Xylella fastidiosa Isolates from Both subsp. *multiplex* and *fastidiosa* Cause Disease on Southern Highbush Blueberry (*Vaccinium* sp.) Under Greenhouse Conditions

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ABSTRACT

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Xylella fastidiosa is a xylem-limited gram-negative plant pathogen that affects numerous crop species, including grape, citrus, peach, pecan, and almond. Recently, *X. fastidiosa* has also been found to be the cause of bacterial leaf scorch on blueberry in the southeastern United States. Thus far, all *X. fastidiosa* isolates obtained from infected blueberry have been classified as *X. fastidiosa* subsp. *multiplex*; however, *X. fastidiosa* subsp. *fastidiosa* isolates are also present in the southeastern United States and commonly cause Pierce's disease of grapevines. In this study, seven southeastern U.S. isolates of *X. fastidiosa*, including three *X. fastidiosa* subsp. *fastidiosa* isolates from grape, one *X. fastidiosa* subsp. *fastidiosa* isolate from elderberry, and three *X. fastidiosa* subsp. *multiplex* isolates from blueberry, were used to infect the southern highbush blueberry 'Rebel'. Following inoculation, all isolates colonized blueberry, and isolates from both *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *fastidiosa* caused symptoms, including characteristic stem yellowing and leaf scorch symptoms as well as dieback of the stem tips. Two *X. fastidiosa* subsp. *multiplex* isolates from blueberry caused more severe symptoms than the other isolates examined, and infection with these two isolates also had a significant impact on host mineral nutrient content in sap and leaves. These findings have potential implications for understanding *X. fastidiosa* host adaptation and expansion and the development of emerging diseases caused by this bacterium.

Xylella fastidiosa is a xylem-limited, fastidious bacterial plant pathogen that infects a wide variety of crop plants (Hopkins and Purcell 2002). Diseases caused by *X. fastidiosa* include Pierce's disease of grapevine, citrus variegated chlorosis of citrus, almond leaf scorch, and the more recently identified bacterial leaf scorch (BLS) of blueberry (Chang et al. 2009). *X. fastidiosa* is transmitted from plant to plant by several leafhopper insects (family Cicadellidae), including the glassy-winged sharpshooter (*Homalodisca vitripennis*) and the blue-green sharpshooter (*Graphocephala atropunctata*) (Janse and Obradovic 2010).

A major component of the infection process of *X. fastidiosa* is xylem plugging, caused by the formation of bacterial biofilm in the host xylem vessels (Chatterjee et al. 2008). This plugging is believed to disrupt water and nutrient flow within the host plant, which ultimately leads to many of the symptoms caused by *X. fastidiosa* infection. In addition to xylem plugging by biofilm, *X. fastidiosa* induces host tylose formation (Sun et al. 2013), host gene expression changes (Carazzolle et al. 2011; Choi et al. 2013), and host ionomic changes (De La Fuente et al. 2013; Navarrete and De La Fuente 2015; Oliver et al. 2014). These changes all contribute to a complex interplay between *X. fastidiosa* and the development of symptoms in the assorted hosts.

Isolates of *X. fastidiosa* can be divided into at least four genetically distinct subspecies (Scally et al. 2005; Schaad et al. 2004) composed of numerous haplotypes (Parker et al. 2012), which show specificity in terms of the host plants from which they are isolated. These subspecies include *fastidiosa*, isolated from grape and almond; *pauca*, isolated from citrus and coffee in South America; *sandyi*, isolated from oleander; and *multiplex*, isolated from an assortment of species, including pecan, plum, peach, almond, elm, and blueberry. Generally, isolates of *X. fastidiosa* are believed to show host specialization (Janse and Obradovic 2010). For example, *X. fastidiosa* subsp. *fastidiosa* isolates, which are prevalent in citrus-growing areas of Florida, Texas, and California, do not appear to be capable of causing disease on citrus (Hopkins and Purcell 2002). By contrast, isolates from both *X. fastidiosa* subsp. *multiplex* and *fastidiosa* are capable of causing disease in almond (Almeida and Purcell 2003). Overall, the basis of *X. fastidiosa* host specificity is not well understood but classical effector proteins do not appear to be involved due to the lack of type III secretion system in *X. fastidiosa* (Killiny and Almeida 2011).

X. fastidiosa was originally identified as the causal agent of BLS, a disease afflicting blueberry in southern Georgia (Chang et al. 2009); and, since then, it has also been found in northern Florida (Harmon and Hopkins 2009). The symptoms of BLS include leaf scorch, dieback, and stem yellowing, which ultimately lead to the death of affected blueberry bushes (Chang et al. 2009). It has been reported that the disease has the most significant effects on southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids), while other blueberry plants, including 'rabbit-eye' cultivars (*V. virgatum*) as well as southern highbush 'Emerald' and 'Millenia' grown in close proximity, have shown little to no effect from the pathogen over many years (Brannen et al. 2008). Previous work with the primary *X. fastidiosa* vector in southern blueberry plants, the glassy-winged sharpshooter, has shown that these differences are not due to differential attractiveness of the cultivars to the vector; therefore, it has been suggested that these cultivars may possess

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*The *e-Xtra* logo stands for "electronic extra" and indicates that two supplementary figures are published online.

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some degree of tolerance or resistance to *X. fastidiosa* (Tertuliano et al. 2012), perhaps due to greater hydraulic conductance potential within the xylem (Holland and Scherm 2012). Though *X. fastidiosa* subsp. *fastidiosa* isolates are prevalent in Georgia and Florida, all *X. fastidiosa* isolates taken from BLS-afflicted blueberry plants to date have been identified as *X. fastidiosa* subsp. *multiplex* (Hopkins et al. 2012; Oliver et al. 2014; Parker et al. 2012) and, heretofore, there has been scant evidence to suggest that *X. fastidiosa* subsp. *fastidiosa* isolates can cause BLS on blueberry (Hopkins et al. 2012).

Prior work by our group has shown that *X. fastidiosa* isolates from blueberry, grape, and elderberry are capable of causing a range of symptoms on the model host *Nicotiana tabacum* 'SR1' (Oliver et al. 2014). Though the *X. fastidiosa* subsp. *multiplex* isolates from blueberry were shown to colonize the host in lower numbers and have effects on the host ionome (i.e., host mineral nutrient content) that were distinguishable from the effects of the *X. fastidiosa* subsp. *fastidiosa* isolates from grape and elderberry, no distinction between the intensity of the visible symptoms caused on tobacco was evident between the two subspecies (Oliver et al. 2014). Based on these results from a model system, and the fact that *X. fastidiosa* subsp. *fastidiosa* isolates are known to be present in the southeastern United States but have not been shown to cause disease on blueberry in the Southeast, the effects of seven southeastern U.S. isolates of *X. fastidiosa* were compared in the southern highbush blueberry 'Rebel'. These seven isolates consist of four *X. fastidiosa* subsp. *fastidiosa* isolates, three from grape and one from elderberry, as well as three *X. fastidiosa* subsp. *multiplex* isolates isolated from blueberry in Florida and Georgia, and include the three isolates shown to be most virulent in the *N. tabacum* model system (Oliver et al. 2014).

Our results indicated that all seven isolates were capable of colonizing blueberry host plants and that isolates from both *X. fastidiosa* subsp. *multiplex* and *fastidiosa* were capable of inducing host symptoms, with the most severe symptoms observed with two of the blueberry isolates. Examination of mineral nutrient changes in the host ionome in *X. fastidiosa*-infected plants also suggested that the magnitude and composition of changes in the host ionome correspond to the severity of the symptoms observed in affected hosts (De La Fuente et al. 2013; Oliver et al. 2014). Overall, these results suggest that *X. fastidiosa* subsp. *fastidiosa* isolates are capable of causing disease on blueberry and that virulence differences exist between *X. fastidiosa* isolates capable of causing BLS on blueberry, two very important pieces of information necessary to manage this emerging blueberry pathogen.

MATERIALS AND METHODS

***X. fastidiosa* isolates and culture conditions.** *X. fastidiosa* isolates consisted of three *X. fastidiosa* subsp. *multiplex* isolates originally isolated from blueberry in Florida and Georgia, as well as four *X. fastidiosa* subsp. *fastidiosa* isolates originally isolated from grape or elderberry in Texas, Georgia, and Florida (Table 1). These isolates represent seven unique haplotypes within *X. fastidiosa* subsp. *fastidiosa* and *multiplex* based on multilocus sequence analysis of environmentally mediated genes conducted previously (Oliver et al. 2014; Parker et al. 2012). For 1 week prior to inoculation, isolates were grown on periwinkle wilt medium at 28°C, then suspended in succinate-citrate-phosphate buffer as previously described (De La Fuente et al. 2013) for subsequent plant inoculations.

Plant and greenhouse conditions. A variety of southern highbush blueberry (*Vaccinium* Rebel [PPA18, 138]) (NeSmith 2008) was used in these experiments. Seedlings were obtained from Agri-Starts (Apopka, FL) and transplanted into a soil mixture in 9.5 liters and 25.7 cm (diameter) pots. Following transplanting, plants were grown in the greenhouse at 20 to 25°C with natural sunlight in a soil mixture consisting of three parts pine bark mulch and one part sand. Plants were initially fertilized with a slow-release fertilizer (Osmocote Outdoor & Indoor Smart-Release Plant Food; The Scotts Company, Marysville, OH) and watered regularly. Between 1 and 2 months after transplanting, blueberry plants were inoculated using a 1-ml tuberculin syringe at the soil line on two main stems of the plant, as described previously (Chang et al. 2009), with droplets of inoculum containing *X. fastidiosa* at approximately 10^8 cells/ml. Fifteen plants were inoculated per isolate treatment, and inoculation with buffer only was used as a control treatment. Three weeks after the original inoculations, all plants were reinoculated in the same manner to ensure infection. Three replicate experiments were performed.

Infection of blueberry plants and symptom monitoring. Following inoculation, plants were monitored for the development of symptoms. Symptoms developing on plants inoculated with *X. fastidiosa* were compared with uninfected control plants inoculated with buffer only (Supplementary Fig. S1). Symptoms, including leaf color changes (yellowing and reddening), leaf necrosis or scorching, and stem yellowing, were recorded as well as the extent of symptom spread within each plant stem. To allow for monitoring of relative disease progression on each plant, the severity of symptoms with respect to each stem of each plant was recorded at approximately 2-week intervals in each experiment after symptoms first became evident on any of the isolate treatment groups. Each stem received a severity score according to a 0-to-7 rating scale (Table 2; Supplementary Figure S2) based on the proportion of leaves on that stem showing symptoms and the predominant symptom present. At each time interval, the total severity score for each plant was calculated by adding together the individual severity ratings given to each of the stems on that plant. Following each experiment, recorded severity scores for each of six time points were used to calculate the area under the disease progress curve (AUDPC) for each inoculated plant based on the midpoint rule method (Campbell and Madden 1990) as follows: $\text{AUDPC} = \Sigma_i^{n-1}[(y_i + y_{i+1})/2](t_{i+1} - t_i)$, where n = the number of assessment times, y = disease severity score for each plant at each assessment, and t = time at each assessment. Plants were maintained and observed in the greenhouse for 140 days after initial

TABLE 1. List of the *Xylella fastidiosa* isolates used in this analysis, the host they were isolated from, the location of their isolation, and the isolate source

X. fastidiosa subsp.	Isolate	Host	Host species	City	County	State
multiplex	AlmaEm3	Blueberry	*Vaccinium* 'Emerald'	Alma	Bacon	Georgia[u]
	BB08-1	Blueberry	*Vaccinium* 'Windsor'	Palatka	Putnam	Florida[v]
	BBI64	Blueberry	*Vaccinium* 'V1'	Hoboken	Brantley	Georgia[w]
fastidiosa	CCPM1	Grape	*Vitis vinifera* 'Petit Manseng'	Dahlonega	Lumpkin	Georgia[x]
	EB92-1	Elderberry	*Sambucus* sp.	Leesburg	Lake	Florida[y]
	GilBec625	Grape	*Vitis vinifera* 'Viognier'	Stonewall	Gillespie	Texas[z]
	WM1-1	Grape	*Vitis vinifera* 'Mourvedre'	Dahlonega	Lumpkin	Georgia[x]

[u] Isolate reference or source: Oliver et al. 2014.
[v] Isolate reference or source: Donald Hopkins (University of Florida).
[w] Isolate reference or source: Harald Scherm (University of Georgia).
[x] Isolate reference or source: Parker et al. 2012.
[y] Isolate reference or source: Hopkins 2005.
[z] Isolate reference or source: Mark Black (Texas A&M University).

inoculation, at which time stems were removed from the plant for sap analysis (see below).

Bacterial detection by quantitative polymerase chain reaction. To determine whether inoculated plants became infected with *X. fastidiosa*, total DNA was extracted from midrib and petiolar portions of three pairs of leaves from above the inoculation point using a modified cetyltrimethylammonium bromide protocol (Doyle and Doyle 1987) shown previously to be an effective DNA isolation method from *X. fastidiosa*-infected blueberry leaves (Christiano and Scherm 2011). Leaves were collected at 112 days post inoculation and sections of the lower petioles and leaf midrib were excised as previously described (De La Fuente et al. 2013). Pairs of leaves consisting of one leaf from each of the two main stems originally inoculated were pooled into single samples. Leaves from three, five, and seven nodes above the soil line were collected. *X. fastidiosa* CFU per gram of extracted leaf tissue were enumerated via quantitative polymerase chain reaction (qPCR) relative to a four-point standard curve prepared from DNA of known CFU from pure cultures, as described previously (De La Fuente et al. 2013). Because phenolic compounds extracted from blueberry leaves are known to interfere with qPCR amplification (Christiano and Scherm 2011), extracted DNA was diluted and different dilutions were used for qPCR amplification to ensure that tested samples were minimally affected by qPCR inhibitors. A redesigned TaqMan probe including a degenerate base, described previously (Oliver et al. 2014), was used for qPCR amplification. Successful infection was defined by the presence of detectable *X. fastidiosa* populations in leaves above the inoculation point. For successfully infected plants from all three experiments, CFU per gram from a single pair of leaves per plant (most basal *X. fastidiosa*-positive leaf pair) was averaged for each *X. fastidiosa* isolate (n = 31 to 44).

Ionome analysis. *Leaf samples.* After petiolar and midrib portions of leaves were removed for DNA extraction, the remainder of each collected leaf was used for ionome analysis by inductively coupled plasma–optical emission spectroscopy (ICP-OES). As described above, paired samples of leaves consisting of one leaf from each of the two inoculated stems were tested. Leaves were prepared for analyses by drying at 65°C for 1 h. Whole leaves were crushed to a fine powder by mortar and pestle and approximately 5- and 10-mg dry weight samples were placed into 1.5-ml microcentrifuge tubes for acid digestion. Samples were digested for at least 1 h at 100°C in 200 µl of mineral-free concentrated nitric acid (OPTIMA, Fisher Scientific). Samples were analyzed by ICP-OES (Perkin Elmer 7300 DV, Waltham, MA) with simultaneous measurement of B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn. Mineral concentrations were determined by comparing emission intensities to a standard curve created from certified mineral standards (SPEX CertiPrep, Metuchen, NJ) according to the methods described previously (De La Fuente et al. 2013). Due to the lengthy sample preparation and analysis times associated with this procedure, the analysis was carried out for leaves (three pairs of leaves per plant) collected from all plants (120 total plants) from one representative experimental replicate.

Sap samples. At the conclusion of the experiment (140 days after initial inoculation), sap was extracted from blueberry plants using a cut-stem pressure chamber method (Liang and Zhang 1997). Briefly, an inoculated stem from each plant was removed via a single cut through the stem and immediately placed into a Model 600 Pressure Chamber (PMS Instrument Company, Corvallis, OR). Sap was removed from the cut end after pressure was applied to the leaves and upper portion of the stem and collected into a 1.7-ml microcentrifuge tube for subsequent analysis. One 100-µl sample of sap was collected from each plant, diluted with 900 µl with ultrapure mineral-free water, and analyzed via ICP-OES as described for the leaf analysis above. Due to the lengthy collection times associated with this procedure, sap was collected from three plants per treatment from two experimental replicates.

Statistical analysis. All analyses were performed in the R language for statistical computing (Ihaka and Gentleman 1996). Linear random effects models were fit to the data from infected plants to model individual leaf mineral element response to isolate type (function lmer from lme4 R package) (Bates et al. 2013). This type of model was used to control for random effects due to plant position and control for pseudoreplication at the plant level (i.e., multiple leaves per plant). The mean values for each isolate–mineral element combination were extracted from the mixed effects models, and were used for multivariate analyses. Principal component analysis (PCA) of the correlation matrix was used to investigate ionome responses to infection by different isolate types. Multiple comparisons between AUDPC means and between *X. fastidiosa* population means from qPCR were carried out using Fisher's LSD method and Tukey's honestly significant difference method, respectively (functions LSD.test and HSD.test from agricolae R package) (de Mendiburu 2014).

RESULTS

Symptoms caused by *X. fastidiosa* isolates. Symptoms of *X. fastidiosa* infection (Fig. 1) began to appear on Rebel blueberry plants at approximately 2 months postinoculation with *X. fastidiosa* isolates. Symptoms began largely as a discoloration (yellowing and reddening) of the leaves toward the base of the stem. The discoloration often appeared in association with the leaf veins until it ultimately spread to the entire leaf (Fig. 1A–C). Leaf necrosis was also observed, often at the leaf margins in typical 'scorch' fashion (Fig. 1D, E, and G). As disease progressed, symptoms generally spread from the base of the stem toward the stem tip, with discolored leaves often ultimately becoming scorched, as had been observed previously in other inoculated blueberry plants (Chang et al. 2009). The most severely infected plants would often show tip dieback (Fig. 1F), stem yellowing, and leaf loss, reminiscent of the disease's initial observations in the field, which led to it being called 'yellow twig' disease (Chang et al. 2009). After inoculation, symptoms appeared on plants in each isolate treatment group, while plants in the control group inoculated with buffer only were free from these symptoms beyond the occasional yellowing leaf. Differences between the observed symptoms caused by the different isolates were apparent in their severity and progression over time (Table 3). Though symptoms generally appeared first on the two originally inoculated stems on each plant, it was common in the most severely affected plants to see severe symptoms develop on additional stems

TABLE 2. Scale for disease severity rating given to each plant stem

Severity rating	Proportion of stem affected[y]	Predominant symptoms[z]
0	None	None
1	1 to 2 leaves	Leaf yellowing appears along leaf margin or along the vein
2	1 to 2 leaves	Previously yellowing leaf areas have begun to turn red
3	>2 leaves, but <50% of total	Previously yellowing leaf areas have begun to turn red
4	>2 leaves, but <50% of total	Leaves are partially yellow with red margins/interveinal areas, or are bright red with necrotic areas developing
5	>50% of leaves	Leaves are partially yellow with red margins/interveinal areas, or are bright red with necrotic areas developing
6	>50% of leaves	Leaves are red/brown with marginal or interveinal necrosis present
7	Entire stem affected	Severe leaf scorching/necrosis with evident stem yellowing/matchstick or extensive leafdrop

[y] Proportion of leaves on an individual stem affected by the accompanying predominant symptom.
[z] Predominant symptoms of leaves on an individual stem.

of the same plant prior to the end of the experiment. In all three experiments, blueberry plants inoculated with *X. fastidiosa* subsp. *multiplex* isolates AlmaEm3 and BB08-1 showed more severe symptoms that often appeared and spread up the stem earlier than those observed on plants inoculated with other isolates. Frequently, plants inoculated with isolates AlmaEm3 and BB08-1 showed symptoms which progressed to marginal necrosis, scorching, and leaf drop prior to the end of the experiment, and marginal necrosis was also noted in plants inoculated with the *X. fastidiosa* subsp. *fastidiosa* isolates from grape (CCPM1, GilBec625, and WM1-1). Symptoms developing on plants inoculated with BBI64 and EB92-1 generally appeared later and were largely limited to discoloration of the lower few leaves of inoculated stems; symptoms further up the stem or symptom progression to marginal necrosis were rarely observed on plants inoculated with these isolates prior to the end of the experiment on day 140 postinoculation. Overall, plants inoculated with isolates BBI64 (an *X. fastidiosa* subsp. *multiplex* isolate from blueberry) and EB92-1 (an *X. fastidiosa* subsp. *fastidiosa* isolate from elderberry) showed the least severe symptoms and, with respect to plants inoculated with isolate EB92-1, as a whole, the mean AUDPC was not significantly different (at $P < 0.05$) than in the control plants that did not receive *X. fastidiosa* (Table 3).

***X. fastidiosa* populations determined by qPCR.** Isolation and amplification of *X. fastidiosa* DNA from infected leaves indicated that all the isolates examined were capable of causing infection in Rebel blueberry. *X. fastidiosa* DNA was detectable in leaves collected from above the initial inoculation point nearly 4 months after inoculation (Table 3). *X. fastidiosa* DNA was detected in a large proportion of all plants initially inoculated, with percentages of infected plants ranging from approximately 72% for some isolates (BBI64 and WM1-1) to 100% for others (AlmaEm3) (Table 3). *X. fastidiosa* populations in infected plants ranged from an average of 5.5×10^2 CFU/g of leaf tissue for isolate BBI64 to 2.5×10^4 CFU/g for isolate AlmaEm3. No significant differences between subspecies (*X. fastidiosa* subsp. *multiplex* isolates versus subsp. *fastidiosa* isolates) with respect to *X. fastidiosa* colonization of plant leaf petioles were evident.

Ionome analysis. Using mineral element concentrations determined by ICP-OES, we applied a random effects model and used PCA to visualize the relationship among isolates due to differences between isolates' effects on the blueberry leaf ionome (Fig. 2). In this visualization of the ionomes, the most virulent isolates (*X. fastidiosa* subsp. *multiplex* AlmaEm3 and BB08-1) were shown to separate dramatically from the other isolates based on their effects on the host ionome, while the other isolates did not show much separation from one another (Fig. 2). Further examination of plant Ca concentrations, previously shown to be modified in *X. fastidiosa*-infected tobacco plants (De La Fuente et al. 2013; Navarrete and De La Fuente 2015; Oliver et al. 2014), indicated that the most virulent isolates also showed the largest increases in Ca concentrations relative to the control (Fig. 3).

DISCUSSION

The factors limiting the host range of *X. fastidiosa* are not well understood, although it is well described that geographical

TABLE 3. Summary of the colonization incidence, area under the disease progress curve (AUDPC), and *Xylella fastidiosa* population observed after inoculation of blueberry plants with each respective *X. fastidiosa* isolate compared with numbers observed in control plants that were inoculated with inoculation buffer only

Isolates	Incidence (%)[x]	AUDPC[y]	*X. fastidiosa* population[z]
AlmaEm3	44/44 (100)	474.48 a	$2.5 \times 10^4 \pm 8.0 \times 10^3$ a
BB08-1	40/45 (88.9)	344.00 b	$3.0 \times 10^3 \pm 1.1 \times 10^3$ bc
GilBec625	37/45 (82.2)	212.63 c	$1.5 \times 10^4 \pm 6.1 \times 10^3$ ab
CCPM1	34/44 (77.3)	187.37 c	$6.2 \times 10^3 \pm 2.0 \times 10^3$ abc
WM1-1	31/43 (72.1)	177.63 c	$4.8 \times 10^3 \pm 2.2 \times 10^3$ bc
BBI64	32/44 (72.7)	52.07 d	$5.5 \times 10^2 \pm 1.5 \times 10^2$ c
EB92-1	39/43 (90.7)	44.10 de	$7.2 \times 10^3 \pm 1.7 \times 10^3$ abc
Control	0/45 (0)	3.45 e	nd

[x] Ratio of plants found to be *X. fastidiosa* infected by quantitative polymerase chain reaction versus those originally inoculated (percentage of total).

[y] Mean AUDPC per treatment group. Different letters represent significant differences between means at $P < 0.05$ according to Fisher's least significant difference test.

[z] Bacterial population of *X. fastidiosa* in CFU/g of leaf material (mean ± standard error of the mean); nd indicates that *X. fastidiosa* was not detected in any control plant. Different letters represent significant differences between means at $P < 0.05$ according to Tukey's honestly significant difference test.

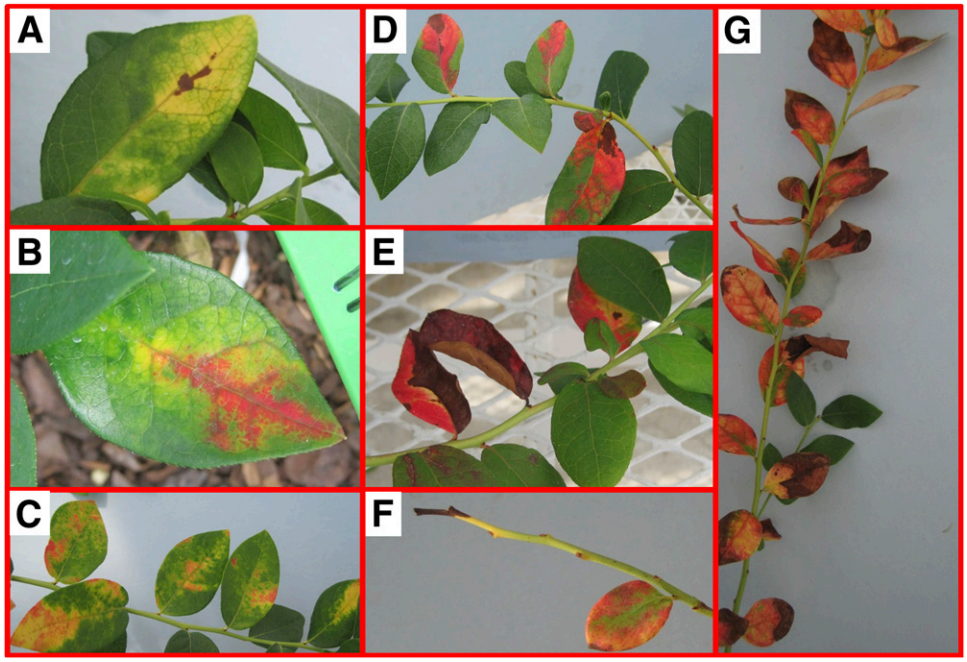

Fig. 1. Symptoms observed in *Xylella fastidiosa*-infected Rebel blueberry after inoculation: **A and B,** early stages of vein necrosis and discoloration; **C,** expanding leaf discoloration; **D and E,** early stages of leaf and petiole scorching; **F,** late-stage stem yellowing and tip dieback ('matchsticks'); and **G,** late stages of severe leaf scorch.

distribution, warm weather, and mild winters are important for disease development (Hopkins and Purcell 2002). In recent years, several diseases caused by *X. fastidiosa* have emerged in diverse hosts, such as pear (Leu and Su 1993), avocado (Montero-Astua et al. 2008), chitalpa (Randall et al. 2009) and other ornamental trees (Hernandez-Martinez et al. 2007), olive (Krugner et al. 2014; Loconsole et al. 2014), and blueberry (Chang et al. 2009). Although diseases caused by *X. fastidiosa* have historically been restricted to the Americas, in recent years there have been reports of outbreaks in Asia (Leu and Su 1993; Su et al. 2013) and Europe (Loconsole et al. 2014). The basis for the appearance of new host specificities is not understood but it has been hypothesized to be caused by genetic recombination after infection of nonhost plants (Nunney et al. 2014a,b). This suggests that the occasions that *X. fastidiosa* has for inter- or intrasubspecific recombination may play a critical role in the emergence of new *X. fastidiosa* diseases. Therefore, characterizing the host-range capabilities of *X. fastidiosa* isolates is necessary to understand the kinds of opportunities for recombination that may exist.

Within recent years, *X. fastidiosa* subsp. *multiplex* has begun to have a significant impact on southern highbush blueberry production in Florida and Georgia (Brannen et al. 2008; Holland et al. 2014); however, little information is available characterizing virulence among BLS-causing *X. fastidiosa* subsp. *multiplex* isolates. Furthermore, *X. fastidiosa* subsp. *fastidiosa* isolates that can also be found throughout the southeastern United States, including Florida and Georgia, have not been isolated from infected blueberry. In this study, in addition to examining the virulence of *X. fastidiosa* subsp. *multiplex* isolates from blueberry, we also investigated the potential of southeastern *X. fastidiosa* subsp. *fastidiosa* isolates to cause disease on southern highbush blueberry. We were particularly interested in isolate AlmaEm3, that was isolated from symptomatic *Vaccinium* Emerald, a cultivar previously described as resistant to *X. fastidiosa*, as well as the biocontrol isolate EB92-1 (Hopkins 2005); and, in our tests, blueberry plants inoculated with AlmaEm3 showed more severe symptoms than plants inoculated with any other isolate, whereas blueberry plants inoculated with EB92-1 were largely asymptomatic. Our findings, which show that isolates from both *X. fastidiosa* subsp. *fastidiosa* and *multiplex* are able to colonize and cause symptoms on blueberry, provide valuable information for assessing the risk of isolate exchange between grape and blueberry with this emerging disease. This is also the first report of discernible virulence variations between BLS-causing *X. fastidiosa* subsp. *multiplex* isolates on blueberry.

Isolates of both *X. fastidiosa* subsp. *fastidiosa* and *multiplex* could efficiently colonize their plant host after pinprick inoculation. Furthermore, no overall difference was apparent between isolates of different subspecies in terms of bacterial populations in petioles. This indicates that isolates of both *X. fastidiosa* subsp. *multiplex* and *fastidiosa* are capable of host colonization in roughly equivalent quantities. No significant correlation was observed between the severity of symptoms and *X. fastidiosa* colonization of the leaf petiole, matching previous reports from *X. fastidiosa* infection of other hosts (Gambetta et al. 2007; Oliver et al. 2014). A recent report indicated that more severely afflicted blueberry plants had higher *X. fastidiosa* populations in extracted xylem sap (Holland et al. 2014) although, in that case, samples were taken from commercial plantings which were asynchronously vector inoculated, making it possible that the observed correlation may have been the result of cocorrelation due to time after infection.

In our study, isolates from both *X. fastidiosa* subsp. *multiplex* and *fastidiosa* were capable of causing symptoms after infection, though two *X. fastidiosa* subsp. *multiplex* isolates from blueberry, AlmaEm3 and BB08-1, caused more severe symptoms than the other isolates examined. Intriguingly, despite the fact that two out of three *X. fastidiosa* subsp. *multiplex* isolates from blueberry caused the most severe symptoms observed, the third isolate from blueberry, BBI64, showed significantly fewer or less severe symptoms than any of the *X. fastidiosa* subsp. *fastidiosa* isolates from grape. Symptoms observed after inoculation with BBI64 were comparable with symptoms observed to be caused by *X. fastidiosa* subsp. *fastidiosa* isolate EB92-1 from elderberry, an isolate which has been suggested to be useful for biocontrol or cross-protection in grape (Hopkins 2005) due to its low symptom severity on grape compared with other *X. fastidiosa* subsp. *fastidiosa* isolates. Though blueberry plants inoculated with BBI64 did show significantly more symptoms or disease progress relative to control plants, and this same stock of BBI64 did not exhibit diminished virulence relative to other *X. fastidiosa* isolates examined in another greenhouse inoculation experiment with *N. tabacum* (Oliver et al. 2014) which concluded concurrently with the start of the first experiment described here, we cannot exclude the possibility that the isolate of BBI64 used here (which also had the lowest detectable populations after blueberry colonization) may have diminished virulence in blueberry relative to its original isolation. Complicating virulence comparisons between isolates, bacterial pathogens maintained in laboratory culture sometimes exhibit reduced virulence compared with their original isolation in some instances (Fux et al. 2005), and *X. fastidiosa* is no exception (Oliver et al. 2014).

Generally, among all *X. fastidiosa*-inoculated blueberry plants, symptoms were most pronounced on the two stems originally inoculated with *X. fastidiosa* although, in the most severely affected plants (especially those inoculated with AlmaEm3 and BB08-1), it was not uncommon to see multiple stems (including those that emerged after inoculation) showing severe symptoms of necrosis and scorching. This indicates that *X. fastidiosa* was readily able to move against the xylem flow from the original point of inoculation and into the other stems of the plant, as has been shown to occur through twitching movement by type IV pili (Meng et al. 2005). This further indicates that contact among *X. fastidiosa* isolates inoculated in different stems of the same plant is possible, increasing the potential for intraisolate recombination in the environment.

The seven isolates used in this study were included among the isolates used in a prior study (Oliver et al. 2014), where they were found to cause differential symptoms on the model host *N. tabacum,* and the symptoms caused by each isolate were correlated with changes in the ionome. The changes observed included a significant

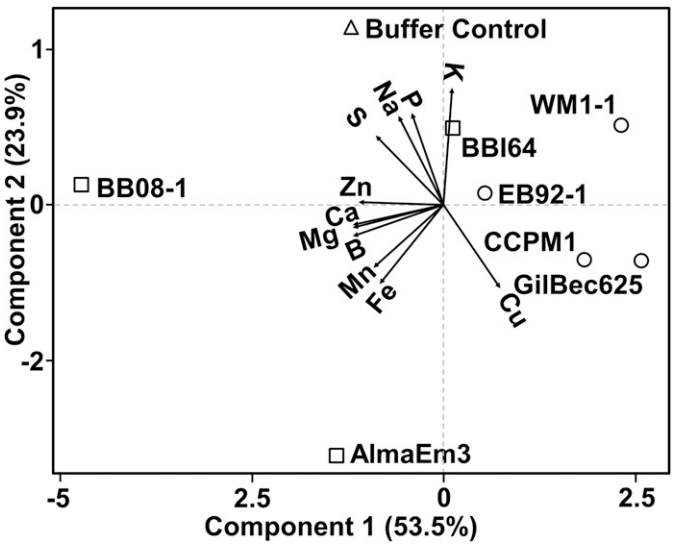

Fig. 2. Principal component analysis of blueberry plant leaf ionomic results by isolate. Shapes indicate whether the isolate mean depicted is buffer control (triangle), *Xylella fastidiosa* subsp. *fastidiosa* (circle), or *X. fastidiosa* subsp. *multiplex* (square). Eigen vectors for each leaf mineral element are shown.

increase in leaf and sap Ca concentrations on the most severely affected plants. To examine whether pronounced effects on the host ionome were observable in infected, symptomatic blueberry plants, leaves and sap were collected and tested. Matching previous results in tobacco (Oliver et al. 2014) and a small field sampling of infected blueberry plants (De La Fuente et al. 2013), plants infected with our two most severe isolates (AlmaEm3 and BB08-1) showed pronounced ionomic changes. These included increases in both sap and leaf Ca concentrations relative to uninfected control plants.

The isolate that caused the most symptoms on tobacco in our prior study, WM1-1, caused relatively fewer symptoms here than several other isolates, whereas the isolate which caused the most severe effects on blueberry in this study, AlmaEm3, caused less severe symptoms on tobacco relative to all but one of the seven isolates used here. Potential causes for the differences in relative virulence between these isolates on these two hosts are not known but undiscovered host adaptation factors may be involved that help a specific isolate to cause disease by allowing it to escape the plant host's defense response (e.g., tylose formation). It should be noted that, although tobacco is not known to form tyloses (Bonsen and Kucera 1990; Mueller and Morgham 1996), blueberry plants are known to form tyloses during infection with other pathogens (Daykin and Milholland 1990; Milholland 1972), and differential host formation of tyloses in response to *X. fastidiosa* is well characterized in grape and is known to play a role in the severity of symptoms observed on that host (Baccari and Lindow 2011; Sun et al. 2013).

The results observed here have implications for the emergence of *X. fastidiosa* on blueberry in the southeastern United States. Certainly, *X. fastidiosa* subsp. *fastidiosa* isolates from grape present in the southeastern United States are capable of both colonizing and causing symptoms on southern highbush blueberry under greenhouse conditions; however, all isolates reported to cause BLS on blueberry thus far have been *X. fastidiosa* subsp. *multiplex*. This raises the question of whether *X. fastidiosa* subsp. *fastidiosa* isolates from grape are capable of infecting blueberry in field settings (and, conversely, whether *X. fastidiosa* subsp. *multiplex* isolates from blueberry are capable of infecting grape). In field settings, *X. fastidiosa* is transmitted from plant to plant by leafhopper insects whereas, in this study, pinprick inoculations were used to transmit *X. fastidiosa*. The

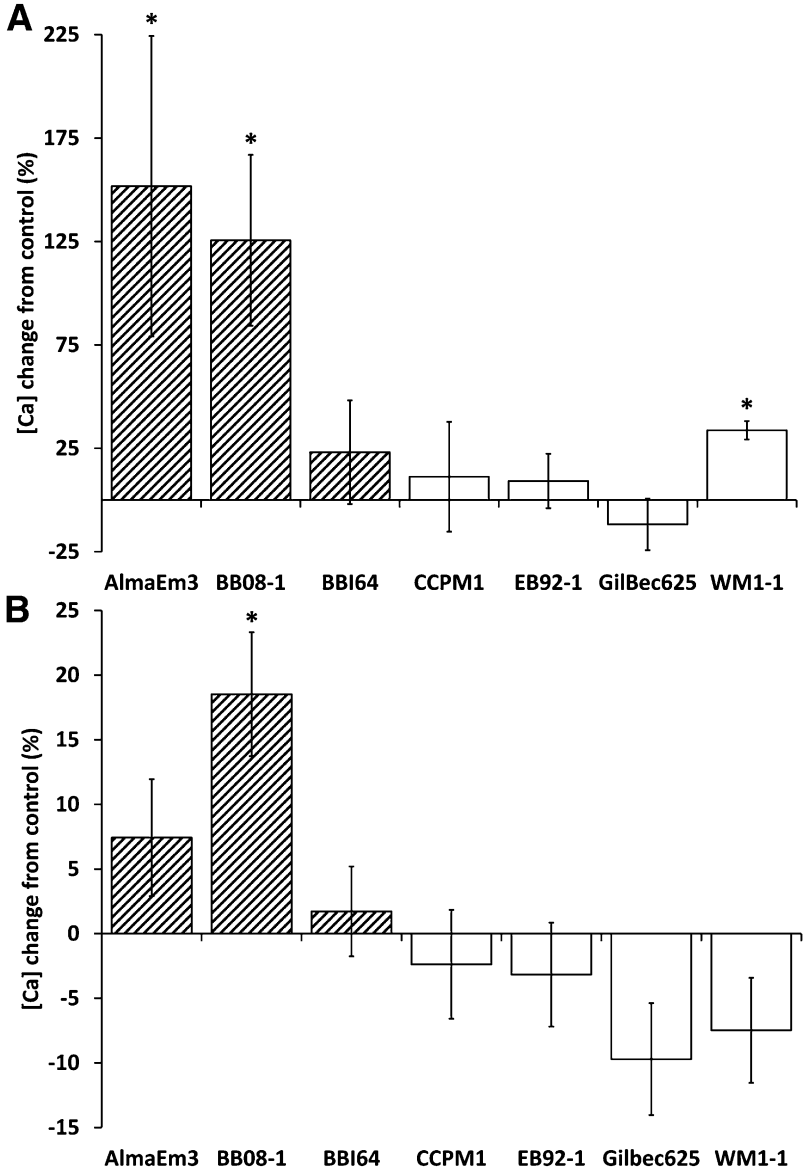

Fig. 3. Calcium concentration changes per isolate expressed as a percentage versus the buffer control. Shaded bars represent *Xylella fastidiosa* subsp. *multiplex* isolates and white bars represent *X. fastidiosa* subsp. *fastidiosa* isolates. Error bars represent standard error of the mean. **A,** Calcium changes in sap and **B,** calcium changes in leaf material. Significant increases versus buffer control (at $P < 0.05$) are indicated by an asterisk (*).

number of bacteria the plant xylem actually receives via the pinprick method is difficult to precisely estimate but it is likely to be higher than the amount introduced from vector inoculations; therefore, due to this difference, it is conceivable that *X. fastidiosa* subsp. *fastidiosa* may be more capable of infecting blueberry in the greenhouse than in a vector-inoculated field situation. The glassy-winged sharpshooter, which is reported to be the most common leafhopper species in southern highbush blueberry plantings in Georgia, is a generalist known to feed on over 100 species of woody and herbaceous plants (Tertuliano et al. 2012); however, host preferences can affect *X. fastidiosa* epidemiology in some cases (Redak et al. 2004). Though rabbit-eye blueberry crops have long been grown in the southeastern United States where *X. fastidiosa* and its vectors are endemic, the acreage of southern highbush blueberry has been rapidly expanding in recent years (Chang et al. 2009). Rabbit-eye blueberry seem to possess some level of resistance or tolerance and, even among southern highbush cultivars, there are apparent differences in *X. fastidiosa* susceptibility (Brannen et al. 2008; Chang et al. 2009); therefore, it is possible that the recent expansion of acreage planted with more susceptible cultivars has led to the recognition of BLS. *X. fastidiosa* disease symptoms can resemble drought stress and can be confused with several other problems which afflict blueberry (Brannen et al. 2008). Furthermore, the *X. fastidiosa* subsp. *fastidiosa* isolates used here caused less severe symptoms than most *X. fastidiosa* subsp. *multiplex* isolates, potentially making it harder to spot infection with these isolates in the field. It has recently been suggested that the *X. fastidiosa* subsp. *multiplex* isolates causing BLS on blueberry are intersubspecific recombinants (Nunney et al. 2014a); therefore, perhaps only after transfer of these intersubspecific recombinants to an appropriately susceptible cultivar was *X. fastidiosa* disease on blueberry recognized. To gain a better understanding of the potential of other *X. fastidiosa* isolates to cause disease on blueberry in field settings, including *X. fastidiosa* subsp. *fastidiosa* isolates, additional studies are necessary.

Overall, our study indicates the potential for *X. fastidiosa* subsp. *fastidiosa* isolates to cause disease on susceptible highbush blueberry cultivars, and indicates that virulence differences between isolates can be apparent, even among *X. fastidiosa* subsp. *multiplex* isolates from blueberry. Because it has been suggested that genetic selection of resistant blueberry cultivars may be a solution to this disease problem (Brannen et al. 2008), it is necessary to have an adequate understanding of the diverse pathogen pressures from divergent *X. fastidiosa* isolates that must be taken into account when identifying and selecting resistant cultivars. Our study, therefore, helps fill a gap in our knowledge of this emerging disease.

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