Diverse Responses of Lettuce Cultivars and Germplasm Lines to Infections of Three Isolates of Xanthomonas campestris pv. vitians

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Abstract. Bacterial leaf spot (BLS) disease, caused by Xanthomonas campestris pv. vitians (Xcv) has become an increasingly damaging disease in the lettuce production areas of the United States. To understand the nature of the outbreaks of this disease, the pathogenic variations for causing disease were evaluated on 29 lettuce cultivars and germplasm lines using three Xcv isolates recovered in different years from the Everglades Agriculture Area (EAA) of Florida. Significant differences were shown in both the BLS incidences and disease severities among the three Xcv isolates, and the rank from high virulence to less severity was L7 > JF196 > NF1. Our results suggest that the pathogenic variations of the isolates may have been associated with the epidemic outbreaks of BLS in EAA. Among the 29 lettuce genotypes, the host plant resistance was characterized by specific host genotype and Xcv isolate interactions. The leaf lettuce PI358001-1 was consistently in the high resistant category to all three Xcv isolates, and is a promising resistant source for development of resistant cultivars.

Lettuce (Lactuca sativa L.) is one of the top ten most valuable crops in the United States with an average annual value of $2.1 billion (USDA, 2014). There are eight types of lettuce grown in the world, whereas four of them including crisphead, butterhead, romaine, and leaf are the major market types in the United States. On average from a 5-year data (2009 through 2013), head, romaine, and leaf lettuce accounted for 55.0%, 30.7%, and 14.3% of the U.S. lettuce production, respectively (USDA, 2014). California and Arizona are the two largest growing states of lettuce, producing 96% of head and romaine lettuce, and 98% of leaf lettuce in the United States (Glaser et al., 2001). Lettuce is an important winter vegetable crop in Florida. The EAA is the main production area of the state with an annual acreage of 10,000–11,000 and a farm gate value of $50–$60 million. The warm and humid subtropical climate in Florida favors occurrence of biotic stresses such as diseases, which have significant, adverse impacts on lettuce production.

Bacterial leaf spot (BLS), caused by Xcv, is a devastating disease of lettuce in Florida. This disease was first reported in South Carolina and Virginia in the United States in 1918 (Brown, 1918). Since 1990s, substantial outbreaks of BLS have been reported with increasing frequency in some of the major lettuce growing regions, such as California (Barak et al., 2001; Bull et al., 2007; Bull and Koike, 2005; Unmesh et al., 1996), Florida (Lu and Raid, 2013; Pernezny et al., 2000) and Ohio (Sahin and Miller, 1997). Currently, there are no chemicals available to control the disease. A severe epidemic usually results in significant crop damage and economic losses.

Initial symptoms of BLS occur on mature, outer leaves with small water-soaked angular leaf lesions, and later become brown to black necrotic and papery (Koike and Gilbertson, 1997). The symptoms then spread to other parts of the lettuce plants, resulting in unmarketable heads or hearts. Epidemiology of the disease has been investigated on a number of factors associated with dispersal and survival of Xcv. The pathogen is thought to be seedborne and can survive on dry seeds for extended periods (Sahin and Miller, 1997). Xcv can be transmitted by lettuce plant debris in soil or leaves of symptomless weed species after the summer and winter fallow periods in California (Barak et al., 2001). Xcv has also been demonstrated to be spread rapidly by overhead irrigation in greenhouse from only a few infected plants (Toussaint, 1999). A broad list of potential host plant species has been provided after inoculation of these plants with Xcv (Tsuchiya et al., 1981). Robinson et al. (2006) identified pepper as a potential crop host of Xcv of lettuce in Florida and also determined that the optimum temperature for Xcv infection is 22.7 °C under growth chamber conditions.

Nevertheless, virulence diversity of Xcv strains and variation of host responses among lettuce genotypes are very important epidemiological aspects for understanding and controlling BLS outbreaks. Structure differences of DNA, RNA, and other biochemical factors among Xcv strains have been investigated (Barak and Gilbertson, 2003; Sahin et al., 2003). Pernezny et al. (1995) found that symptoms in plants inoculated with the five Florida strains individually were more severe than other three Xcv reference strains from outside of the state, whereas, although the three lettuce genotypes tested were susceptible to Xcv, the disease reactions of crisphead cv. South Bay was less severe than romaine cv. Tall Guzmaine and butterhead breeding line B-1190. Different levels of host resistance have been reported in commercial lettuce cultivars or germplasm by inoculation with single Xcv strain (Carisse et al., 2000; Lu and Raid, 2013; Pernezny et al., 2000) or a mixture of Xcv strains (Bull et al., 2007; Hayes et al., 2014; Sahin and Miller, 1997). However, information is limited on responses of resistant lettuce genotypes to different Xcv strains. In addition, there have been no reports on change of pathogenic virulence of Xcv strains recovered in severe epidemic years and nonepidemic years in the same region.

The objectives of this study were to 1) investigate pathogenic differences of Xcv strains isolated in various years in the EAA of Florida with BLS outbreaks or without a severe disease occurrence and 2) determine reactions of susceptible and resistant lettuce genotypes to the different Xcv strains.

Materials and Methods

Pathogenicity tests were conducted according to the method reported by Lu and Raid (2013). The tests were repeated twice. For the first trial, seeds were sown on 2 Oct. 2014, seedlings were inoculated with three Xcv isolates on 30 Oct. 2014, and plants were scored on 17 Nov. 2014. For the second trial, seeds were sown on 8 Oct. 2014, seedlings were inoculated on 5 Nov. 2014, and plants were scored on 24 Nov. 2014. The experiment design was a split-plot design with Xcv isolate being main plots and lettuce genotypes being subplots and had three replications.

Plant materials. A total of 30 lettuce genotypes consisting of 10 crisphead, 13 romaine, 4 butterhead, 2 leaf, and 1 latice lettuce as a resistant control were used for pathogenicity evaluation (Table 1). Seeds of each genotype were sown in a single row with 10 rows for 10 genotypes in a 30 × 50 cm flat tray filled with Fafard growing mix containing 65.75% Canadian sphagnum peat, moss, perlite, and composted tree bark (Sun Gro
The disease incidence was calculated using the 0–4 rating system as reported by Bull et al. (2007) with modifications: 0 = no symptoms, 1 = one to 10 lesions with each diameter less than 3 mm, 2 = more than 10 lesions with each diameter less than 3 mm, 3 = lesions of which diameter larger than 3 mm, and 4 = coalesced lesions. In each row, the average disease severity value was calculated from five random plants. Genotypes with an average score of 0 to 0.9 for disease severity were classified as highly resistant, 1.0 to 1.9 was moderately resistant, 2.0 to 2.9 as moderately susceptible, and higher than 3.0 as susceptible.

Data analysis. Data were analyzed using SAS software (SAS/Stat procedures, Version 9.3; SAS Institute, Inc., Cary, NC). Analysis of variance (ANOVA) using the PROC GLM command in SAS was conducted to calculate the effects of trials, lettuce genotypes and Xcv isolates on BLS disease incidence and severity. Data on disease incidences (%) were transformed by arcsine square root before ANOVA. Least significant difference method at the 5% significant level was used for multiple comparisons for disease incidence and severity among 29 genotypes in each trial within the three Xcv isolates and also among three Xcv isolates in each trial.

Results and Discussion

Pathogenicity difference among Xcv isolates. Significantly different BLS incidence and severity were observed among three Xcv isolates which were collected in different years in EAA, Florida (Table 2). Variances of interactions between trial and Xcv isolate and between genotype and Xcv isolate were also significant for both BLS incidence and severity rate, whereas variance of trial x genotype x Xcv isolate interactions was significant only for the BLS incidence (Table 2). Based on overall means of 29 genotypes, L7 was the most virulent Xcv isolate, causing symptoms on 81.1% of plants in trial 1 and 87.6% of plants in trial 2 (Table 2). Plants had an average disease severity of 1.75 in trial 1 and an average disease severity of 2.91 in trial 2. Disease incidences were 69.0% in trial 1 and 74.0% in trial 2 and disease severities were 1.16 and

### Table 1. Lettuce genotypes used for pathogenicity evaluation.

| Serial No. | Cultivar name | Horticultural type | Source |
|------------|---------------|--------------------|--------|
| 1          | 10157         | Romaine            | This study |
| 2          | 10160         | Crisphead          | This study |
| 3          | 10168         | Romaine            | This study |
| 4          | 10176         | Romaine            | This study |
| 5          | 10178         | Romaine            | This study |
| 6          | 50100         | Romaine            | This study |
| 7          | 50664         | Crisphead          | This study |
| 8          | 60157         | Crisphead          | This study |
| 9          | 60167         | Crisphead          | This study |
| 10         | 60182         | Romaine            | This study |
| 11         | 60185         | Romaine            | This study |
| 12         | 70096         | Romaine            | This study |
| 13         | 8074          | Crisphead          | GMI (unpublished data) |
| 14         | 9085          | Crisphead          | GMI (unpublished data) |
| 15         | Floricos83    | Romaine            | Guzman and Zitter (1983) |
| 16         | Gator         | Crisphead          | Seminis Vegetable Seeds, Inc. (USDA, 2015a) |
| 17         | Glades        | Romaine            | 3 Star Lettuce, LLC (USDA, 2015a) |
| 18         | hi078         | Crisphead          | unknown |
| 19         | Lantana       | Crisphead          | 3 Star Lettuce, LLC (USDA, 2015a) |
| 20         | Little Gem    | Lettuce            | Burpee Gardens (USDA, 2015b) |
| 21         | Manatee       | Romaine            | 3 Star Lettuce, LLC (USDA, 2015a) |
| 22         | Okeechobee    | Romaine            | 3 Star Lettuce, LLC (USDA, 2015a) |
| 23         | PI274373-1    | Butterhead         | USDA-ARS germplasm collection (USDA, 2015c) |
| 24         | PI289016-2    | Leaf               | USDA-ARS germplasm collection (USDA, 2015c) |
| 25         | PI289025-1    | Butterhead         | USDA-ARS germplasm collection (USDA, 2015c) |
| 26         | PI342512-1    | Butterhead         | USDA-ARS germplasm collection (USDA, 2015c) |
| 27         | PI342532-1    | Butterhead         | USDA-ARS germplasm collection (USDA, 2015c) |
| 28         | Raleigh       | Crisphead          | Guzman (1984) |
| 29         | Terrapin      | Romaine            | Seminis Vegetable Seeds-Genecorp (USDA, 2015b) |
| 30         | PI358001-1    | Leaf               | USDA-ARS germplasm collection (USDA, 2015c) |

### Table 2. Analysis of variance for 29 lettuce genotypes infected with three isolates of Xanthomonas campestris pv. viticola in two trials.

| Source      | DF  | Mean Square | F value | Pr > F | Mean Square | F value | Pr > F |
|-------------|-----|-------------|---------|--------|-------------|---------|--------|
| Trial (T)   | 1   | 7,235.58    | 36.51   | <0.0001| 64.91       | 194.78  | <0.0001|
| Rep/T       | 1   | 296.43      | 1.50    | 0.2230 | 0.61        | 1.84    | 0.1762 |
| Isolate (I) | 2   | 28,964.19   | 146.14  | <0.0001| 55.50       | 166.55  | <0.0001|
| Genotype (G)| 28  | 3,033.05    | 15.30   | <0.0001| 3.53        | 10.60   | <0.0001|
| T x I       | 2   | 1,029.48    | 5.19    | 0.0065 | 1.90        | 5.70    | 0.0040 |
| T x G       | 28  | 353.59      | 1.78    | 0.0136 | 0.99        | 2.99    | <0.0001|
| G x I       | 56  | 419.22      | 2.12    | 0.0001 | 0.64        | 1.93    | 0.0007 |
| T x G x I   | 56  | 432.83      | 2.18    | <0.0001| 0.32        | 0.96    | 0.5649 |
| Error       | 170 | 198.19      |         |        | 0.33        |         |        |
Table 3. Average disease incidences and severities of 29 lettuce genotypes infected with three isolates of *Xanthomonas campestris pv. vititana* in two repeated trials.

| Isolate | Disease incidence1 (%) | Disease severity2 | Disease incidence (%) | Disease severity |
|---------|------------------------|-------------------|------------------------|-----------------|
| JF196   | 60.55 b [69.03] b       | 1.16 b            | 65.45 b [74.03] b      | 1.94 b          |
| L7      | 71.28 a [81.11] a       | 1.75 a            | 77.96 a [87.65] a      | 2.91 a          |
| NF1     | 35.17 c [36.49] c       | 0.62 c            | 50.98 h [57.97] c      | 1.27 c          |

1. Disease incidence was expressed as percentage of plants exhibiting infection.
2. Disease severity was evaluated using the rating system as described in Bull et al. (2007) with modifications: 0 = no symptoms on a plant; 1 = 1–10 lesions of less than 3 mm; 2 = more than 10 lesions of less than 3 mm; 3 = more than 3 mm; and 4 = coalesced lesions.
3. Means in the column followed by the same letter are not significantly different (a = 0.05) using a least significant difference test (Version 9.3; SAS Institute, Cary, NC).

1.94 in the two trials when plants were inoculated with Xcv isolate JF196. The Xcv isolate NF1 caused symptoms on 36.5% of plants with an average disease severity of 0.62 in trial 1 and 57.9% of plants with a disease severity of 1.27. Therefore, L7 was the most virulent Xcv isolate to lettuce and NF1 was the least virulent isolate. This indicated that there were pathogenic differences among the Xcv isolates collected from EAA. Our results were in accordance with those of Sahin et al. (2003) and Barak and Gilbertson (2003). Sahin et al. (2003) tested 44 Xcv strains on a susceptible romaine lettuce cv.绿 and conducted biochemical and molecular tests. They found that the Xcv strains could be divided into two distinct groups based on the pathogenic variation and structure diversity. Genetic diversity was measured among 48 Xcv strains preliminarily recovered in California in 1990s through pathogenic testing on a susceptible crisphead type lettuce cv. Salinas, and through analyses of the restriction fragment length polymorphisms (RFLPs) and plasmid DNA probe (Barak and Gilbertson, 2003). The genetic variability existed not only among strains from different geographical regions, but also among strains from the same location. The authors demonstrated that the population structure of Xcv strains were not clonal, but composed of relatively homogeneous groups.

The three Xcv isolates were recovered from the fields in EAA area in different years when there were different levels of BLS outbreaks. The Xcv isolate L7 was recovered from lettuce samples with BLS symptoms in 1993 when a severe BLS outbreak occurred. JF196 was isolated from lettuce leaf samples with viable BLS disease symptoms in late season of 2012 when the lettuce production was already finished. NF1 was recovered from lettuce samples in a field with a minor outbreak in Feb. 2014, which was in the middle season of lettuce production in EAA, FL. Our results suggested that much virulent Xcv pathogen (L7) may have led to the severe BLS epidemic in 1993. On the contrary, NF1 was the least virulent isolate and only caused light disease (disease severity 0.62 in trial 1 and 1.27 in trial 2), which was probably the main reason why the disease occurring in the middle season (Feb. 2014) did not spread to other lettuce fields to cause an extensive outbreak. However, it is not known what has caused significantly different pathogenicity in the same location. Because Xcv can be seedborne, infected seed transmission from different geographic regions could have resulted in change of strains in EAA. Genetic exchanges between different Xcv strains, other pathovars and species, and mutation in Xcv were also possible. Using RFLP markers, high genetic diversity of the cassava (Manihot esculenta Crantz) bacterial pathogen *Xanthomonas campestris pv. manihotis* strains were detected within most of the field samples in less than one year, as significant changes of strain genotypes in pathogen population occurred during a single crop cycle (Restrepo et al., 2004). Pathogen races of X. a. pv. *vesicatoria* changed within a season in pepper fields (Kousik and Ritchie, 1996; Vauterin et al., 1996). Host ranges and environmental factors such as rainfall and temperature may also have a critical influence on the strain shifts (Scortichini, 2005).

Responses of lettuce genotypes to different Xcv isolates. A wide range of susceptible and resistant lettuce genotypes representing five horticultural types were tested in this study. Significant differences of BLS incidence and severity rate were observed among the 29 genotypes (Table 2). In addition to the interaction effects of genotype × Xcv isolate and trial × genotype × Xcv isolate described previously, trial × genotype interaction was significant for the BLS incidence and severity rate (Table 2). Disease incidence percent and severity rate generally were higher in the second trial than the first one, indicating that the plants and environmental factors in the second trial were more favorable for infection of Xcv and for disease development. Differences in disease occurrence between the two repeated trials have been previously observed in the greenhouse studies (Pempezy et al., 2000) and in the field evaluations (Bull et al., 2007). Taking overall results of plant disease incidence percent and severity rate for individual genotypes in the two trials (Figs. 1 and 2), leaf lettuce ‘PI358001-1’ expressed the highest resistance to all three Xcv isolates, having disease incidence 18% and disease severity 0.3 in trial 1 and disease incidence 22% and disease severity 0.4 in trial 2 when inoculum was JF196, disease incidence 20% and disease severity 0.3 in trial 1 and disease incidence 25% and disease severity 0.4 in trial 2 when infected with L7, and disease incidence 5% and disease severity 0.1 in trial 1 and disease incidence 15% and disease severity 0.2 in trial 2 when inoculated with NF1. ‘PI358001-1’ had much stronger disease resistance than the resistant control ‘Little Gem’ and it was classified as a highly resistant lettuce (Fig. 2). This confirmed a previous observation of Lu et al. (2014) who reported that ‘PI358001-1’ was a source of high resistance. In four cases (Fig. 2), ‘Little Gem’ had a disease severity higher than 1.0 but lower than 2.0, indicating it was a moderately resistant cultivar. When plants were inoculated with isolate L7, the BLS severity rates for ‘Terrapin’, ‘Lantana’, ‘Okeechobee’, ‘Manatee’, ‘8074’, ‘70096’, ‘Gator’, ‘Glades’, and ‘Raleigh’ were significantly higher than that for ‘Little Gem’ (Fig. 2). These results were in agreement with those of Lu and Raid (2013). Because NF1 was a weak Xcv isolate, all but five lettuce genotypes (‘10178’, ‘50100’, ‘h1078’, ‘Okeechobee’, and ‘Terrapin’) exhibited various degrees of resistance (Fig. 2C). A total of 12 genotypes (2A) had high or moderate resistance to the Xcv isolate JF196 which had virulence between L7 and NF1. In addition to ‘PI358001-1’, the butterhead lettuce ‘PI342512-1’ was also found to have higher levels of resistance to all three Xcv isolates than ‘Little Gem’ (Fig. 2). However, the disease severities of ‘PI342512-1’ were lower than 1.0 in some cases and higher than 1.0 in other case. In an early study (Lu et al., 2014), ‘PI342512-1’ was classified as a moderately resistant lettuce. Further evaluations are needed to determine whether ‘PI342512-1’ is a highly resistant or a moderately resistant lettuce.

All crisphead and romaine lettuces were moderately susceptible or susceptible to L7 (Table 4). Romaine lettuces were generally more susceptible to L7 than crisphead lettuces. However, four resistant sources were identified among the butterhead, leaf, and latine lettuce types. The results were in agreement with the BLS reactions of lettuce types to the Xcv isolate L7 discovered in Pempezy et al. (2000), Lu and Raid (2013), and Lu et al. (2014). According to Mikel (2007), out of the 328 lettuce cultivars which were registered in the United States from 1970 through 2004, 61.6% were crisphead, 19.5% loose leaf, 13.7% romaine, 4.9% butterhead, and 0.3% latine lettuce types. Majority of lettuce cultivars in the United States are likely to be vulnerable to the severe BLS epidemics. Therefore, new cultivars with improved resistance to BLS should be developed by incorporating resistant genes from resistant leaf, butterhead, and latine types into crisphead and romaine lettuce. On the other hand, it was demonstrated that 30% of crisphead lettuce and 23.1% of romaine lettuce were moderately resistant to Xcv isolate JF196, and even higher percent of these two types showed resistance to Xcv isolate NF1 (Table 4), indicating that plants respond differently to different Xcv isolates. We found that some commercial cultivars such as Gator and Glades had low disease severities under...
infections of the two less virulent isolates NF1 and JF196 (Fig. 2A and C). It is thus possible to identify commercial cultivars that are resistant to multiple Xcv strains with lower virulence. Growing such cultivars in the areas where BLS is frequently a problem can provide sufficient protection of the crop in most of years because in those years the Xcv strains present in the fields are less virulent.

Strategies of resistant cultivar development.

The goal of breeding BLS-resistant lettuce cultivars is to develop cultivars with high level resistance that can protect lettuce from damage by Xcv strains that are highly virulent. The germplasm line ‘PI358001-1’, which is a leaf lettuce, had low disease incidences and disease severities under infection of any of the three Xcv isolates (Figs. 1 and 2). These observations were consistent with a previous report by Lu et al. (2014). All results indicate that this line has high level resistance and the resistance is stable across Xcv strains. It appears that the line is a good source for development of resistant cultivars. The butterhead lettuce ‘PI342532’ performed similarly as ‘PI342512-1’ in reaction to Xcv isolates. It is not known whether a single one of the two butterhead lettuces can be used to develop resistant cultivars that will provide sufficient protection of the crop under severe BLS epidemic conditions. The resistant control ‘Little Gem’ has been found to be moderately resistant based on disease severities in many studies (Bull et al., 2007; Lu et al., 2014; Lu and Raid, 2013). In the present study, it was moderately resistant to all three Xcv isolates and its resistance was stable across the isolates (Fig. 2). Like the two resistant butterheads,

Fig. 1. Disease incidence of 29 lettuce genotypes infected by 3 isolates of Xanthomonas campestris pv. viticans (Xcv) in two repeated trials [(A) Xcv isolate JF196, (B) Xcv isolate L7, and (C) Xcv isolate NF1]. The solid line on each bar is the standard deviation.
damage will occur under BLS epidemic conditions if resistance in ‘Little Gem’ is used for protection of the crop. Because high resistance with disease severity less than 1.0 on the 0–4 scale in single lettuce genotypes is uncommon, other breeding strategies should be considered. In a recent paper, Hayes et al. (2014) reported that the BLS-resistance in lettuce cultivar La Brillante was controlled by a single gene named Xarl on linkage group (chromosome) 2 and resistance in cultivars Pavane and Little Gem was likely controlled by the same gene. There were evidences showing that chromosome 2 may carry gene(s) for BLS-resistance in many germplasm lines that were evaluated by Lu et al. (2014). Lu et al. (2014) also found that chromosome 4 may harbor gene(s) for high level resistance in ‘PI358001-1’. If future research identifies different genes on chromosome 2 for resistances

Fig. 2. Disease severity of 29 lettuce genotypes infected by 3 isolates of Xanthomonas campestris pv. viticola (Xcv) in two repeated trials [(A) Xcv isolate JF196, (B) Xcv isolate L7, and (C) Xcv isolate NF1]. Disease severity was evaluated using the rating system as described in Bull et al. (2007) with modifications: 0 = no symptoms on a plant; 1 = 1–10 lesions of less than 3 mm; 2 = more than 10 lesions of less than 3 mm; 3 = lesions of greater than 3 mm; and 4 = coalesced lesions. The solid line on each bar is the standard deviation.
in different lettuce cultivars or germplasm lines, gene pyramiding approach can be used for development of high resistant cultivars. Likewise, if the resistance in 'PI358001-1' is indeed conferred by gene(s) on chromosome 4, lettuce cultivars with even higher resistance can be developed by combining the resistant gene(s) on chromosomes 2 and 4 into a single cultivar background.

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