Bird's Eye Lesions of Tomato Fruit Produced by Aerosol and Direct Application of *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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Development of the bird's eye fruit lesion of tomato was studied by inoculating flowers and the surface of young tomato fruit with strains of *Clavibacter michiganensis* subsp. *michiganensis*. Flowers were sprayed once or twice with *C. michiganensis* subsp. *michiganensis* at 10^8 CFU/ml. The maximum incidence (80%) and severity (12 spots/fruit) of spotted fruit resulted when inoculum was sprayed twice, 3 days apart. Flowers were most susceptible to infection 2 days after anthesis. When a paintbrush was used to apply inoculum to the surface of small fruit, a large number of fruit spots (≤ 456 spots/fruit) resulted. Even strains determined to be avirulent based on a tomato stem inoculation assay and a hypersensitive response on four-o'clock leaves (*Mirabilis jalapa*) were able to produce fruit spots, although at a reduced level. The inoculation methods developed in this study can provide opportunities to observe subtle host-pathogen interactions between *C. michiganensis* subsp. *michiganensis* strains and tomato and to help formulate methods to quantify infection.

Additional keywords: avirulence, bacterial canker, rep-PCR

Bacterial canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis*, is a concern for tomato growers because the disease can drastically reduce tomato yield and quality (4,11,13). Symptoms of disease caused by the systemic, gram-positive pathogen include necrosis of the leaf margin, stunting, wilting, vascular discoloration, and plant death (17). Disease symptoms on the fruit include small, raised, superficial necrotic spots surrounded by white halos. These lesions, referred to as bird's eye lesions (3,17), can cause economic losses in fresh market tomatoes due to reduced fruit quality (3).

Studies in which flowers and fruit were inoculated with bacterial pathogens have demonstrated the importance of natural openings (5,7-9) and injured tissue (16) in fruit infection. Bacterial speck lesions, caused by *Pseudomonas syringae* pv. *tomato*, on tomato fruit developed following the external inoculation of flowers during and after anthesis (7,8). Watermelon fruit lesions caused by *Acidovorax avenae* subsp. *citrulli* were reduced when infection

courts on the fruit were covered with wax (9). Pepper fruit, wounded by sandblasting and inoculated with *Xanthomonas campestris* pv. *vesicatoria*, showed an increased number of spots per fruit when compared to nonwounded, inoculated fruit (16). A study by Ehrig and Griesbach (5) reported that bird's eye lesions of tomato are the result of external infection rather than internal systemic infection. However, their report did not describe the role of inoculum in relation to flower and fruit development.

Prevention of bacterial canker includes the use of certified seed, healthy transplants, disinfested greenhouse materials and facilities, and rotation away from tomatoes for at least 2 years (12). Although the application of bactericides to seedlings in the greenhouse was shown to reduce yield losses due to *C. michiganensis* subsp. *michiganensis* infection (13), a management program to reduce incidence of fruit spotting has not been developed. Therefore, to control bird's eye lesions, a better understanding of the infection process is required. The objective of this study was to determine the earliest stage of flower or fruit development that is susceptible to infection by *C. michiganensis* subsp. *michiganensis* in order to devise strategies that may reduce fruit spotting in the field.

MATERIALS AND METHODS

Inoculum preparation and flower inoculation. The *C. michiganensis* subsp. *michiganensis* strains used in each experi-

ment represent distinct polymerase chain reaction (BOX-PCR) fingerprint types designated as type A (Cmm 292) or type B (Cmm 68) (15). Cmm 68 and Cmm 292 were isolated from fruit lesions from Ohio (in 1987) and Michigan (in 1993) fields, respectively. Pathogenicity tests on tomato plants and the hypersensitive response (HR) on four-o'clock leaves (*Mirabilis jalapa*; 10) indicated that both strains were virulent (15). Both strains were stored in glycerol stock cultures (1 ml of broth culture per 0.5 ml of 40% glycerol) kept at -80°C and recovered at the start of each experiment by streaking onto nutrient broth yeast extract agar (NBY) plates (6).

Inoculum was prepared by inoculating NBY broth with 5 ml of a 48-h-old culture in NBY broth of either bacterial strain and placing it on a shaker at 200 rpm at room temperature for 48 h. Using NBY broth as the dissolvent, a serial dilution of each culture was conducted and spectrophotometer readings were performed at 600 nm to each dilution series. The serial dilution that corresponded to 50% transmittance was used as a reference to calculate the volume of the original culture necessary to prepare a diluted culture of approximately 10^8 CFU/ml. The diluted culture was centrifuged in a desktop clinical centrifuge (IEC Clinical Centrifuge, Needham Hts., MA) for 15 min at maximum speed and the pellet was suspended in sterile distilled water, recentrifuged, and the cells resuspended in 100 ml of sterile distilled water. The final concentration of the inoculum used for all tomato flowers inoculations ranged from 3×10^7 to 3×10^8 CFU/ml. Prior to flower inoculation, each bacterial suspension prepared was tested for its ability to induce an HR, a condition assumed to correlate with virulence of the pathogen (15).

Two tomato transplants of the susceptible processing cultivar Heinz 8704 (H. J. Heinz Co., Stockton, CA) were set in 31-cm-diameter clay pots filled with commercially prepared potting media (Baccto; Michigan Peat Co., Houston, TX). Tomato plants were grown in the greenhouse for approximately 8 weeks under sodium vapor lights with a photoperiod of 18 h. Greenhouse temperatures ranged from 25 to 30°C (day) and 18 to 22°C (night). Tomato plants were fertilized twice each week with a 200-ppm solution of Peters 20-20-20 (Scotts, Allentown, PA).

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Inoculations were performed by applying approximately 0.7 ml of the washed *C. michiganensis* subsp. *michiganensis* suspension per flower using an air-pressurized sprayer (Preval, Precision Valve Co., Yonkers, NY); sterile distilled water was used as a control. A small circle, approximately 25 mm in diameter and large enough to cover a single flower with a wide open corolla, was cut in the bottom of a clear plastic cup (60 ml). Individual flowers were inserted into the bottom of the cup before applying the inoculum or sterile distilled water, to direct the fine mist toward each flower and to limit the amount of aerosol landing on adjacent flowers and tissues.

Inoculation of flowers and bagging.

The first treatment consisted of a single inoculation of two to four flowers per inflorescence when the corollas were fully expanded. The second treatment consisted of two inoculations, the first when the corollas were fully expanded and the second 3 days later. The third and fourth treatments were inoculated in the same manner as treatments 1 and 2 above and, following each inoculation, individual clusters of flowers were covered with clear plastic bags (18 by 18 by 6 cm) for 16 to 18 h. For every treatment mentioned above, sterile distilled water was sprayed as a control. The experiment was conducted twice, and the repetitions are hereafter referred to as experiments 1 and 2.

An average of 66 and 30 flowers were used for experiments 1 and 2, respectively. *C. michiganensis* subsp. *michiganensis* strains (Cmm 68 and Cmm 292) were used as inocula in experiment 1 and 2, respectively. Three weeks after the second inoculation for treatments 3 and 4, all fruit were inspected for bird's eye lesions. The number of fruit infected and the number of spots per fruit were recorded. The average number of spots per fruit for each treatment from both experiments were analyzed by using Kruskal-Wallis analysis of variance (ANOVA) by ranks ($P < 0.05$) and differences between treatments were identified using pairwise comparison by Dunn's method (SigmaStat, SPSS, Inc., Chicago, IL). These methods were used because data were not normally distributed and there was no appropriate transformation (14).

A subsample of fruit spots was randomly sampled to confirm that developing lesions were caused by the specific inoculum sprayed. Fruit spots were excised using a sterile scalpel and placed in a droplet of sterile distilled water for 5 s. Each droplet was streaked onto NBY plates and incubated for 96 h at room temperature. After repeated streaking to obtain pure cultures, the colonies resembling *C. michiganensis* subsp. *michiganensis* were subjected to repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting (15) to demonstrate that the BOX-PCR genotype

was the same as that of the inoculum used in each experiment.

Inoculation of fruit. Bacterial suspensions were prepared as described above and directly applied to the entire surface of young green fruits 10 to 15 mm in diameter using a #2, artist-style, camel's hair paintbrush. Ten *C. michiganensis* subsp. *michiganensis* strains were selected based on their BOX-PCR fingerprint type and virulence rating (15). *C. michiganensis* subsp. *michiganensis* strains 299 (type A), 68R (type B), 68 (type B), and 285 (type D) are classified as virulent; T33 (type A), 236 (type C), and 56 (type C) represent strains with intermediate virulence; and 363 (type A), 208 (type A), and 133 (type A) are classified as avirulent (15). Each bacterial suspension was applied to a maximum of five fruit, with only one bacterial strain per fruit. The suspensions were simultaneously tested for HR on four-o'clock leaves. Five fruit were brushed with sterile distilled water as a control. This experiment was conducted three times. Four weeks after fruit inoculation, the number of infected and noninfected fruit and the number of spots per fruit were recorded. The average number of spots per fruit for each strain was statistically analyzed as described above. Five fruit spots per fruit, representing each bacterial strain used in the study, were arbitrarily selected and excised as described above. Pure culture colonies resembling *C. michiganensis* subsp. *michiganensis* were subjected to rep-PCR fingerprinting to confirm the BOX-PCR genotype of the inocula.

Flower development stage in relation to fruit spots. A bacterial suspension (approximately 10^8 CFU/ml) of strain Cmm 68 was prepared and applied to five

flower developmental stages as defined using anthesis (pollen maturity) as the reference point. Each flower at the desired developmental stage was inoculated once and its inflorescence was covered with a clear plastic bag (18 by 18 by 6 cm) for 16 to 18 h. Bhadula and Sawhney (1) established that tomato calyces 11.0 to 12.0 mm in length with an open corolla corresponded to flowers at anthesis. Therefore, the first stage of flower development, considered to be about 5 days prior to anthesis, consisted of buds with closed calyces approximately 11 to 12 mm in length. The flower developmental stages inoculated in this experiment were five (–5) and three (–3) days preanthesis, anthesis (0), two (+2) and four (+4) days postanthesis, corresponding to closed calyx, open calyx, partially open corolla, fully open corolla, and half-closed corolla, respectively (Fig. 1). For each flower developmental stage, sterile distilled water was used as a control. The experiment was conducted three times with an average of 57, 65, and 66 flowers available for each experiment. When the youngest fruit reached 27 mm in diameter, the number of infected and noninfected fruit and the number of spots per fruit were recorded. The average number of spots per fruit for each experiment was statistically analyzed as previously mentioned. Colonies resembling *C. michiganensis* subsp. *michiganensis* were isolated from fruit spots and subjected to rep-PCR fingerprinting.

RESULTS

Inoculation of flowers. At 6 to 8 days after inoculation of flowers with open corollas, white lesions appeared on fruit that were 10 to 12 mm in diameter. At 3 to 5 days after the white lesions appeared, they

Fig. 1. The five developmental stages of tomato flowers inoculated with *Clavibacter michiganensis* subsp. *michiganensis* to determine the susceptibility of flowers to infection: –5 = 5 days preanthesis (closed calyx), –3 = 3 days preanthesis (open calyx), 0 = anthesis (partially open corolla), +2 = 2 days postanthesis (fully open corolla), and +4 = 4 days postanthesis (half-closed corolla).

became necrotic (Fig. 2), resembling the typical bird's eye lesion. Some fruit spots were atypical of bird's eye lesions in that they developed necrosis but lacked a white halo. All lesions were circular, approximately 2 mm in diameter, and superficial, with a raised, blister-like appearance. Rep-PCR assays confirmed that bacteria isolated from the fruit spots were the same *C. michiganensis* subsp. *michiganensis* strains used as inoculum.

The percent of fruit with lesions and the number of lesions per fruit were a function primarily of the number of inoculations each flower received (Table 1). Fruit developing from flowers that were inoculated twice had the highest percent of fruit with lesions ($\geq 53\%$) and the highest number of spots (≥ 7) per fruit. When flowers were inoculated once, a maximum of 32% of fruit exhibited an average of 0.2 to 2 spots per fruit. Bagging flowers to maintain high humidity following inoculation did not increase the number of spots per fruit; however, the incidence of symptomatic fruit increased in the 1 $\times$ -inoculated treatment with bagging (Table 1). Similar results were observed with all strains tested. No fruit spots developed on control flowers sprayed with sterile water.

Inoculation of fruit. Fruit spotting occurred after green fruit were inoculated using a paintbrush dipped in *C. michiganensis* subsp. *michiganensis* (Table 2). Strains determined to be virulent in previous studies (16) and reacting positively in the HR assay in this study caused spots on 80 to 100% of the inoculated fruit. Strains classified as intermediate in virulence and causing positive reactions in HR assays resulted in 67 to 100% of the inoculated fruit developing spots. Strains determined to be avirulent in previous studies and producing negative reactions in HR assays resulted in 60 to 100% of the inoculated fruit developing spots. No spotted fruit resulted when the surfaces of fruit were brushed with sterile water. Generally, the number of spots per fruit was higher on fruit inoculated with virulent and intermediate strains than on fruit inoculated with avirulent strains. No significant fruit abortion occurred as a result of brushing the young fruit with the paintbrush (*data not shown*).

Flower development stage in relation

to fruit spot development. A minimum of 15% of the fruit produced from flowers inoculated 2 or 4 days after anthesis developed characteristic bird's eye lesions (Table 3). Inoculation at these times also

resulted in the highest numbers of spots per fruit. Flowers inoculated prior to anthesis rarely developed fruit spots. No fruit spot lesions were observed when flowers were inoculated with sterile water.

Fig. 2. Tomato fruit showing bird's eye lesions **A**, 14 days after *Clavibacter michiganensis* subsp. *michiganensis* inoculum was sprayed on stage 0 flowers and **B**, 10 days after inoculum was applied using an artist's paintbrush to the surface of young green fruit (13 mm in diameter). Arrow indicates a fully developed bird's eye lesion, where dark necrotic tissue is surrounded by a white halo. Scale bars = 1 cm.

Table 2. Fruit spot development after brushing *Clavibacter michiganensis* subsp. *michiganensis* on the surface of young tomato fruit^a

Virulence, strain ^z	Fruit with lesions (%)			No. spots/fruit ^y		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Virulent						
Cmm 299	100	100	100	60 a	132 a	212 a
Cmm 285	...	100	100	...	225 a	141 a
Cmm 68R	100	100	100	66 a	326 a	210 a
Cmm 68	...	100	80	...	456 a	178 a
Intermediate						
Cmm T33	67	100	100	45 a	178 a	241 a
Cmm 236	100	...	100	109 a	...	148 a
Cmm 56	100	...	100	24 a	...	389 a
Avirulent						
Cmm 363	...	100	60	...	24 a	32 b
Cmm 208	80	100	100	15 a	40 a	179 a
Cmm 133	75	...	60	27 a	...	56 a

^a Five green fruit were included in experiments 1 and 3, and three fruit in experiment 2; ... indicates strain not included in experiment.

^y Statistical analysis using Kruskal-Wallis analysis of variance by ranks and pairwise multiple comparison Dunn's method. Values for each experiment were not significant at $P \leq 0.05$. $n = 35$, 21, and 40 for experiment 1, 2, and 3, respectively.

^z Virulence rating as described by Louws et al. (15): Virulent = producing canker and wilt; Intermediate = producing canker only; and Avirulent = producing no symptoms by stem inoculation assays.

Table 1. Fruit spot development after inoculating open corollas of flowers with strains of *Clavibacter michiganensis* subsp. *michiganensis*

Treatment		No. flowers inoculated ^x		Total fruit		Fruit with lesions (%)		No. spots/fruit ^y	
Inoculation	Bag ^z	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
1 $\times$	–	31	81	29	46	14	9	0.4 a	0.2 a
2 $\times$	–	31	128	30	61	80	70	12 b	7 b
1 $\times$	+	36	64	31	29	32	28	2 a	1 a
2 $\times$	+	29	122	23	72	78	53	7 b	7 b

^x Flowers with open corollas sprayed with *C. michiganensis* subsp. *michiganensis* strains Cmm 68 and Cmm 292 for experiments 1 and 2, respectively.

^y Statistical analysis using Kruskal-Wallis analysis of variance by ranks and pairwise multiple comparison Dunn's method. Values for both test were significant at $P \leq 0.05$.

^z Incubation of *C. michiganensis* subsp. *michiganensis* on open corollas after covering individual inflorescences with clear plastic bags for 16 to 18 h after inoculation.

Table 3. Fruit spotting after inoculating flowers at various developmental stages with *Clavibacter michiganensis* subsp. *michiganensis*

Flower stage ^z	Total no. fruit			Fruit with lesions (%)			Average no. spots/fruit ^y		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
-5	30	12	22	0	0	0	0.0 a	0.0 a	0.0 a
-3	23	14	20	0	0	5	0.0 a	0.0 a	0.05 a
0	48	29	16	6	0	0	0.1 a	0.0 a	0.0 a
+2	54	47	57	30	15	42	1.7 b	0.8 a	2.8 b
+4	46	59	35	30	20	37	0.4 b	0.5 a	1.9 b

^y Statistical analysis using Kruskal-Wallis analysis of variance by ranks and pairwise multiple comparison Dunn's method.

^z Flower developmental stages were five (-5) and three (-3) days preanthesis, anthesis (0), two (+2) and four (+4) days postanthesis, corresponding to closed calyx, open calyx, partially open corolla, fully open corolla, and half-closed corolla, respectively.

DISCUSSION

This is the first report on the development of bird's eye tomato fruit lesions following inoculation of flowers or fruit with *C. michiganensis* subsp. *michiganensis*. Two greenhouse protocols, one where the flowers were sprayed with the pathogen and the other where the pathogen was placed on young fruit with a paintbrush, were developed in this study to obtain fruit lesions.

In the first protocol, spraying tomato flowers twice with a bacterial aerosol produced a higher percentage of fruit with lesions as well as a higher number of spots per fruit than a single inoculation, whether or not flowers were bagged after inoculation. When inoculating once, bagging the flowers and thereby keeping humidity high resulted in an increased number of spotted fruit.

Significant fruit spotting did not occur unless bacteria were applied after anthesis, suggesting that the bacterial population on the flower must be relatively high at the latter stages of the flower development in order to cause significant fruit spotting. This could be one reason why the bird's eye lesion is the most inconsistent of the bacterial canker symptoms observed during field epidemics (M. K. Hausbeck and D. W. Fulbright, *personal observation*). Although the application of 10⁸ CFU/ml of *C. michiganensis* subsp. *michiganensis* to preanthesis and anthesis flowers should have been adequate to establish epiphytic populations that could serve as inoculum to the developing ovaries, perhaps petal abscission reduced the population and bacterial cells were unable to colonize the ovary tissue. Perhaps, in the field, where rain, wind, and splashing water are likely to enhance infection and epiphytic bacterial populations and facilitate infection, preanthesis and anthesis flower infection may occur more readily than in our study.

A few spotted fruit developed from flowers inoculated before or during anthesis. If a large pathogen population was present a few days before the optimum infection stage, this population might still be viable at the optimum time for infection (3 to 5 days later). However, this possibility was not evaluated. Also, bagging of inoculated flowers may have provided the high relative humidity necessary to sustain pathogen populations long enough for inoculations before and during anthesis to result in infection.

In the second protocol, a large number of fruit spots resulted from external inoculation of young green fruit using a paintbrush. In a similar experiment, Volcani et al. (18) used a brush and carborundum to achieve similar results when applying *C. michiganensis* subsp. *michiganensis* to pepper. In our study, physical abrasion was not required for fruit spot production; the paintbrush alone may have produced the needed abrasive action or the brush may have efficiently delivered the pathogen to the natural openings in the epidermis of the tomato fruit (2,7). It was interesting to note that strains not having the ability to produce wilt, canker, and marginal necrosis of leaves in previous pathogenicity assays (16), and verified as negative in HR assays, caused bird's eye lesions, suggesting that we do not have a good understanding of pathogenesis in this pathosystem.

This study supports our general understanding that bacterial fruit spots result from inoculation through stomata or other natural openings on ovaries or young fruit (2,7). Reducing the pathogen populations at the critical stages of postanthesis and early fruit development through copper applications may decrease incidence of spotted fruit. Management strategies utilizing biological controls or genetically engineered hosts could also benefit from knowledge of the development of fruit spots. Effective biocontrol agents might specifically colonize flowers and ovaries, thereby reducing the pathogen populations that might cause fruit lesions. Genetically modified tomatoes could also deliver pathogen-inhibiting compounds in targeted cells, such as trichomes on the ovary.

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