Behavior of *Ralstonia solanacearum* Race 3 Biovar 2 During Latent and Active Infection of Geranium

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ABSTRACT

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Southern wilt of geraniums (*Pelargonium hortorum*), caused by the soilborne bacterium *Ralstonia solanacearum* race 3 biovar 2 (R3bv2), has inflicted significant economic losses when geranium cuttings latently infected with this quarantine pest were imported into the United States. Little is known about the interaction between *R. solanacearum* and this ornamental host. Using UW551, a virulent R3bv2 geranium isolate from a Kenyan geranium, we characterized development of Southern wilt disease and R3bv2 latent infection on geranium plants. Following soil inoculation, between 12 and 26% of plants became latently infected, carrying average bacterial populations of 4.8×10^8 CFU/g of crown tissue in the absence of visible symptoms. Such latently infected plants shed an average of 1.3×10^5 CFU/ml in soil run-off water, suggesting a non-

destructive means of testing pools of asymptomatic plants. Similarly, symptomatic plants shed 2×10^6 CFU/ml of run-off water. A few hundred *R. solanacearum* cells introduced directly into geranium stems resulted in death of almost all inoculated plants. However, no disease transmission was detected after contact between wounded leaves. Increasing temperatures to 28°C for 2 weeks did not convert all latently infected plants to active disease, although disease development was temperature dependent. Holding plants at 4°C for 48 h, a routine practice during geranium cutting shipment, did not increase frequency of latent infections. *R. solanacearum* cells were distributed unevenly in the stems and leaves of both symptomatic and latently infected plants, meaning that random leaf sampling is an unreliable testing method. UW551 also caused potato brown rot and bacterial wilt of tomato, surpassing race 1 strain K60 in virulence on tomato at the relatively cool temperature of 24°C.

Additional keyword: bioterrorism.

Southern wilt of geranium (*Pelargonium hortorum*) is caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al., a soilborne bacterium that typically invades plants through the roots and colonizes host xylem vessels. Symptoms include leaf yellowing, wilting, and necrosis, as well as vascular browning. *R. solanacearum* is best known as the cause of bacterial wilt, a disease that affects diverse plants ranging from solanaceous crops like tomatoes and tobacco to peanuts, bananas, ginger, and eucalyptus. The pathogen is widely distributed in tropical and warm-temperate regions, where it causes large crop losses (21). *R. solanacearum* is a species complex, a large heterogeneous group of related strains (13). It has been subdivided into races based on host range and biovars based on carbohydrate utilization (21). A phylogenetically and phenotypically homogenous cluster known as race 3 biovar 2 (R3bv2) contains cool-tolerant strains believed to have originated in the Andes (14,31).

R3bv2 strains cause potato brown rot, which ranks among the most destructive diseases of potato in Africa, Asia, and Central and South America (6). The pathogen commonly forms latent (asymptomatic) infections in the cool tropical highlands, but when infected seed tubers are planted in warmer lowland fields, the resulting plants quickly wilt and die (1). R3bv2 was introduced into northern Europe in the late 1980s and caused both economic and political problems when it appeared on potatoes in 1995 (23). Despite ongoing eradication efforts, the bacterium is still present in a number of fields and waterways of northern Europe, although it has caused only minor direct crop losses (11). Recent ecological

studies found that an R3bv2 strain could survive at least 12 months in temperate field soil and for at least 110 days in surface water at 12°C (39,40). Although the host range of R3bv2 was once thought to be limited to potatoes, eggplant, and tomato, it is now known that these strains can potentially infect many ornamental and weed species as well (24,32,38,42). *Solanum dulcamara*, a common semi-aquatic weed, is an important survival refuge and source of inoculum in Europe (10). R3bv2 is not known to be established in North America.

Geraniums sold in Europe and North America are commonly grown from cuttings produced in the highland tropics of Africa and Central America, where R3bv2 is endemic. Southern wilt was reported on U.S. geraniums grown from imported cuttings as early as 1981 (35). The volume of geranium cuttings imported to the United States has grown dramatically, increasing from an estimated 10 million cuttings per year in 1985 to an estimated 100 million in 2003 (M. Klopmeier, Ball FloraPlant, *personal communication*). Perhaps not coincidentally, *R. solanacearum* has been repeatedly introduced to Europe and North America in geraniums in recent years (18,24,27,43). Most geranium isolates of *R. solanacearum* that have been classified belong to R3bv2 (24,27,43), though one outbreak was caused by a race 1 biovar 1 strain (33). Southern wilt is a minor disease of geranium, but any introduction of R3bv2 to North America raises concerns because the presence of R3bv2 on potato could cause quarantine and trade-related losses to the potato industry. R3bv2 was among 10 plant pathogens listed in the Agricultural Bioterrorism Protection Act of 2002, so any finding of this organism in the United States is now subject to the strictest eradication requirements and the most stringent security regulations (28).

In February 2003, *R. solanacearum* R3bv2 was detected in the United States in geraniums imported from Kenya; later that year

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the pathogen was also found in geraniums from Guatemala and Costa Rica (3,18). The resulting response cost growers and regulators an estimated \$10 million and involved the destruction of around 5 million plants. To address this outbreak, the Animal and Plant Health Inspection Service (APHIS) prepared a *Ralstonia* Action Plan specifying measures that growers and regulators must take when race 3-infected plants are detected (2). However, these measures were based on best guesses and reasonable extrapolations from other hosts such as potato, because very little research has been done on the geranium–*Ralstonia* interaction. The regulatory problem is complicated by the ability of the bacterium to form latent infections, which are difficult to detect but can generate inoculum that leads to disease spread. Understanding R3bv2 pathogenesis and latency in geranium plants is key to designing rapid, accurate diagnostic methods and rational control responses.

We initiated this research to address some of the many unanswered questions about this plant–pathogen interaction. How virulent are R3bv2 strains on geranium? How easily do they form symptomatic and latent infections of this host? What triggers latently infected plants to become actively symptomatic? Is the disease transmitted easily by casual contact during shipping? How is the pathogen distributed in the tissue and rhizosphere of infected plants?

MATERIALS AND METHODS

Culture conditions and diagnostic testing. *R. solanacearum* strains were grown in casamino acids, peptone, glucose (CPG) broth (22) for rich medium and Boucher's minimal medium (BMM) broth for minimal medium (4). Broth cultures were grown at 28°C with 250 rpm agitation for aeration. To grow cultures on solid medium, we used CPG plus 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride (TZC) (26). Extracts from plants or effluent were plated on modified semi-selective agar medium (SMSA) (12) containing rifampicin (50 µg/ml) to select UW551-rif; all dilution platings were triplicated. *R. solanacearum*-typical colonies were counted after incubation for 48 to 72 h at 28°C. Rapid diagnostic assays were conducted with immunoassay strips specific for *R. solanacearum* according to the manufacturer's instructions (AgDia, Inc., Elkhart, IN). Strains were classified to biovar by inoculating 1×10^7 cells into test tubes filled with 3 ml of Hayward medium containing either 1.0% glucose, 1.0% maltose, 1.0% mannitol, 1.0% dulcitol, or no carbon source as a negative control (20). A color change from green to yellow after 21 days at 28°C indicated the ability of a given strain to produce acid from the carbon source. Diagnostic DNA fragments were amplified by polymerase chain reaction (PCR) using the *R. solanacearum*-specific primer pair 759/760 (5' GTC GCC GTC AAC TCA CTT TCC 3' and 5' GTC GCC GTC AGC AAT GCG GAA TCG 3') and the race 3-specific primer pair 630/631 (5' ATA CAG AAT TCG ACC GGC AC 3' and 5' ATT CAC ATG CAA TTC GCC TAC 3') as described (14,30).

Bacterial strains. *R. solanacearum* strain UW551 was isolated in February 2003 from the stem of a wilted *P. hortorum* (geranium) cv. Americana Dark Red plant growing in a Wisconsin greenhouse. The plant had been grown from a cutting shipped from a production facility near Naivasha, Kenya, at an altitude of 2,000 m. Ooze from the stem was streaked on TZC medium and yielded a pure culture of fluidal irregular cream-colored colonies with pink centers; one colony was identified as *R. solanacearum* by diagnostic immunoassay strip. The identity of the isolate was confirmed by *R. solanacearum*-specific PCR and by sequencing of the 16S rDNA gene, which was amplified using universal prokaryotic primers 27F and 1492R (29). This isolate was further classified as R3bv2 by biovar test and race 3-specific PCR, and was named UW551. To allow selection against the bacterial contaminants present in plant material, we created a rifampicin-resistant derivative of UW551 by moving a spontaneous mutation con-

ferring rifampicin resistance from strain K60-rif into UW551 by means of natural transformation (25). The resulting strain, UW551-rif, was indistinguishable from its parent in terms of growth in minimal medium, growth in rich medium, biovar test profile, and virulence on tomatoes and geraniums. Control strains for virulence, biovar, and PCR analyses were *R. solanacearum* strains K60 (R1bv1) (26); GMI1000 (R1bv3) (4); UW141 (R1bv4); UW143 (R1bv3); UW145 (R3bv2); UW344 (R3bv2); UW361 (R1bv5); *Erwinia carotovora* subsp. *carotovora* (WPP1) (46); *Xanthomonas hortorum* pv. *pelargonii* (UW553); and *Escherichia coli* DH5- α (19). Strains without listed references were from the Allen lab strain collection at the University of Wisconsin-Madison.

Host plant inoculations. Virulence assays on tomato (cv. Bonny Best), potato (cv. Russet Norkotah), and tobacco (cv. Bottom Special) were performed by soil-soak inoculation, in which a bacterial suspension was poured over the soil of unwounded plants. To make inoculum, overnight cultures of *R. solanacearum* strains were pelleted and resuspended in water. Dilution plates were used to confirm the inoculum cell densities. Tomato disease progress curves for strains K60 and UW551 at 24 and 28°C were measured as previously described (37). Briefly, bacterial suspensions were poured into the soil of 14- to 15-day-old unwounded tomato plants to obtain a final density of 2×10^7 CFU/g of soil. Plants were maintained at either 28°C 12-h light/12-h dark or 24°C 14-h light/19°C 10-h dark for 14 days. Plants were assessed daily by a rater blind to strain identity on a scale of 0 to 4 where 0 = no wilt, 1 = 1 to 25% leaf area wilted, 2 = 26 to 50% wilted, 3 = 51 to 75% wilted, and 4 = 76 to 100% wilted or dead. Sixteen plants were inoculated for each treatment and the entire experiment was repeated three times. Virulence of UW551 on potato and tobacco was confirmed by soil-soak inoculation of 10 potato plants grown from cuttings and five tobacco plants grown from seed. Geranium plants (cv. Americana Dark Red) were grown from rooted geranium cuttings (supplied by Goldsmith Plants, Gilroy, CA). Rooted cuttings were transplanted in their rooting plugs into 4-in.-round pots containing about 80 g of Scotts seedling potting mix (Scott's, Marysville, OH) and were given at least 1 week to recover from transplanting before inoculation. Unless otherwise noted, geraniums were inoculated with strain UW551-rif and grown at 24°C 14-h light/19°C 10-h dark, environmental conditions that correspond to those found in commercial production. Geranium plants were inoculated by pouring 50 ml of a bacterial cell suspension over the soil, with the exception of the petiole scar inoculation experiment described below. Inoculum cell densities for each experiment are given below.

Quantifying bacteria from plant tissue. To determine the size of *R. solanacearum* populations in geranium plants, we harvested two slices of about 0.1 g each from the base of the stem just at the soil line (the crown) or from other plant parts as specified below. One slice was tested for presence of *R. solanacearum* using the immunostrip; since the strip's limit of detection is around 10^5 CFU of bacteria, this allowed rough estimation of bacterial population size for calibration of the dilution plating. The second slice was ground with a mortar and pestle and suspended in 1 ml of water, serially diluted 10-fold in water, and plated on SMSA plus rif agar plates.

Quantifying bacteria in geranium effluent. The size of *R. solanacearum* populations in effluent (run-off) water and crown tissue from inoculated plants was determined using inoculated plants from the temperature effect experiments. Symptomatic plants were visually rated as stage 1 (1 to 33% of leaves yellowed, wilted), stage 2 (34 to 66% of leaves yellowed or wilted), stage 3 (67 to 100% of leaves yellowed or wilted), or stage 4 (dead and decaying). To obtain effluent water, geranium pots were placed on two glass stirring rods over a clean petri dish. Water was poured over the soil of the geranium plant until 5 to 15 ml of effluent flowed from the bottom of the pot into the petri dish. Two hun-

dred fifty microliters of effluent was mixed with 250 μ l of AgDia BEB1 immunoassay buffer and after 5 min this mixture was tested with an immunostrip to determine if more than 10^5 CFU was present. The effluent was then dilution plated on SMSA plus rif. Crown tissue was also harvested, ground, and dilution plated. To study bacterial population sizes in effluent from geranium plants that had no residual bacterial inoculum in their soil, 60 geranium plants were directly inoculated in the stem. A lower leaf was snapped off at the stem, generating an open wound where the petiole had been attached (petiole scar). A 2- μ l *R. solanacearum* suspension containing either 180 cells (30 plants) or 1,800 cells (30 plants) was applied to the petiole scar; this droplet was immediately absorbed into the stem. Plants were monitored for wilt symptoms, effluent was collected from plants at various disease stages, and bacteria were enumerated by plating.

Determining *R. solanacearum* distribution in plants. Twelve symptomatic and 12 latently infected, soil-inoculated geranium plants were sampled by randomly taking cross sections of crown tissue, stems, petioles, and flower stalks. Each sample was cut in half lengthwise. One half was used for an immunostrip test, and the other half was weighed, ground with sterile distilled water, and dilution plated on SMSA plus rif. Plant material giving a positive strip test was ground and dilution-plated at 10^{-4} , 10^{-5} , and 10^{-6} , and material giving a negative strip test was plated at 10^{-1} . If no bacteria were detected at 10^{-1} dilution, a sample was reported as 0. If no bacteria were detected at 10^{-4} dilution (and no lower dilutions were available), a sample was reported as undetected.

Determining effects of temperature increase on frequency of latent infection. Forty-five geranium plants were soil-inoculated to a final density of 6.25×10^5 CFU/g of soil and 45 plants were inoculated to a final density of 6.25×10^6 CFU/g of soil. Eight water controls remained uninoculated. After 25 days at 24°C 14-h light/19°C 10-h dark, all symptomatic plants were de-

stroyed by autoclaving. From the remaining asymptomatic plants, 16 were randomly harvested from each treatment, and their crown tissue was plated as described previously to test for latent infection. The remaining unharvested asymptomatic plants were moved to 28°C 12-h light/12 h-dark. After an additional 14 days at the higher temperature, all diseased plants were destroyed by autoclaving and the asymptomatic plants were harvested and tested for latent infection. This experiment was replicated three times.

Determining effects of temperature decrease on frequency of latent infection. Sixty geranium plants were soil-inoculated to a final density of 4.27×10^7 CFU/g of soil. After 48 to 72 h incubation at 24°C/19°C, 30 plants were moved to 4°C while 30 control plants remained at 24°C/19°C. After 48 h, the plants were moved back to 24°C/19°C. All the plants were then monitored for an additional 31 days. After this time, all plants were harvested as described previously. This experiment was replicated three times.

Measuring pathogen transmission via co-mingling of plants. Fifty rooted geranium cuttings, still in their shipping flat, were lightly wounded by running two gloved hands back and forth over them for four passes. A symptomatic wilting geranium was cut from its pot and held by the stem as it was brushed over the geranium cuttings four to five times. The cuttings were then transplanted into 9-oz plastic cups with no drain holes containing moist potting mix and incubated at 24°C 14-h light/19°C 10-h dark. After 14 days, a 0.1-g slice of crown tissue was removed from each plant and incubated overnight in 3 ml of SMSA broth at 28°C with shaking to enrich for *R. solanacearum* cells. One hundred microliters from each tube was spread on duplicate SMSA plus rif plates to detect any *R. solanacearum* colonies. Stems were considered positive if the broth was cloudy or if any *R. solanacearum* colonies grew on the SMSA plates. This experiment was replicated three times.

RESULTS

***R. solanacearum* UW551 is a virulent R3bv2 isolate.** *R. solanacearum* strain UW551 was isolated from a wilting geranium plant derived from cuttings shipped from Kenya, which is the presumed origin of this isolate. PCR amplification of a race 3-specific band (13) and the biochemical biovar test (20) both indicated that UW551 belonged to R3bv2 (data not shown). UW551 has a broad profile of antibiotic resistances. It is fully resistant to ampicillin at 25 μ g/ml, chloramphenicol at 50 μ g/ml, gentamycin at 50 μ g/ml, streptomycin at 60 μ g/ml, and spectinomycin at 20 μ g/ml; it had partial resistance (slow growth) on ampicillin at 50 μ g/ml and kanamycin at 12.5 μ g/ml. UW551 was pathogenic on geranium, potato, and tomato, but not on tobacco (data not shown). R3bv2 strains of *R. solanacearum* are considered more cold tolerant than race 1 strains (9). To compare the effect of temperature on virulence of R3bv2 strain UW551 and R1bv1 strain K60, we inoculated tomato plants by soil-soak and incubated them at either 24 or 28°C. At the warmer temperature, strain K60 killed all plants by 12 days after inoculation, whereas UW551 never caused 100% disease. At 24°C, both strains caused less disease on tomato than at 28°C; however, UW551 generated a significantly higher mean disease index than K60 at the cooler temperature (Fig. 1).

***R. solanacearum* commonly forms latent infections on geraniums.** Following each of our soil-soak inoculations of geranium plants, we identified a significant number of plants that had no detectable Southern wilt symptoms but contained large populations of *R. solanacearum* in their crown tissue (the area just above the soil line where the stem transitions to root). These were classified as latently infected. In multiple experiments in which geranium plants were inoculated with 1×10^7 to 5×10^7 CFU/g of soil and incubated at 24°C, an average of approximately 20% of the plants became latently infected. However, the proportion of plants that developed active infections, latent infections, and those that

Fig. 1. Disease progress on tomato caused by *Ralstonia solanacearum* UW551 and K60 at 24 and 28°C. Bacterial suspensions were poured over the soil of 15-day-old tomato plants (cv. Bonny Best) to a final density of 3.88×10^7 CFU/g of soil for race 1 biovar 1 strain K60 and 3.53×10^7 CFU/g of soil for race 3 biovar 2 strain UW551. For each treatment in each replicate, 16 tomato plants were incubated at 24°C and 16 were incubated at 28°C. Disease progress was rated daily for 14 days on a disease index scale of 0 to 4 (0 = no wilt, 1 = 1 to 25% leaves wilted, 2 = 26 to 50% leaves wilted, 3 = 51 to 75% leaves wilted, and 4 = 76 to 100% leaves wilted). Squares represent K60, circles represent UW551, filled symbols represent incubation at 28°C, and open symbols represent incubation at 24°C. This graph shows the combined data from three independent experiments; thus, each point represents the mean disease index of 48 plants. Bars represent standard error.

remained uninfected varied considerably across experiments. Latently infected geraniums generally carried large populations of bacteria in their crown tissue, averaging 4.8×10^8 CFU/g (Fig. 2). These numbers were comparable to the populations found in the crowns of actively symptomatic geraniums (Fig. 2). We rarely observed more than 60% symptomatic plants, suggesting that under our inoculation conditions *R. solanacearum* was not an especially aggressive pathogen of geraniums. This result is in agreement with reports from geranium growers, who have not experienced serious direct losses to Southern wilt disease (M. Klopmeier, Ball Flora-Plant and D. Snow, Goldsmith Plants, *personal communication*).

Geranium plants infected with *R. solanacearum* shed large numbers of bacteria from their roots. *S. dulcamara* plants infected with *R. solanacearum* R3bv2 shed bacteria abundantly from their roots (10). To test the hypothesis that both geraniums showing disease symptoms and geraniums that were latently infected would have high bacterial populations in their effluent, we dilution-plated run-off water from the pots of both symptomatic and asymptomatic geranium plants. Any plant containing more than 10 CFU of *R. solanacearum* per gram of crown tissue was considered infected. In practice, the distribution of population sizes in plants was bimodal; nearly all plants contained either no detectable *R. solanacearum* cells or more than 10^5 CFU/g of crown tissue. Latently infected plants generated significantly larger effluent populations than uninfected plants, and diseased plants generated the highest effluent populations (Fig. 2A). Overall, the soil run-off water from both symptomatic and latently infected geraniums contained large numbers of bacteria. Latently infected plants shed an average of 1.3×10^5 CFU/ml of effluent, while symptomatic plants shed an average of 2×10^6 CFU/ml of effluent (Fig. 2A). As disease symptoms progressed and the geraniums began to die and decay, the bacterial populations inside the plant decreased sharply, but the effluent populations remained relatively large. To verify that the numbers of bacteria we ob-

served in effluent were not due to residual soil-inhabiting *R. solanacearum* cells left from the initial soil-soak inoculation, we inoculated plants directly into the stem with either 180 or 1,800 CFU. This direct introduction of *R. solanacearum* into the plant's vascular system generated no latently infected plants: 59 out of 60 inoculated plants developed symptomatic infections and the one asymptomatic plant contained no detectable bacteria 86 days after inoculation. However, these directly inoculated plants generated bacterial populations in their effluent water as large as those collected from soil-soak inoculated plants (Fig. 2B), suggesting that the effluent population sizes shown in Figure 2A derive primarily from the infected plant, not from residual inoculum in the soil.

Increasing temperature did not reliably convert latent infections of geranium into active symptomatic disease. To test the hypothesis that increasing temperatures will trigger symptoms and thus identify all latently infected geranium plants, we inoculated 45 geranium cuttings for each experiment with UW551-rif and incubated the plants at 24°C 14-h day/19°C 10-h night. After 25 days, emergence of new disease had leveled off. At this point all diseased plants were discarded, and a random sample of 16 asymptomatic plants were harvested and tested for latent infection to determine the baseline frequency of latent infection. The remaining plants were maintained at 28°C for 14 days to significantly exceed the time-temperature guidelines stipulated in the 2003 APHIS *Ralstonia* Action Plan (2), and then all remaining geraniums were harvested and tested for latent infection. This increase in ambient temperature was followed by wilting of many previously asymptomatic geranium plants, confirming the importance of temperature in Southern wilt disease development (Fig. 3). However, after 14 days at 28°C there were still asymptomatic plants that harbored large numbers of bacteria in their crown tissue.

Exposing geranium plants to cold temperatures postinoculation did not increase frequency of latent infection. To reduce spoilage, geranium cuttings are chilled to about 4°C immediately

Fig. 2. Population sizes of strain UW551 in the crown tissue and the effluent water of geraniums at different stages of infection. Geranium plants were inoculated with strain UW551-rif **A**, by soil soak (6×10^6 to 5×10^7 CFU/g of soil) or **B**, by directly introducing 180 or 1,800 bacterial cells into a petiole scar, and given at least 45 days to develop symptoms. Water was poured over the soil and effluent was collected as it flowed from the bottom of the pot. Stem tissue was sampled by grinding and dilution plating a cross section of crown tissue; effluent water was dilution-plated directly. Gray bars represent bacterial populations in the crown tissue as CFU per gram of tissue and open bars represent bacterial populations in effluent as CFU per milliliter. Within each type of sample, bars marked with different letters differ at the $P < 0.05$ significance level (Student's *t* test), and *n* represents the number of plants tested for each group. **B**, Effluent population data are missing from 8 of the 60 plants: three from disease stage 1, two from disease stage 2, and two from disease stage 3.

after harvest and held at this temperature when possible during shipping and while awaiting quarantine inspection at port in the United States; this process takes approximately 2 days. To test the hypothesis that brief exposure to low temperatures increases likelihood of latent infection, we inoculated geranium plants with *R. solanacearum* UW551-rif and incubated them at 24°C 14-h day/19°C 10-h night for 48 to 72 h. Half the plants were then transferred to 4°C (without light) for 48 h and then returned to 24°C/19°C. Thirty-five days after inoculation, all the plants were tested for latent infection. We found no significant differences in the proportion of latently infected plants between the cold-shocked group and the untreated group (Fig. 4). This result suggests that current shipping and handling practices involving holding geranium cuttings at low temperatures do not increase the likelihood that *R. solanacearum*-infected plants will develop latent rather than symptomatic infections.

***R. solanacearum* was distributed variably throughout above-ground parts of symptomatic and latently infected geraniums.** We observed that Southern wilt symptoms often develop on one side of a geranium plant well before the other side shows disease. To test the hypothesis that this pathogen is not uniformly distributed in its host, we took tissue samples from various parts of symptomatic and latently infected geranium plants, ground them and dilution plated them to measure bacterial population size in each plant part. Samples were taken from symptomatic petioles, healthy petioles, stem tissue, crown tissue, and flower stalks. The plant was considered infected if the crown tissue contained significant populations of *R. solanacearum*. However, the density and presence of R3bv2 in different parts of symptomatic geraniums was highly variable (Fig. 5A). Within a single plant, some tissue samples contained no detectable bacteria while other parts carried large populations. Similar results were found for the latently infected plants (Fig. 5B), although among these plants a larger proportion of sites contained zero or no detectable bacteria. This result demonstrated that sampling for bacteria by removing random stem pieces or petioles is not a reliable method of diagnosing *R. solanacearum* infections of geranium.

Southern wilt does not spread via casual contact between above-ground geranium plant parts. The 2003 USDA-APHIS

action plan for dealing with the *R. solanacearum* R3bv2 outbreak in American greenhouses involved an initial hold order and eventually destruction of all plants that had been “co-mingled” with suspect plants; these included any that had been within 1 m of symptomatic geraniums and any plants that “may have come into contact with suspect geraniums during shipping” (2). To study the frequency of disease transmission between geranium cuttings after casual contact, we simulated the minor wounds and plant-to-plant contact likely to occur during shipping by brushing a wilting geranium plant (infected with UW551-rif) several times over the leaves of lightly damaged, rooted, geranium cuttings still

Fig. 4. Effect of cold shock on latent infections. Sixty geranium plants were inoculated with *Ralstonia solanacearum* UW551-rif to a final concentration of about 4×10^7 CFU/g of soil. Thirty plants (gray bar) were held at 4°C for 48 h at 2 days postinoculation. The other 30 plants (open bars) received no cold treatment. After 32 to 35 days at 24°C 14-h light/19°C 10-h dark, crown tissue of asymptomatic plants was harvested by grinding and dilution plating to determine whether they were latently infected. The graph represents the means of three independent experiments. Bars represent standard error.

Fig. 3. Effect of increased temperature on latent infections. Forty-five geranium plants were inoculated with *Ralstonia solanacearum* UW551-rif to a final density of either **A**, 6.25×10^5 CFU/g of soil or **B**, 6.25×10^6 CFU/g of soil and incubated at 24°C 14-h light/19°C 10-h dark. After 25 days, populations in 16 asymptomatic plants were determined and the remaining asymptomatic plants were moved to 28°C 14-h light/28°C 10-h dark. After 14 additional days, bacterial populations in the remaining asymptomatic plants were measured to identify those latently infected. The graph shows the percentage of total plants inoculated at each inoculum level that became symptomatic, latently infected, and actively infected. Open bars represent plants tested at day 25 and black bars represent plants tested at day 40. The graph shows the result of three independent experiments for each inoculum level.

in their shipping trays. After 14 days incubation at 24°C, a slice of crown tissue from each brushed plant was incubated overnight at 28°C in modified SMSA enrichment broth, which was then plated on SMSA. We tested a total of 150 cuttings in this manner in three separate experiments and never detected *R. solanacearum* cells in the SMSA broth (data not shown). When slices of stem tissue from symptomatic geraniums were incubated in SMSA broth as a positive control, they invariably resulted in cloudy cultures and produced detectable *R. solanacearum* colonies after plating on TZC agar. We concluded that casual contact between above-ground plant parts did not result in detectable transmission of *R. solanacearum*.

DISCUSSION

We undertook these studies to better understand the interactions between geranium plants and the Southern wilt pathogen, *R. solanacearum* R3bv2. These interactions are of particular concern for regulatory scientists and geranium producers working to develop detection methods and offshore production standards that will reliably prevent further introductions of this quarantine pathogen on imported geranium cuttings. Our studies demonstrated that interactions between R3bv2 and geraniums are complex, with no single environmental or biological factor explaining latent infections. Moreover, we found that large numbers of the bacterium are shed from the roots of both latently infected and symptomatic geraniums, a finding that identified a worrisome source of pathogen dispersal but also suggested a useful diagnostic method.

Infected but symptomless plants pose a vexing problem for producers of vegetatively propagated plants. This problem of latent infection is exacerbated in the case of geraniums and *R. so-*

lanacearum R3bv2 because the pathogen is a highly regulated, zero tolerance quarantine pest in western Europe and North America. In addition, producers and regulatory agencies are confronted by an overwhelming volume of imported cuttings (an estimated 100 million in 2003 in the United States alone). It is therefore of considerable interest to identify the factors that cause *R. solanacearum* to form a latent rather than a symptomatic infection in a geranium plant. Latent infections were common under our experimental conditions, ranging from 12 to 26% of all inoculated plants. We observed significant variability in the percentage of inoculated plants that developed latent infections across multiple experiments. Frequency of latency appeared to be affected by inoculum density and the age of the geraniums at inoculation, but other as-yet unknown factors must also affect development of latent infections. These may include light duration and intensity, plant nutritional status, and size of plants at the time of inoculation. In addition, host plant genetics probably affect the infection process, although nothing is known about resistance to Southern wilt in geranium. Most host resistance to *R. solanacearum* is quantitative and multigenic, and resistance often results from reduced pathogen colonization (34). The bacterium colonizes stems of susceptible tomato lines more effectively than those of resistant lines, and tubers of resistant potato plants are less likely to be latently infected (7,17); these findings suggest that plant host resistance will also affect frequency of latent infection in geranium.

It was particularly important to investigate the effect of temperature on this pathosystem because it is a key parameter for development of both bacterial wilt disease and latent infection by *R. solanacearum* in other hosts (21). Temperature has been repeatedly shown to influence latent infection of potato tubers by R3bv2, and in particular, high temperatures can trigger development of brown rot disease in the field, unmasking potato plants

Fig. 5. Distribution of *Ralstonia solanacearum* race 3 biovar 2 throughout infected geranium plants. Tissue samples were taken from various regions of infected geranium plants, indicated on the x axis, to determine bacterial CFU per gram of tissue (fresh weight). Each point represents one sample from one plant, and points of the same color represent samples taken from different parts of a single plant. Samples listed as “undetectable” generated no colonies at a detection limit of about 1×10^5 CFU/g of tissue, while those listed as zeros generated no colonies at a detection limit of about 1×10^2 CFU/g of tissue. Total samples in each of these two categories are represented in brackets as [number of zeros, number of undetectables]. We sampled **A**, 12 symptomatic plants and **B**, 12 latently infected plants. Latent infection was defined as the presence of bacteria in the crown tissue in the absence of visible symptoms.

latently infected by *R. solanacearum* R3bv2 (7,9,15,16,21,34,36). We found that geranium plants were more susceptible to R3bv2 strain UW551 at 28°C than at 24°C (data not shown). On tomato plants, UW551 was not as aggressive at 28°C as R1bv1 strain K60, but it was notably more aggressive than K60 at 24°C (Fig. 1). Similar comparative studies on potato plants also found that R3bv2 strains were more virulent at 16, 20, and 24°C than race 1 strains, although the strains caused similar levels of disease at 28°C (8). Together, these results support the idea that R3bv2 strains of *R. solanacearum* are better adapted to cause disease at moderately cool temperatures than the more tropical races 1 and 2. However, it should be noted that in our assay, UW551 was not an especially successful tomato pathogen, even on highly susceptible cv. Bonny Best. Neither strain tested killed all tomato plants at 24°C and at 28°C, race 1 strain K60 was significantly more virulent than UW551 (Fig. 1).

A time-temperature table in the 2003 USDA-APHIS *Ralstonia* Action Plan required growers to hold geraniums suspected of harboring *R. solanacearum* at warm temperatures for a certain period before they could be released for sale (2). Our results demonstrated that although raising temperatures did generally increase disease incidence in geranium plants inoculated with *R. solanacearum* R3bv2, holding latently infected geranium plants at high temperature for 2 weeks did not reliably cause all latently infected geraniums plants to develop visually detectable symptoms (Fig. 3). Given that there is zero tolerance for this pathogen, this result suggests that it is not useful to place greenhouses containing suspect geraniums under a hold order until they reach an arbitrary time-temperature target.

An interval at low temperature did not affect the course of disease caused by UW551 on geranium. After harvest, geranium cuttings are usually kept at 4°C for several days during shipping and inspection. *R. solanacearum* R3bv2 strain 1609 reportedly enters a viable-but-nonculturable state following exposure to 4°C in water; cells revived from this state could colonize tomato plants but were no longer virulent (41). However, we found that holding inoculated geranium plants at 4°C for 48 h did not affect frequency of either latent or symptomatic infections (Fig. 4). It should be noted that these bacteria were in soil or plant tissue when exposed to 4°C, which may have altered their response to low temperature. Moreover, our experiments involved a relatively brief exposure to 4°C. It is possible that sustained incubation of inoculated geranium plants at slightly warmer temperatures that permit limited bacterial multiplication (such as 16°C) or exposure to 4°C at a different time or stage of infection would have a different effect on Southern wilt disease development. Finally, while this experiment was conducted on small geranium plants growing in potting mix, regulatory concerns focus on unrooted cuttings from latently infected plants, which may respond differently to chilling.

We found that latently infected geranium plants and those showing symptoms both shed large numbers of *R. solanacearum* cells from their roots, and that these bacteria could be easily collected and detected in the effluent flowing from their pots (Fig. 2). This finding suggests the possibility of a nondestructive, low-technology method for testing large numbers of pooled plant samples for latent infection with *R. solanacearum*. Run-off water from many plants could be combined and concentrated by filtration to allow detection of one or a few latently infected plants by commercial immunostrip or PCR. However, our effluent data were obtained from young geranium plants growing in peat-based potting mix. To understand how *R. solanacearum* cells are shed from geranium plants under typical cutting production conditions, these studies should be repeated on large mother plants growing in the volcanic scoria potting medium that is usually used for off-shore cutting production. The abundant release of bacteria from roots of infected plants also indicates that ebb-and-flow subirrigation systems can be a very effective means of disseminating this pathogen and should therefore be avoided by geranium producers.

Some plants remained latently infected for at least 120 days under our conditions, suggesting that geraniums may be able to maintain this symptomless but infected condition indefinitely.

Geraniums are propagated vegetatively by rooting developing branches cut from large mother plants. Diagnostic sampling of geranium plants to detect *R. solanacearum* often involves testing a single leaf or stem chosen at random. However, despite its relevance for cutting production and testing, nothing is known about the distribution of *R. solanacearum* in infected plants. We found *R. solanacearum* cells were not uniformly distributed in infected geranium plants, which we defined as carrying bacterial populations of more than 10 CFU/g of crown tissue. Bacterial populations varied wildly within a single infected plant, ranging from 5×10^{10} CFU/g of tissue to undetectable (Fig. 5). Symptomatic plants generally had higher populations than latently infected plants, and latently infected plants were more likely to have regions free of the pathogen. However, we also often found parts of symptomatic plants that contained no detectable bacteria. These results indicate that it is inappropriate to randomly test a single geranium stem or leaf for *R. solanacearum*. The patchy distribution of this pathogen in above-ground geranium parts is also consistent with our finding that *R. solanacearum* was not transmitted by casual leaf-to-leaf contact of the kind that occurs during shipping or between plants adjacent on greenhouse benches. In contrast, direct inoculation of the petiole scar on geranium stems with even a few hundred *R. solanacearum* cells quickly killed 59 of the 60 plants tested (the remaining plant was an apparent escape, containing no detectable bacteria). This finding suggests that latent infection and resistance to Southern wilt are circumvented when the pathogen is introduced directly into host xylem tissue rather than invading the plant through roots. Contaminated cutting knives are thus likely to be a very efficient means of pathogen transmission. Growers frequently pinch off developing flower stalks from geranium plants by hand, which could also transmit disease. However, pinching may be slightly less of a threat than infested cutting knives. We detected *R. solanacearum* in only 5 of 10 flower stalks from symptomatic plants and 5 of 12 flower stalks from latently infected plants, which suggests that flower stalks may be less frequently colonized than other above-ground plant parts.

It is of both applied and basic interest to understand how large populations of *R. solanacearum* can live in plant tissue without triggering symptoms. One key to understanding the biology of latency may lie in pathogen gene expression. *R. solanacearum* expresses a subset of its genes specifically during wilt disease development (5). In vivo expression technology or microarray analysis could be used to determine if a different set of bacterial genes are expressed during latent infection than during symptomatic disease. From the other side of the interaction, measuring expression of plant defense genes could reveal whether *R. solanacearum* cells living latently in geranium plants trigger host defense responses. Together with direct studies of such parameters as plant age, cultivar, and temperature, these experiments are needed to elucidate the complex relationship between latent infection by *R. solanacearum* and active Southern wilt disease in geranium plants.

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