Ingress of the Watermelon Fruit Blotch Bacterium into Fruit

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ABSTRACT

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Bacteria were observed with the scanning electron microscope to be located randomly on watermelon (*Citrullus lanatus*) fruit surfaces 2 hr after inoculation with drops of suspensions of the fruit blotch bacterium. For the next 4 days, bacteria were observed around and in stomata. Nine days after inoculation, masses of rod-shaped bacteria were observed in stomatal chambers. The incidence of disease decreased with age of the fruit at the time of inoculation. The incidence of plugging, or covering, of stomata in the fruit with wax progressively increased with fruit age and appeared to provide a morphological barrier to bacterial ingress. The observations were consistent with the concept that the pathogen enters the fruit through stomata and that immature fruit are the most likely to be infected.

Additional keywords: *Pseudomonas pseudoalcaligenes* subsp. *citrulli*

Watermelon fruit blotch (WFB) was first confirmed in the United States in 1989 (8,14). The disease was reported that year in Delaware (3), Florida (14), and Indiana (8). In addition, cultures of bacteria from watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) plants in Arkansas, Georgia, Iowa, Maryland, North Carolina, and South Carolina were identified as the WFB bacterium by fatty acid analyses (N. C. Hodge, *personal communication*). In Florida and Indiana, crop losses were as high as 50–90% (8,14).

Symptoms of disease occur on seedlings as well as on leaves and fruit. On seedlings, water-soaked lesions occur on both hypocotyls and cotyledons. Symptoms on leaves are relatively inconspicuous, and plants are not defoliated. The lesions are light brown to reddish brown and tend to spread along the midrib of the leaf. Fruit lesions begin as small, raised, gray spots or as small, water-soaked areas with irregular margins that may expand to cover the upper surface of the fruit. The lesions remain localized in the upper layers of the rind. Fruit in this stage are still edible but are not marketable because of their unsightly appearance. Eventually, lesions may open and fruit may rot if invaded by saprophytic organisms.

Watermelon fruit blotch is caused by a white-colonied, gram-negative, aerobic, oxidase-positive, nonfluorescent bacterium that gives a positive hypersensitive reaction in tobacco (14). A similar bacterium from watermelon, *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, was described by Schaad et al (13) and caused lesions on watermelon cotyledons and, in some instances, seedling death. The fruit blotch disease was reported in Guam and Tinian in 1987, and the causal bacterium was called *P. pseudoalcaligenes* subsp. *citrulli* (15). However, the WFB pathogen from Florida differs from the type strain of the seedling blight pathogen by being positive for hypersensitivity in tobacco, by producing fruit blotch symptoms, and by having dissimilar fatty acid profiles (14). The taxonomic significance of those differences has not been determined. Recently, *P. p. citrulli* was renamed *P. avenae* subsp. *citrulli* (6) and then *Acidovorax avenae* subsp. *citrulli* (16).

Fruit blotch appeared on mature watermelon fruit in the 1989 epidemic in Florida, and the leaf spot phase of the disease was not observed. Speculation that the bacterium developed systemically in the plants arose because a large amount of necrosis developed rapidly in the fruit near maturity. The lack of infection of mature watermelon fruit by the causal bacterium, except after some sort of injury to the melon (14), seemed to support the systemic development of the disease. Fruit blotch did develop, however, when immature melons were sprayed with inoculum in the field (14).

The purpose of this study was to study the ingress of the WFB bacterium into watermelon fruit. The presence of stomata in watermelon fruit at various maturities was documented and the plugging and covering of stomata by wax was correlated with disease incidence.

MATERIALS AND METHODS

Bacterial strain and inoculum production. A bacterial strain, WFB 89-1, was isolated from fruit in a commercial watermelon field in Florida and used in all inoculation tests. Bacterial cultures on nutrient agar plates were removed after 2 days of growth at 29 C and suspended in sterile tap water. Concentrations of cells were adjusted to $A_{600} = 0.250$ OD in a Spectronic 2000 spectrophotometer. The bacterial concentration was determined to be 1×10^8 cfu/ml by dilution plating. Other concentrations were obtained from such suspensions by appropriate dilutions with sterile tap water.

Test plants. The watermelon cultivar Charleston Gray was planted in the field at the Central Florida Research and Education Center, Leesburg. The planting consisted of five rows, each 60 m long with 3 m between rows. Seeds were planted at approximately 1-m intervals in each row. In a summer planting, plants were divided into groups for tests of stomatal entry, wax production, and fruit susceptibility. The fruit susceptibility test was repeated in a fall planting.

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Stomatal entry. Twenty watermelon fruit were tagged when approximately 2–3 wk old. Three circles, each 8–10 mm in diameter, were marked on the upper surface of each fruit. The fruit surface inside the circles was then covered with a suspension of strain WFB 89-1 at 10^6 cfu/ml at about 11:00 a.m. The inoculum was allowed to air-dry, which took approximately 2 hr. At 0 (2 hr after inoculation), 1, 2, 3, 4, 7, and 9 days after inoculation, a rind sample 5–8 mm in diameter and 1–2 mm thick was removed from inside each of three circles with a razor blade. Samples, one from each of three different fruit, were taken from fruit still attached to the plant. Through 7 days after inoculation, samples were from areas in which lesions had not formed; however, there were small lesions in the 9-day samples. The samples were stored in vials containing 2.5% glutaraldehyde. The samples were examined for the presence of bacteria with the aid of a scanning electron microscope (SEM).

Fruit susceptibility. Watermelon fruit were inoculated at 1, 2, 3, 4, and 5 wk post anthesis. Each of the five age groups inoculated on different dates was in a separate block with buffer zones between the blocks. The fruit were inoculated by atomizing a suspension of bacteria containing 10^6 cfu/ml of the WFB bacterium on the upper surface to runoff. Fruit were evaluated for disease symptoms 7 days after inoculation. This test was completed twice, with 30 fruit for each age group tagged at anthesis in a summer test and with 50 fruit in each age group tagged at anthesis in a fall test. In some age groups, fruit aborted prior to inoculation, resulting in fewer inoculated fruit. In the summer test, groups 1, 2, and 5 were inoculated in bright, sunny weather with daily temperatures ranging from a high of 35 C to a low of 25 C and groups 3 and 4 were inoculated in a rainy period with temperatures ranging from 30 C to 22 C. In the fall test, all groups were inoculated in partly cloudy weather with daily temperatures ranging from a high of 33 C to a low of 22 C.

Wax production on fruit surface. Ten watermelon fruit were tagged at the time of anthesis, and from these, three samples, each 5–8 mm in diameter and 1–2 mm thick, were removed randomly from the fruit surface at 1, 2, 3, 4, and 5 wk post anthesis. Samples were placed immediately into vials containing 2.5% glutaraldehyde and stored at 4 C until processed for observations with a SEM. One hundred stomata on each sample were scored for wax coverage or plugging.

SEM. Samples for SEM were prepared by a method similar to that of Corey et al (1). The rind pieces stored in glutaraldehyde were washed three times with sodium cacodylate buffer and fixed for 1 hr to overnight in 1% OsO_4. After at least 1 hr, the rind samples were washed with buffer and then with distilled water. Samples were dehydrated in an ethanol series consisting of 15-min washes in each series. The samples were critical-point-dried, placed on aluminum mounts, and sputter-coated with gold. Samples were observed in a Hitachi S-450 SEM.

RESULTS

Stomatal entry. A few characteristic water-soaked lesions 1 to 2 mm in diameter were observed on fruit 9 days after inoculation. With the SEM, bacteria could be seen on the surface of the rind tissue collected at day 0. Bacteria were observed around the stomata at day 1 and within the stomatal chamber on day 2 (Fig. 1A). Only a few bacteria were seen on days 3 and 4, and those were in the stomatal chamber. No bacteria were observed in stomata or on the surface of the symptomless samples on day 7, but bacteria were abundant within stomata on day 9. Bacteria were seen only in association with stomata in all samples except at day 0, when bacteria were distributed widely over the surface of the fruit.

Fruit susceptibility. Good results were gathered in the summer test from inoculations of fruit in age groups 1, 2, and 5, but heavy cloud cover and rain caused many fruit of groups 3 and 4 to abort. Groups 3 and 4 were inoculated during a period of frequent rains that could have washed the inoculum off the fruit and caused the low level of symptom development (Table 1). Many small localized lesions, which resembled those resulting from a defense reaction, developed on the fruit in group 1. Not all of the lesions on fruit of group 1 stayed localized, as some expanded across the fruit surface by 14 days after inoculation. Lesions occurred in 92% of the fruit, however. Disease occurred in 97% of fruit in group 2, and the symptoms were more characteristic of the disease. The lesions were small, water-soaked, and dark green with irregular margins. Disease developed in only 4% of the inoculated fruit in group 5, but the symptoms were characteristic of WFB.

Again in the fall test, a high percentage of fruit in group 1 developed disease (Table 1), but the lesions mostly remained localized. A high percentage of fruit in group 2 also developed disease, and the symptoms were characteristic of WFB. Disease incidence decreased as maturity of fruit at inoculation increased. No disease occurred in fruit of group 5.

Wax production. A layer of wax that covered the surface of the fruit developed as the fruit matured. The wax also covered or plugged stomatal pores (Figs. 1B and 1C). The wax layer first appeared 2 wk after anthesis, and 2–4% of stomatal pores were covered or plugged in the three replicate samples. The ranges of percentages of stomata covered or

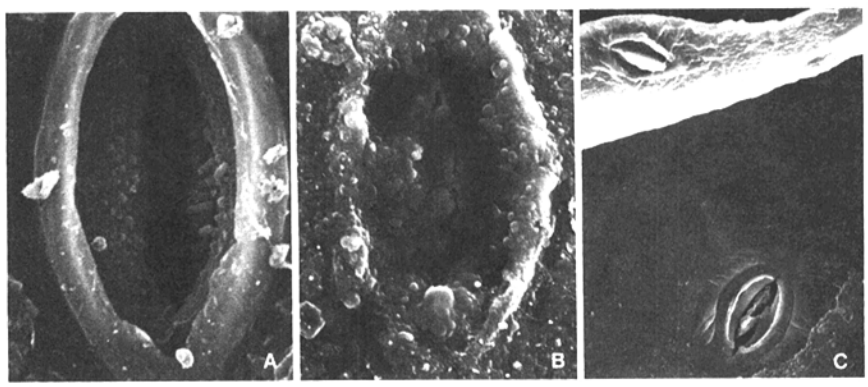

Fig. 1. Scanning electron micrographs of stomata on watermelon fruit surface: **(A)** Rod-shaped bacteria within stomatal chamber, **(B)** wax formed over stomate, and **(C)** layer of wax rolled back revealing imprint of wax-covered stomate.

Table 1. The relationship between fruit age and disease incidence after inoculation of fruit with the watermelon fruit blotch bacterium[a]

Fruit age[b] (wk)	Summer test		Fall test	
	No. of inoculated fruit	Percent diseased	No. of inoculated fruit	Percent diseased
1	25	92	46	91
2	29	97	45	93
3	8	13[c]	36	72
4	28	7[c]	27	48
5	24	4	18	0

[a]The summer test was conducted during May–June and the fall test during September–October.
[b]Interval between anthesis and inoculation of fruit by misting with a 10^6 cfu/ml suspension of the bacterium.
[c]A 2-wk period of heavy rain and cloud cover occurred during inoculations.

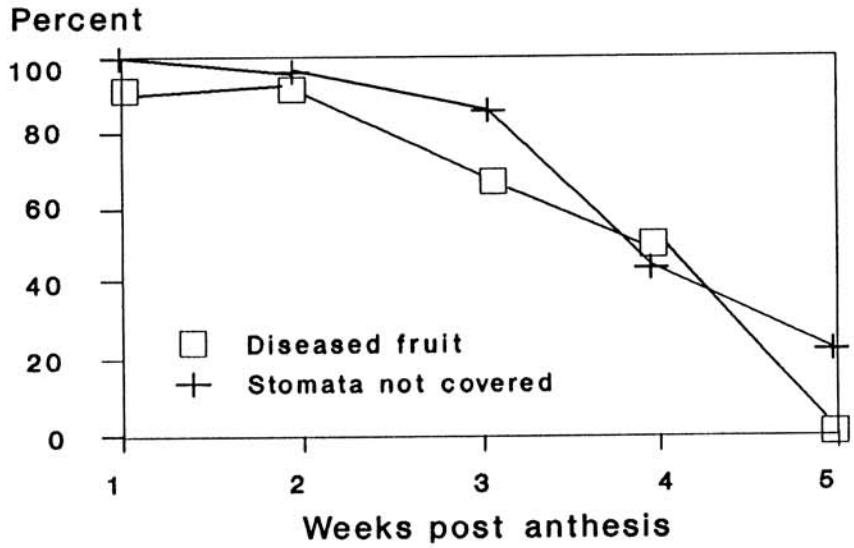

Fig. 2. Percentages of diseased fruit and of stomata not covered by wax in relation to fruit age at inoculation.

plugged with wax were 9–20%, 48–65%, and 66–73% at 3, 4, and 5 wk post anthesis, respectively. Wax coverage or plugging of stomata increased with fruit maturity and the incidence of fruit blotch decreased (Fig. 2). In fruit inoculated at different maturity levels, the percentage of stomata not covered or plugged with wax was directly correlated with the percentage of fruit with disease symptoms, with a correlation coefficient (r) of 0.95.

DISCUSSION

Most plant-pathogenic bacteria penetrate their hosts through stomata. Gitaitis et al (5) documented the ingress of *P. alboprecipitans* (= *Acidovorax avenae* subsp. *avenae*) through stomata on corn leaves. Miles et al (10) presented evidence for stomatal entry of *Xanthomonas pruni* (= *X. campestris* pv. *pruni*) in peach leaves, and Daub and Hagedorn (2) studied the entry of *P. syringae* into bean leaves through stomata. Stomata occur in watermelon fruit, and bacteria were associated with them after inoculations with the WFB bacterium in the field. The high incidence of WFB in fruit inoculated at immaturity vs. the low incidence of WFB in fruit inoculated at maturity was correlated with the covering and plugging of stomata by wax. In previous work, disease incidence was related to stomatal numbers (11) and stomatal pore size (9), but the relationship of disease incidence with natural plugging and covering of stomata with wax seems to be a new concept (7).

Evidence for systemic development of the WFB bacterium in the plant was not obtained in other experiments (4,12). Apparently, the fruit blotch bacteria are harbored in inconspicuous leaf lesions in nature and then spread to fruit surfaces. The bacteria enter stomata in immature fruit, and small water-soaked lesions form. At first, the lesions are very small and may be overlooked, but they expand very rapidly and may cover the entire upper surface of the watermelon fruit in a 2-wk period.

The information from this study may be useful in developing control strategies for WFB. The period favorable for ingress of the causal bacterium into fruit appears to be relatively short. The timing of sprays of bactericides to coincide with the periods favorable for ingress of bacteria into fruit and to control the leaf infections could reduce inoculum levels to below those necessary for infection of fruit and result in control of the disease.

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