communication).

Neither *P. cinnamomi* nor ringspot symptoms were associated with *X. fastidiosa* infection status. It is possible that some *X. fastidiosa*-positive plants in very advanced stages of decline once harbored *P. cinnamomi* but no longer supported detectable populations of the pathogen. Soil from around two of the three plants in the most advanced stages of decline tested negative for *P. cinnamomi*. Although *P. cinnamomi* can survive in soil for two or more years without a living host under some conditions, it appears that populations decrease when living roots are absent (Zentmyer, 1980). Had it been possible to sample soil at the time the plants were infected by *Xylella*, it may have been possible to determine if an interaction took place at a critical time. If *P. cinnamomi* predisposes plants to infection by *X. fastidiosa*, practices that help prevent Phytophthora root rot problems might help prevent *X. fastidiosa* infection, as well.

Logistic analyses did not support hypotheses that levels of ring nematodes, soil pH, or concentrations of most soil nutrients or Na were related to infection with *X. fastidiosa*. The P value for Cu, however, was significant at $\alpha = 0.05$, and that for Zn was significant at $\alpha = 0.10$. Because these soil nutrient levels were naturally occurring rather than applied treatments, it is possible that the associations between higher levels of these two nutrients with infection status

are coincidental, or that infected plants took up lesser amounts of them, leaving higher concentrations in the soil. Also, while results are shown for logistic analyses using Cu, Zn, and P, the fact that these are all associated with the same component in a principal components analysis shows that they are not independent of each other. It is possible that one predisposes plants to infection while the concentrations of the others are simply correlated with the concentration of that predisposing factor. The mean soil Cu and Zn concentrations for both *X. fastidiosa*-positive and *X. fastidiosa*-negative plants were well below the levels considered toxic for crops in general (Hardy et al., 2014). However, most recommendations related to Cu and Zn fertility in blueberries are based on foliar tissue levels (Hart et al., 2006; Puls, 1999), which were not analyzed in this study. Additional research is needed to show any causal relationship between soil Cu or Zn concentration and plant susceptibility to *X. fastidiosa* infection and should take foliar concentrations into account.

Dutta et al. (2015) formulated models that associated plant tissue concentrations of Cu^{2+} and Zn^{2+} , along with Fe^{2+} and Mn^{2+} , and severity of bacterial leaf spot (causal agent: *Xanthomonas euvesicatoria*) in pepper. In a related work, Rooks et al. (2015) found that the soil Cu:Fe ratio was associated with tomato spotted wilt virus severity in tobacco. In both cases, it is suggested that a relationship between concentrations of these nutrients and disease severity may be mediated by elements of the systemic acquired resistance response, including the expression of certain superoxide-dismutase genes and the *NPR1* gene (Dutta et al., 2015; Rooks et al., 2015). These studies provide some evidence of a possible relationship between soil Cu and plant predisposition to disease, and it would be interesting to see if the observed relationship between soil Cu concentration and *X. fastidiosa* infection status is more than coincidental.

Research and extension workers, as well as growers, should be aware of *X. fastidiosa* as a potential yield- and survival-impacting factor in rabbiteye blueberry. Spread of infection between established plants was not detected but might occur more rapidly among younger plants. Besides additional studies on yield loss in naturally infected orchards, investigation of a possible predisposing effect of Cu or Zn on susceptibility of plants to infection by *X. fastidiosa* is warranted.

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