Ralstonia solanacearum Race 3, Biovar 2 Strains Isolated from Geranium Are Pathogenic on Potato

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ABSTRACT

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Ralstonia solanacearum race 3, biovar 2 is a soilborne bacterium that causes potato brown rot disease in temperate and subtropical climates. Recent outbreaks of this disease in Europe have caused serious losses, but the pathogen had not been identified in the United States. However, in 1999, strains of *R. solanacearum* were isolated from wilting geraniums growing in Wisconsin greenhouses. Physiological and biochemical tests of the Wisconsin strains and a similar strain from South Dakota demonstrated that the strains belong to *R. solanacearum* subgroup biovar 2, which is largely synonymous with the race 3 subgroup, a classification based on host range. These results were confirmed by polymerase chain reaction analyses in which race 3, biovar 2-specific primers amplified a fragment of the expected size. This is the first report of race 3, biovar 2 in the United States, and it is the first known occurrence of race 3, biovar 2 in Wisconsin. The geranium strains were highly pathogenic on both geranium and potato. The presence of *R. solanacearum* race 3, biovar 2 in the United States raises concern that the bacterium could move from ornamental plants into potato fields, where it could cause both direct economic damage and quarantine problems. A commercial indirect enzyme-linked immunosorbent assay for *R. solanacearum* produced some false negatives for these strains, indicating that current indexing may not be sufficient to identify this destructive pathogen.

Ralstonia solanacearum (synonyms: *Pseudomonas solanacearum* and *Burkholderia solanacearum*) is a widespread and economically important bacterial plant pathogen (15,24). It causes bacterial wilt, a major disease that limits production of diverse crops such as potato, tomato, peanut, and banana. *R. solanacearum* has an extended host range that includes hundreds of plant species in 44 families (15). The pathogen is widely distributed in tropical, subtropical, and warm temperate climates. The species is highly variable and heterogeneous.

Strains of *R. solanacearum* are differentiated into five races according to host range (3) and five biovars according to utilization of various disaccharides and hexose alcohols (14,16). The race and biovar classifications do not correspond, except that race 3 is generally equivalent to biovar 2 (15). Biovars fall into two geographically distinct groups (23,27). In general, division 1, comprising biovars 3, 4,

and 5, predominates in Australia and Asia. Division 2, including biovars 1 and 2, predominates in the Americas, although it should be noted that biovar 2, until now, has not been reported in the United States. Polymerase chain reaction (PCR) diagnostic tests have been developed for this bacterium, with 16S rRNA and ITS primers that differentiate between divisions 1 and 2 and additional primers that are specific for biovar 2 (2,23,31).

A distinct group of *R. solanacearum* (race 3, biovar 2) causes brown rot, a serious disease of potatoes (15,17). This group has a host range primarily limited to potato, tomato, and common solanaceous weeds, although it has also been reported on stinging nettle where roots were growing in contaminated water (32) and on other non-solanaceous weeds under favorable conditions (6,17,28). The strain is adapted to the cooler temperatures found at higher elevations and higher latitudes (4,6,26). Intermittent but increasingly frequent brown rot outbreaks in northern Europe since 1972 have resulted in significant losses due both to disease incidence and to quarantine restrictions that require destruction of infected crops and fallowing of infested fields (6,17). The bacteria were probably disseminated by latently infected tubers and contaminated irrigation water (6,17,32). Although some reports claim

that the bacterium does not survive long-term in soil (6,12), it has overwintered in soils, waterways, and/or in roots of a perennial weed host, *Solanum dulcamara*, in Sweden, France, the UK, and the Netherlands (8,21,30). To our knowledge, race 3 strains of *R. solanacearum* have not been reported in the United States.

In the spring of 1999, we isolated *R. solanacearum* from zonal geraniums, *Pelargonium × hortorum*, that were imported into Wisconsin from Guatemala. Geranium is known to be a natural host of *R. solanacearum* race 1 (13,14,25) but only a casual report has identified race 3 on geranium to date (17). Our goals were to identify the race and biovar of *R. solanacearum* strains recovered from geranium and to characterize the pathogenicity of these strains.

MATERIALS AND METHODS

Bacterial strains. In April 1999, wilt symptoms were observed on geraniums obtained from greenhouses in Jefferson and Milwaukee counties in Wisconsin. The plants had been propagated from vegetative material imported from Guatemala. Bacteria were isolated from infected leaves and stems of the host plants, yielding strain BB-Feutz from Jefferson County, and BB-Esser from Milwaukee County. Similar geranium strains of *R. solanacearum* (strains 12-4-5 WI, 12-4-6 SD, and 12-4-9 NJ) were collected by the Long Island Horticultural Research Laboratory. These strains were compared with various strains of *R. solanacearum* (race 1/biovar 1 strain K60; race 1/biovar 4 strains UW130 and GMI1000; race 2/biovar 1 strain UW9; and race 3/biovar 2 strains UW72, UW257, UW272, UW276, and UW344) from a collection at the Department of Plant Pathology, University of Wisconsin, Madison (Table 1).

Cultural and physiological tests. Tests for Gram stain, and oxidase and catalase activity followed procedures described in Schaad et al. (22) and Gerhardt et al. (10). The strains were grown on yeast dextrose calcium carbonate medium (YDC) to assess color and on King's B medium amended with 100 ppm cycloheximide (KB+C) for fluorescence (22). The isolates were routinely cultured on casamino acids-peptone-glucose (CPG) and triphenyltetrazolium chloride medium (TZC) (18).

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To test for oxidation of sugars and sugar alcohols, 5 ml each of glucose, trehalose, maltose, or mannitol (10% wt/vol) solutions were filter sterilized and added to 45 ml of Hayward's medium, and 10-ml volumes of the resulting amended medium were dispensed into test tubes (14). Hayward's medium without a sugar or sugar alcohol carbon source served as a control. A suspension was prepared of each test strain (BB-Feutz, BB-Esser, and 12-4-6 SD) and control strains of biovar 1 (K60), biovar 2 (UW72 and UW272), and biovar 3 (UW130) by inoculating 300 µl of sterile water with a wire loopful of cells from each strain grown on CPG for 48 h at 28°C. The test tubes of Hayward's media were inoculated with 30 µl of the prepared suspensions and incubated at 24°C for up to 5 weeks.

Hypersensitive reaction. Ability to induce a hypersensitive reaction (HR) on tobacco was determined for BB-Feutz and 12-4-6 SD following the method of Lozano and Sequeira (19). Tobacco (*Nicotiana tabacum* L. 'Bottom Special') leaves were infused with a bacterial suspension spectrophotometrically adjusted to $A_{600} = 0.16$ to 0.18 (approximately 1.7×10^8 CFU/ml). A similar suspension of GMI1000, an *R. solanacearum* strain known to induce an HR on tobacco, served as a positive control and sterile water as a negative control. Observations were taken at 12-h intervals for the first 3 days and at 24-h intervals for an additional 4 days.

PCR identification of race 3 strains. Ten strains of *R. solanacearum* were tested with race 3, biovar 2-specific primers. For each strain, a biovar 2-specific DNA fragment was amplified by PCR using a modification of the protocol of Weller et al. (31) originally adapted from the PCR protocol of Fegan et al. (9). Oligonucleotide primers B2-I-F and B2-II-R were used. Reactions were conducted in thin-walled 250-µl tubes and contained 50 ng of template chromosomal DNA; 10 pmol of each primer; 1 unit of *Taq* DNA Polymerase (Promega Corp., Madison, WI); 0.25 mM each of ATP, CTP, GTP, and TTP; and 2.5 mM Mg^{++} in a 30-µl volume of 1× Promega *Taq* Polymerase Buffer without magnesium.

The PCR reactions were amplified in an MJ Research Model PTC-200 thermocycler under the following conditions: a 5-min hot start at 94°C was followed by 40 cycles of 30 s at 94°C, 30 s at 70°C, and 1 min at 72°C. The reaction was completed with 10 min at 72°C, and then reactions were stored at 4°C. Because DNA yield was typically low, two separate reactions were combined, precipitated with ethanol, and resuspended in 20 µl of water before being separated on a 2.0% (wt/vol) agarose gel. DNA product bands were visualized under UV light after staining with ethidium bromide.

Indirect enzyme-linked immunosorbent assay (ELISA). Test strains BB-Feutz and 12-4-6 SD, and control strains K60, UW276, and UW344, were sent in 1999 to Agdia Testing Services (Agdia Inc., Elkhart, IN; 219/264-2014) for their indirect ELISA for *R. solanacearum*. Tests were performed twice and repeated in spring 2002 with a new antiserum.

Pathogenicity testing. Koch's postulates were performed with 'Americana Reds' geranium and 'Bonny Best' tomato as hosts. After a 24-h period without water, roots of each plant were cut 1 cm from the stem on one side, and approximately 10 ml of an aqueous suspension of BB-Feutz spectrophotometrically adjusted to $A_{600} = 0.1$ to 0.5 (approximately 1 to 5×10^8 CFU/ml) was poured around the base of the stem. Controls were mock inoculated with water. Plants were placed in a greenhouse at 20 to 31°C and observed daily.

BB-Feutz and 12-4-6 SD were tested for pathogenicity on potato (*Solanum tuberosum* 'Atlantic') and geranium (*Pelargonium × hortorum* 'Melody'). Potatoes were multiplied in tissue culture and transplanted to 10-cm pots containing Scotts Redit-earth plug and seedling mix (Scotts-Sierra Horticultural Products Co., Marysville, OH). Commercially indexed, rooted cuttings of geraniums were similarly planted. Plants were grown for 1 month in a 28°C growth chamber and watered with Hoagland's solution (5).

After a 24-h period without water, groups of 26 potato plants were inoculated with one of the following treatments: BB-

Feutz, 12-4-6 SD, UW 344 (a positive control), or sterile water (a negative control). Roots were cut 1 cm from the stem on one side, and approximately 20 ml of an aqueous suspension of each strain, spectrophotometrically adjusted to $A_{600} = 0.8$ (approximately 8×10^8 CFU/ml), was poured around the base of the stem (33). Inoculated plants and controls mock inoculated with water were placed in a growth chamber at 28 to 30°C with a 12-h photoperiod and watered every second or third day with Hoagland's solution. Treatments were arranged in a randomized complete block design with 26 replications of each treatment. Plants were subsequently recoded so they could be rated for disease blindly. The same experimental design was used for geraniums, except that 30 ml of an aqueous suspension of each strain was poured around the base of the stems.

Plants were evaluated daily for the percentage of the leaves that were wilted (0 = no leaves wilted, 100 = all leaves wilted or dead). Data were converted to proportions and then transformed using an arcsine square root transformation. Transformed values were subsequently analyzed using a repeated measures analysis of variance (ANOVA) (20).

RESULTS

Cultural and physiological characteristics. Typically for *R. solanacearum*, the three geranium strains (BB-Feutz, BB-Esser, and 12-4-6 SD) were gram negative, oxidase positive, and catalase positive. On CPG medium, colonies were irregular, smooth, creamy white, and fluidal, and produced a brown pigment after 48 h. Colony morphology was similar on TZC medium, except that colonies were off-white with red centers. All strains produced cream-colored colonies on YDC; none of the strains fluoresced on King's B medium.

On Hayward's medium, strains BB-Feutz, BB-Esser, and 12-4-6 SD produced acid, causing a complete indicator change with glucose in 2 to 6 days and maltose in 11 to 18 days, but they did not utilize trehalose or mannitol even after 28 days (Table 2). No reaction was produced in inoculated media without a carbohydrate source. Based on these results, all three strains were classified as biovar 2 in accordance with Hayward (14) and He et al. (16).

Koch's postulates. Seven to 10 days after inoculation, geraniums inoculated with BB-Feutz and 12-4-6 SD were wilted and chlorotic, and 3 weeks after inoculation they were largely dead. One week after inoculation, tomato plants were fully wilted, and all inoculated plants were dead after 2 weeks. Bacteria reisolated from wilted plants had cultural characteristics typical of *R. solanacearum*. No wilting was observed in the noninoculated control plants. These results demonstrated that strains BB-Feutz and 12-4-6 SD were the

Table 1. *Ralstonia solanacearum* strains used in this study

Strain	Race	Biovar	Host	Origin
K60	1	1	Tomato	NC, USA
UW130	1	3	Tomato	Peru
GMI1000	1	4	Tomato	French Guyana
UW9	2	1	Heliconia	Costa Rica
UW72	3	2	Potato	Greece
UW257	3	2	Potato	Costa Rica
UW272	3	2	Potato	Costa Rica
UW276	3	2	Potato	Mexico
UW344	3	2	Potato	Brazil
BB-Esser	3	2	Geranium	WI, USA
BB-Feutz	3	2	Geranium	WI, USA
12-4-5 WI	3	2	Geranium	WI, USA
12-4-6 SD	3	2	Geranium	SD, USA
12-4-9 NJ	3	2	Geranium	NJ, USA

pathogens responsible for the wilt disease originally observed in geranium plants.

Hypersensitivity. Neither BB-Feutz nor 12-4-6SD elicited a classic rapid HR on tobacco leaves, although they did elicit a slower tissue collapse typical of compatible interactions. Tobacco leaves infiltrated with BB-Feutz were mottled light green by 15 h after inoculation; this color change gradually spread to the entire infiltrated area by 48 h. By 60 h, the infiltrated leaf areas were fully collapsed and brown. They were either sharply delimited from the healthy green noninoculated tissue or surrounded by a narrow light-green halo. Strain 12-4-6 SD elicited a more rapid response; infiltrated leaves remained symptomless until 48 h after inoculation, but then infiltrated areas became brown, necrotic, and surrounded with a narrow light-green halo. After 5 days, lesions of both strains were similar and had not spread to adjoining tissue. In contrast, tobacco leaves infiltrated with strain GMI1000, which is known to elicit an HR on tobacco, became collapsed, tan, and papery within 15 h after inoculation. Areas infiltrated with water were unaffected.

PCR. The race 3, biovar 2-specific PCR primers of Weller et al. (31) amplified the predicted 68-bp DNA product from the geranium strains BB-Feutz, BB-Esser, 12-4-5 WI, 12-4-6 SD, and 12-4-9 NJ (Fig. 1,

lanes 1 to 5). This 68-bp product derives from the highly variable internal transcribed spacer region between the 16S and 23S rDNA genes. The 68-bp band was not amplified from DNA of the biovar 1 and 4 control strains (K60 and GMI1000) (Fig. 1, lanes 9 and 10) but was consistently amplified from all of the known race 3, biovar 2 strains (Fig. 1, lanes 6 to 8). Thus, these five geranium isolates of *R. solanacearum* contain DNA sequences unique to race 3, biovar 2, strongly supporting their classification in this group.

Indirect ELISA. Neither geranium strains BB-Feutz and 12-4-6 SD, nor known race 3, biovar 2 control strains UW276 and UW344, were identified in 1999 as *R. solanacearum* by indirect ELISA in tests conducted by Agdia Testing Services. However, these tests did identify the race 1, biovar 1 positive control K60 as *R. solanacearum*. A second round of Agdia ELISAs with a new antiserum in spring 2002 did identify most, but not all, geranium strains as *R. solanacearum*.

Pathogenicity. Geranium strains BB-Feutz, 12-4-6 SD, and race 3, biovar 2 control strain UW344 were all highly pathogenic on both potato and geranium. Lower leaves of both hosts began to wilt by approximately 5 days after inoculation.

On potato, disease development was significantly different among treatments,

and between inoculated and noninoculated controls (Fig. 2). Disease progressed most rapidly on potatoes inoculated with BB-Feutz. By 28 days postinoculation, 100% of potato plants inoculated with BB-Feutz, 90% of potato plants inoculated with UW344, and 70% of potato plants inoculated with 12-4-6 SD were severely wilted (80 to 100% of leaves wilted) or dead (Fig. 2), whereas none of the mock inoculated potato controls were wilted.

On geranium, disease development was significantly different between inoculated and noninoculated controls, but not significantly different among bacterial strains (Fig. 3). By 28 days postinoculation, 88% of geraniums inoculated with BB-Feutz, 84% of geraniums inoculated with UW344, and 96% of geraniums inoculated with 12-4-6 SD were severely wilted or dead (Fig. 3). At 28 days, one of the mock inoculated geranium controls had wilt symptoms.

DISCUSSION

Strains of *R. solanacearum* were isolated from wilted geraniums obtained from

Table 2. Oxidation of carbohydrates by strains of *Ralstonia solanacearum*

Strain	Acid production from carbohydrates				
	Glucose + control	Water – control	Trehalose	Maltose	Mannitol
BB-Feutz	+++ ^a	–	–	++	–
BB-Esser	+++	–	–	+	–
12-4-6 SD	+++	–	–	+	–
UW72 (biovar 2)	+++	–	–	+	–
K60 (biovar 1)	+++	–	++	–	–
UW130 (biovar 3)	+++	–	++	w	+

^a +++ = Complete indicator change 2 to 6 days; ++ = complete indicator change 6 to 14 days; + = complete indicator change 14 to 21 days; – = no indicator change up to 28 days; w = partial indicator change at 14 to 21 days.

Fig. 1. Electrophoretic analysis of polymerase chain reaction (PCR)-amplified DNA from *Ralstonia solanacearum* isolates using the primers B2-I-F and B2-II-R of Weller et al. (31). Lane 1, geranium isolate 12-4-6 NJ; lane 2, geranium isolate 12-4-5 WI; lane 3, geranium isolate BB-Esser; lane 4, geranium isolate 12-4-6 SD; lane 5, geranium isolate BB-Feutz; lane 6, UW276 (race 3, biovar 2); lane 7, UW257 (race 3, biovar 2); lane 8, UW344 (race 3, biovar 2); lane 9, GMI1000 (race 1, biovar 4); lane 10, K60 (race 1, biovar 1).

Fig. 2. Pathogenicity of *Ralstonia solanacearum* on potato. Plants were evaluated for the percentage of leaves wilted (0 = no leaves wilted, 100 = all leaves wilted or dead). The "average % wilt" represents the average of 26 ratings from individual plants in each treatment. Disease values that are labeled with different letters are significantly different from each other ($P = 0.05$) based on a repeated measures ANOVA of arcsine square root transformed proportions derived from these data, followed by comparisons of transformed means using protected LSDs.

Wisconsin, New Jersey, and South Dakota in 1999. Physiological tests demonstrated that the strains belong to biovar 2, which is essentially synonymous with race 3. Pathogenicity tests revealed that these strains are highly pathogenic on potato and tomato, suggesting that they have the host range typical of race 3. Finally, race 3, biovar 2-specific PCR primers amplified a DNA fragment of the expected size from all five geranium strains tested. Collectively, these results conclusively demonstrate that the causal agent of the geranium wilt is *R. solanacearum* race 3, biovar 2.

This is the first report of race 3, biovar 2 in the United States, and it is the first known occurrence of race 3, biovar 2 in Wisconsin. Previously, only *R. solanacearum* race 1, biovar 1 had been reported in the United States. Geranium is known to be a natural host of *R. solanacearum*. However, this host has not generally been associated with race 3, biovar 2; the previous report of *R. solanacearum* on geranium in the United States did not identify the pathogen to biovar (25). The one European report that identified the *Pelargonium hor-*

torum pathogen as race 3 called the occurrence a "remarkable fact that has largely gone unnoticed" (17).

It is of considerable concern that the race 3, biovar 2 strains isolated from geranium were aggressively pathogenic on potato and tomato in greenhouse conditions. This finding raises the disturbing possibility that the bacterium could move from ornamental plants originally grown in the tropics to potato fields in temperate North America. It is not known how active race 3, biovar 2 would be under field conditions in Wisconsin, or whether it could successfully overwinter here.

Experience with *R. solanacearum* race 3, biovar 2 in Europe suggests what could happen in the United States. Prior to the 1970s, brown rot was thought to be exclusively a disease of the tropics and subtropics. However, sporadic brown rot outbreaks were reported in Sweden in 1972, in Belgium in 1989, in the Netherlands and the UK in 1992, and in France, Portugal, and Italy in 1994 (17); further sporadic outbreaks have continued. Epidemiological studies demonstrated that the bacterium can overwinter in buried potato tubers, in the roots of *S. dulcamara*, a common perennial weed that grows along waterways (6,21,17), in soil, and in surface water (29,30). Investigations in European countries suggested that the brown rot disease outbreaks were mainly caused by contaminated irrigation water or by latently infected seed potatoes. In cases associated with contaminated water, the source of bacteria may have been effluent from potato processing or water-treatment plants that handled diseased potatoes (6,17). In addition, the pathogen infected *S. dulcamara*, leached from diseased roots, and subsequently infected potatoes irrigated with the water. It is unlikely that *R. solanacearum* can now be eradicated from European countries, since eradication may depend on removal of host weeds and decontamination of rivers and irrigation canals. *S. dulcamara*, introduced from Europe, is naturalized throughout the northeastern United States (11) and is thus a potential weed host in Wisconsin. As it is common practice for greenhouses to dispose of diseased plants by tossing them into an open waste container or a compost pile, it is not difficult to imagine ways in which the pathogen could enter surface water and become established in the northeastern United States.

The increasing number of disease outbreaks caused by *R. solanacearum* race 3, biovar 2 in temperate environments emphasizes the need for efficient diagnostic tests. Current indexing in the United States, which commonly depends on ELISA, may not be sufficient to identify this destructive race/biovar. As with any serological test, antibodies used in indirect ELISA may not react with all strains of a species. In our case, indirect ELISA con-

ducted by Agdia Testing Services produced false negatives for the race 3, biovar 2 strains tested, indicating that this test may be unable to detect all race 3, biovar 2 strains. Dialogue is continuing with Agdia, and a reconstituted test for *R. solanacearum* is underway. Further research is necessary to determine the reliability of ELISA for detecting race 3, biovar 2, and to determine whether Wisconsin strains are antigenically distinct from other race 3, biovar 2 strains.

Improved detection of race 3, biovar 2 may be possible by using a combination of techniques. Several effective diagnostic methods for *R. solanacearum* have been developed in recent years, including detection of DNA sequences following PCR amplification (2,23,31), fluorescent in situ hybridization (FISH) (34), and modified semi-selective SMSA media (7). These methods, as well as ELISA, have advantages and disadvantages in sensitivity, specificity, and practicality (7). The test scheme currently used in the European community to detect *R. solanacearum* incorporates a combination of methods. Initial screening may be made by a rapid diagnostic strip ELISA or colony immunofluorescence antibody staining (IFAS) using antiserum with the demonstrated ability to recognize race 3, biovar 2, or by selective plating with modified SMSA, in all cases with additional confirmation using ELISA, PCR, and other tests. Final identification uses one or more of the following techniques: nutritional and enzymatic tests, IFAS, PCR, FISH, protein profiling, or fatty acid profiling. Finally, pathogenicity is tested on tomato or eggplant (1). We recommend that a similar testing scheme using a combination of methods be followed in the United States to more reliably detect *R. solanacearum*, including race 3, biovar 2.

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Fig. 3. Pathogenicity of *Ralstonia solanacearum* on geranium. Plants were evaluated for the percentage of leaves wilted (0 = no leaves wilted, 100 = all leaves wilted or dead). The "average % wilt" represents the average of 26 ratings from individual plants in each treatment. Disease values that are labeled with different letters are significantly different from each other ($P = 0.05$) based on a repeated measures ANOVA of arcsine square root transformed proportions derived from these data, followed by comparisons of transformed means using protected LSDs.

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