Induction of a Hypersensitivelike Reaction in Four-o'clock by *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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Clavibacter michiganensis subsp. *michiganensis* produced an apparent hypersensitive reaction (HR) in four-o'clock (*Mirabilis jalapa*) when suspensions containing 10^8 cfu of bacteria per milliliter of diluent were infiltrated into the intercellular spaces of leaf panels. A typical HR occurred in response to 34 virulent *C. m.* subsp. *michiganensis* strains and was independent of temperature within the range tested (18–43 C). Injection of four-o'clock with bacterial suspensions was an accurate and rapid way to distinguish *C. m.* subsp. *michiganensis* from other bacteria with similar colony morphology recovered from standard or semiselective bacteriological media. Strains of *Erwinia herbicola, Pseudomonas fluorescens,* and coryneformlike bacteria that failed to elicit HR in four-o'clock were nonpathogenic to tomato (*Lycopersicon esculentum*), whereas all strains eliciting HR caused typical symptoms of bacterial canker on tomato and had the characteristics of *C. m.* subsp. *michiganensis*.

Infiltration of the intercellular spaces of plant leaves with aqueous suspensions of bacteria to determine whether a hypersensitive response (HR) occurs is recognized as a way of determining phytopathogenicity and is useful for identifying certain plant-pathogenic bacteria (8,9,14). The reaction is invaluable for the characterization of phytopathogenic bacteria recovered from outside their host (on farm machinery; in insects, soil, or water; or as residents on weeds) or recovered from the host when disease signs are absent (latent infections, epiphytic survival, or infested seed) (10).

Unfortunately, most plants fail to respond or, like tobacco, are inconsistent in the expression of HR when their leaves are infiltrated with suspensions of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al, the causal agent of bacterial canker of tomato (*Lycopersicon esculentum* Mill.). Furthermore, a host that developed HR regardless of temperature would be more useful to regulatory agents and others who may not have access to growth chambers or other means of controlling environmental conditions than a host whose response varied with temperature.

Bacterial canker of tomato has been of particular concern to the tomato transplant industry and the plant certification program in Georgia since an outbreak in 1984 was traced to "certified" plants. Plant inspectors are faced with the dilemma of visually inspecting healthy plants that may harbor epiphytic populations of the pathogen. Likewise, because the incubation period of bacterial canker is long (2–4 wk, depending on conditions), visual inspections are not appropriate for tomato plants with latent infections caused by *C. m.* subsp. *michiganensis*.

Traditional microbiological tests are also of limited value for the identification of this pathogen. Vidaver and Starr (17) emphasized the use of pathogenicity tests for the identification of most phytopathogenic coryneform bacteria; however, the routine use of host inoculations to identify bacteria recovered from plant samples in a certification program is impractical because of the long incubation period. Consequently, the present work was conducted to develop a method that could be used in the Georgia plant certification program for the rapid identification and determination of pathogenicity of *C. m.* subsp. *michiganensis*. An abstract of this research has been published (4).

MATERIALS AND METHODS

Inocula were prepared by growing bacteria as a shake-culture in nutrient broth for 48–72 hr. Cells were harvested by low-speed centrifugation at 2,000 *g* for 15

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min, and the bacterial pellet was suspended in sterile tap water. The suspension was adjusted with sterile tap water to 50% transmittance at a wavelength of 600 nm measured with a Spectronic 20 colorimeter (Bausch & Lomb, Rochester, NY). Inocula of plant-pathogenic bacteria prepared in this manner contain approximately 10^8 cfu per milliliter (3,14). Inoculum densities were routinely checked ($n = 33$) by spreading 0.1-ml aliquots from a 1:9 serial dilution onto the surface of plates of CNS semiselective medium (6). Colony counts were made after incubation at 30 C for 7 days.

Strain CM-O of *C. m.* subsp. *michiganensis* was used for routine screening to evaluate plant species as indicator hosts. Fully expanded leaves of plants grown in the greenhouse were inoculated by infiltration of the bacterial suspension into the intercellular spaces with a hypodermic syringe and 26-gauge needle. Temperatures in the greenhouse fluctuated, ranging from 18 to 43 C throughout the 6 mo that plants were tested. Test plants were evaluated after 36–48 hr for HR and daily for 2 wk for any later developing disease reactions.

Plants were selected for evaluation based on their availability at the time or their relative ease of injection. Screening of plants was planned to continue until a suitable indicator host was found. The following plants were evaluated for their reactions to a challenge injection of strain CM-O: avocado (*Persea americana* Mill.), *Begonia* spp., bush violet (*Browallia speciosa* Hook.), cabbage (*Brassica oleracea* L.), cockscomb (*Celosia argentea* L.), 17 cultivars of cowpea (*Vigna unguiculata* (L.) Walp.), *Dieffenbachia picta* (Lodd.) Schott, *Fatsia japonica* (Thunb.) Decne. & Planch., four-o'clock (*Mirabilis jalapa* L.), geranium (*Pelargonium* × *hortorum* L. H. Bailey), nasturtium (*Tropaeolum majus* L.), pepper (*Capsicum annuum* L.), periwinkle (*Catharanthus roseus* (L.) G. Don), *Phlox drummondii* Hook., pothos (*Epipremnum aureum* (Linden ex André) Bunt.), radish (*Raphanus sativus* L.), schefflera (*Brassaia actinophylla* Endl.), *Spathiphyllum* spp., tobacco (*Nicotiana tabacum* L.), velvetbean (*Mucuna deeringianum* (Bort) Merr.), and *Zinnia elegans* Jacq.

Aqueous suspensions of approximately 10^8 cfu/ml were prepared as above for 35 strains of *C. m.* subsp. *michiganensis*. The strains and their origins were as follows: CM-O (D. Emmatty, Heinz U.S.A., Ohio); CM-1, CM-2, and CM-3 (D. Cuppels, Ontario, Canada); CM-4, CM-5, and CM-6 (G. Bonn, Ontario, Canada); CM-7 through CM-18 (M. Ricker, Campbell Institute for Research and Technology, Ohio); CM-19 through CM-31 (R. Gitaitis, Georgia; strains originated in Florida, Georgia, North Carolina, and Ohio and from seed produced in Taiwan and Thailand); and CM-32, CM-33, and CM-34 (H. Bolkan, Campbell Institute for Research and Technology, Davis, CA; strains originated in People's Republic of China). In addition, one strain each of *Pseudomonas fluorescens* (Trevisan) Migula, *P. syringae* pv. *syringae* van Hall, *Erwinia herbicola* (Lohnis) Dye, and *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye were used as representative pathogenic and saprophytic bacteria for comparison.

Suspensions of all strains were injected into leaves of *M. jalapa* as outlined above. In addition, about 200 unidentified but coryneformlike bacteria recovered on SCM (1) and CNS semiselective media from seed or soil or as epiphytes from tomato transplants or weeds were grown in pure culture, prepared as above, and tested on four-o'clock leaves. Data on the ability of all strains to induce HR were recorded after 48–72 hr. Gram reactions (16), response in litmus milk (2,15), growth and appearance on CNS (6) modified by omitting lithium chloride (13), and growth and appearance on SCM (1) were used to make preliminary characterizations of all test strains.

Butyrous or fluidal colony types on SCM were selected from the strains of *C. m.* subsp. *michiganensis* described above, subcultured, and evaluated for their ability to induce HR in four-o'clock and typical bacterial canker symptoms in tomato. Each strain selection was incubated as a broth culture, harvested, adjusted photometrically, and injected into leaf panels of four-o'clock as above. Tomato (cv. H-722) plants grown in the greenhouse to the four- to five-leaf stage were used for pathogenicity tests. Stems of plants were punctured about 2–3 cm above the soil line with sterile toothpicks whose points were coated with visible amounts of bacteria picked from agar media. Tomato stems were split lengthwise and examined for internal symptoms after 4–5 wk to assess disease.

The effect of temperature on the expression of HR was evaluated to test the effectiveness of four-o'clock as an indicator plant. Because tobacco has been the preferred indicator plant for other bacterial pathogens (8,9,11,12), it was compared with four-o'clock at two incubation temperatures (25 and 38 C). Individual plants of tobacco (cv. McNair 944) and four-o'clock (mixed colors, Ferry-Morse Seed Co., Fulton, KY) were grown in the greenhouse until four to five leaves had expanded fully, at which time they were moved to incubators at 25 and 38 C. Incubators were constantly illuminated by three 15-W "cool-white" fluorescent tubes placed about 20 cm above the tops of the plants. After a 24-hr exposure to acclimate plants, intercellular areas of leaf panels were infiltrated with suspensions of strain CM-O of *C. m.* subsp. *michiganensis* prepared as described above. Plants were maintained for 48 hr at their respective temperatures, and the presence or absence of HR was then determined. All experiments were replicated four times and repeated once.

RESULTS AND DISCUSSION

Mean bacterial populations of strain CM-O adjusted photometrically and used for the initial screening for an HR host were 6.5×10^8 cfu per milliliter of water ($n = 33$). This exceeded the minimum inoculum concentration of 5×10^6 cfu/ml necessary for HR induction in tobacco (8,9).

Confluent necrosis, typical of HR, occurred in infiltrated areas of four-o'clock within 36–48 hr when leaves were challenged with strain CM-O. Infiltrated areas of leaves of all other test plants were unchanged and apparently healthy after 36–48 hr. Pepper leaves became chlorotic after incubation for 2 wk, but no HR, disease, or discoloration was evident after 2 wk in any other test plant.

All 35 strains of bacteria previously identified as *C. m.* subsp. *michiganensis* grew on CNS and SCM media, were gram-positive, and slowly (2–3 wk) reduced litmus milk with the formation of a yellow pellicle. Thirty-four of the 35 strains produced confluent necrosis within 36–48 hr and produced typical bacterial canker symptoms in tomato. The strain that was negative for HR also failed to produce symptoms in tomato and therefore was considered either avirulent or misidentified.

No compatible bacterial pathogen was on hand to use in studies to compare HR with the interaction of a virulent pathogen with four-o'clock as a suscept. However, reactions resulting from challenge inoculations with *C. m.* subsp. *michiganensis* gave no indication that the bacteria colonized leaf areas beyond those infiltrated with inoculum. Collapsed tissues were dry and papery and were similar in appearance to the HR induced by *P. s.* pv. *syringae,* which induced HR in tobacco as well as in four-o'clock. *P. fluorescens, E. herbicola, X. c.* pv. *vesicatoria,* and numerous coryneformlike bacteria (more than 200) from weeds, soil, plants, and seed were negative for HR in four-o'clock. All strains (except *X. c.* pv. *vesicatoria)* that failed to elicit HR were also nonpathogenic to tomato. Several wild-type strains recovered from seed induced HR in four-o'clock, produced typical bacterial canker symptoms when inoculated into tomato, and were identified as *C. m.* subsp. *michiganensis.*

Colonies of *C. m.* subsp. *michiganensis* are typically mucoid (fluidal), irregular in form, somewhat viscous, and either pale yellow on CNS or mottled gray on SCM. However, other colony characteristics, such as darker pigmentation, circular form, smooth surface, and

butyrous texture, were also observed. A comparison of the various colony types subcultured from 20 strains showed no differences in their ability to elicit HR in four-o'clock, indicating the need to test these types of "atypical" colonies in a certification program.

Hildebrand and Riddle (7) divided HRs into several general types, including type I (temperature has no effect on HR), type II (high temperatures enhance HR), and type III (low temperatures enhance HR). Four-o'clock responded to challenge inoculations in a type I manner; temperature had no effect within the range tested. Tobacco, however, responded to challenge inoculations with *C. m.* subsp. *michiganensis* in a type III manner; confluent necrosis occurred only at 25 C and not at 38 C. High temperatures in the greenhouse probably explain why tobacco did not respond with HR during the initial screening phase. In addition to its insensitivity to temperature, four-o'clock is the preferred choice as an indicator plant for routine pathogenicity testing because of its rapid growth and ease of culture compared to tobacco. Moreover, HR in tobacco was variable in that some strains that produced HR in four-o'clock and disease in tomato failed to elicit HR in tobacco at either 25 or 38 C.

Within the last 2 yr, bioassay with four-o'clock has been used successfully in the Georgia plant certification program to aid in the identification of *C. m.* subsp. *michiganensis*. The method has been a valuable aid in the certification program and to date has been 100% accurate (R. D. Gitaitis, *unpublished*).

Although other methods may be developed that are faster and more specific for the detection and identification of bacteria, the new methods might not confirm pathogenicity. Whether other subspecies of *C. michiganensis* react with four-o'clock in a similar manner is not known; however, other subspecies of this bacterium are relatively unimportant in Georgia and are not commonly associated with tomato or pepper. Although testing for HR in tobacco remains a primary method for determining pathogenicity (5,10), it is not used to identify specific pathovars of *P. syringae*. HR provides an initial characterization that often determines what other tests, if any, will be used to identify a bacterium. Four-o'clock provides a similar system, which until now was unavailable, for screening for HR in response to *C. m.* subsp. *michiganensis*.

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