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Authors

Vallad, G E
Qin, Q M
Grube, R
et al.

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Characterization of Race-Specific Interactions Among Isolates of *Verticillium dahliae* Pathogenic on Lettuce

Gary E. Vallad, Qing-Ming Qin, Rebecca Grube, Ryan J. Hayes, and Krishna V. Subbarao

First, second, and fifth authors: Department of Plant Pathology, University of California, Davis, c/o United States Agricultural Research Station, Salinas, CA 93905; third author: Cooperative Extension and Department of Plant Biology, University of New Hampshire, Durham 03824; and fourth author: U.S. Department of Agriculture–Agricultural Research Service, Salinas, CA 93905.
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ABSTRACT

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Verticillium wilt, caused by *Verticillium dahliae*, poses a major threat to lettuce (*Lactuca sativa*) production in California. Incorporation of resistance into commercial lettuce cultivars offers the least expensive technique of sustaining production in infested areas. To test the breadth of the resistance identified in field experiments, a pair of susceptible ('Salinas' and 'Sniper') and resistant ('La Brillante' and 'Little Gem') lettuce cultivars were used as differentials and individually inoculated with 29 isolates of *V. dahliae* and two isolates of *V. albo-atrum* from several hosts, including lettuce, in replicated greenhouse experiments. The reactions of the four cultivars were determined based on the disease severity at maturity. None of the *V. albo-atrum* isolates or *V. dahliae* isolates from cruciferous hosts caused significant disease on lettuce. Both Salinas and Sniper were susceptible to many isolates of *V. dahliae* (21 of 23) from noncruciferous hosts, and the isolates varied in their overall virulence. However, of these, only three isolates caused significant disease on the resistant cvs. La Brillante and Little Gem. These three isolates also were distinct from the other *V. dahliae* isolates based on sequence data from the intergenic spacer (IGS) region of the nuclear ribosomal RNA gene, suggesting that they form a phylogenetically distinct subgroup that differs in virulence toward specific lettuce genotypes. Accordingly, isolates of *V. dahliae* virulent on all tested cultivars, including the resistant La Brillante and Little Gem, were designated as race 2, whereas those virulent only on the susceptible Salinas and Sniper were designated as race 1. Although a range of virulence among isolates has been described in other hosts, this is the first description of distinct virulence phenotypes in *V. dahliae* since a similar race structure was described in tomato in the 1960s.

Lettuce (*Lactuca sativa* L.) is a major crop in California, with production from spring through fall in the cool coastal valleys of Santa Maria, Santa Cruz, San Benito, and Monterey Counties, and in the San Joaquin and Imperial Valleys during the winter. However, the bulk of the lettuce industry is located in the Salinas Valley, accounting for 42% of the total acreage in the United States, with an estimated value of $1 billion in 2004 (1,16). Verticillium wilt, a common disease on a variety of crops grown in this area caused by the soilborne fungus, *Verticillium dahliae* Kleb., was not a problem in lettuce until 1995, when the disease appeared suddenly on lettuce in a field in Watsonville, CA and several isolates of *V. dahliae* were identified (23,28). The disease has since spread to nearly 400 ha, including lettuce production areas located in the heart of the Salinas Valley posing a serious threat to the California lettuce industry.

V. dahliae can colonize the vascular tissues of a broad range of plants (17). This fungus produces nonseptate conidia borne on verticillate phialides and microsclerotia that can survive dormant in the soil for 10 to 15 years. Microsclerotia germination stimulated by root exudates gives rise to root colonization and initiates the disease (29). Disease incidence and severity generally increases with the buildup of microsclerotia in the soil. Early disease symptoms, only revealed by vertical sectioning of the plant, begin at the apical end of the taproot with the appearance of vascular discoloration that progresses up through the taproot into the stem, coinciding with the appearance of foliar symptoms on basal leaves. Leaves develop areas of chlorosis and angular necrotic lesions along the leaf margins prior to wilting; these symptoms progress acropetally and eventually lead to plant death. Other key foliar symptoms include stunting, defoliation, and other developmental abnormalities, such as a delay or induction of reproductive growth. Prior to the onset of foliar symptoms, the vascular discoloration of root and stem tissues are the only other diagnostic feature of the disease (17).

Symptoms of Verticillium wilt are absent in most hosts until they reach vegetative maturity or initiate reproductive growth. In lettuce, symptoms develop quickly near harvest when the plant reaches vegetative maturity (23). These symptoms are most devastating to crisphead cultivars, because the lower leaves envelope the entire head and, as the disease progresses, other leaves underneath also begin to wilt, leading to plant collapse. In heavily infested fields, the losses are staggering; an entire lettuce crop that appeared healthy only a week earlier is lost to Verticillium wilt. Growers often are forced to harvest their crop early and submit to a loss in yield. Nonetheless, the lower leaves of infected lettuce plants become lined with microsclerotia before death, a feature unique to Verticillium wilt of lettuce, leading to the rapid and abnormally high buildup of microsclerotia in the soil (23,28). Microsclerotia levels associated with infested lettuce fields have ranged from 150 to 2,500 microsclerotia g^{-1} of dry soil (23), compared with the 20 to 80 microsclerotia g^{-1} of dry soil described for a field continuously cropped with cotton over 7 years (18). Control of *V. dahliae* through cultural practices is limited due to the existence of cross-pathogenic isolates capable of infecting most crops grown in rotation with lettuce, including strawberry and artichoke (4,19), and the considerable economic and environmental costs that would be associated with the routine use

Corresponding author: K. V. Subbarao; E-mail address: kvsubbarao@ucdavis.edu

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of soil fumigants. The demonstrated capability of *V. dahliae* to reside in natural flora and invasive weeds and its ability to contaminate the seed of various plants are additional concerns (26). Because of the longevity of microsclerotia in soil, *V. dahliae* is quite invasive and new host-adapted isolates become part of the soil fungal community upon their introduction (17), raising concerns of long-term agronomic and economic sustainability of lettuce production in coastal California. Because of the limitations in available cultural control practices and the economic and environmental burden associated with chemical soil fumigants, the development of lettuce cultivars resistant to *V. dahliae* seems to be the most practical and viable means of controlling Verticillium wilt and maintaining agronomic sustainability within the region.

Race-specific (vertical) resistance to Verticillium wilt is best exemplified by the *Ve* locus in tomato (*Lycopersicon esculentum* Miller), which is composed of two closely linked genes, *Ve1* and *Ve2*, and confers resistance to race 1 isolates of *V. dahliae* (8,15). Tomato plants with the *Ve* genes are able to limit the colonization of root tissues by the pathogen, preventing the pathogen from reaching foliar tissues (11,25). However, race 2 isolates are not limited by the *Ve* resistance genes. Outbreaks of Verticillium wilt linked to race 2 isolates appeared shortly after the release of tomato cultivars with the *Ve* resistance genes in the United States and, subsequently, worldwide (2,17,21). The source of these race 2 isolates has never been clearly established. It was speculated that their appearance was due to the spread of a new recombinant or mutant isolate or to the selection of endemic race 2 isolates, because of the widespread use of the *Ve* resistance genes in tomato production (10,17,24). Regardless of the race, a continuum of virulence exists among isolates of *V. dahliae* pathogenic on tomato, suggesting that considerable genetic variation at loci other than those critical to the specificity of *Ve* genes also exists (10,17,24). Similar findings regarding the existence of a continuum of virulence among isolates of *V. dahliae* also were made in crops like cotton (3) and potato (14).

Several lettuce cultivars, including the heirloom cvs. La Brillante and Little Gem, were identified as potential sources of resistance to Verticillium wilt in field trials (R. J. Hayes, G. E. Vallad, Q.-M. Qin, R. Grube, and K. V. Subbarao, *unpublished data*). Concurrent phylogenetic studies based on sequence analysis of the β-tubulin gene and the IGS region of the nuclear ribosomal RNA gene found that these sequences were useful for classifying isolates of *V. dahliae*, *V. albo-atrum* Reinke and Berth., and *V. tricorpus* I. along taxonomic lines, and that considerable diversity existed among *V. dahliae* isolates from lettuce and other noncruciferous crops, allowing for the division of these isolates into four phylogenetically distinct subgroups (*Vd*NC1, *Vd*NC2, *Vd*NC3, and *Vd*NC4) (19). Isolates of *V. dahliae* from lettuce grouped into subgroups *Vd*NC1 and *Vd*NC3 along with isolates from other noncruciferous crops, including pepper, strawberry, and artichoke, that are grown in rotation with lettuce in the Salinas Valley (19). In view of these findings, our objectives were to evaluate isolates of *V. albo-atrum* and *V. dahliae* from lettuce and other crops for pathogenicity and virulence on the resistant lettuce cvs. La Brillante and Little Gem and the susceptible cvs. Salinas and Sniper, and compare the responses of such isolates to previously obtained phylogenetic data (19). Such evaluations are imperative to predicting the utility and durability of any resistance. Parts of this research were presented previously (27).

MATERIALS AND METHODS

Isolate preparation. Twenty-nine isolates of *V. dahliae* from various crops, including the majority from lettuce and two isolates of *V. albo-atrum* from alfalfa, were used in the virulence tests. The hosts and geographic origin of these isolates are presented in Table 1. The identities of all single-spore isolates were confirmed on the basis of colony morphology, conidiophore formation, conidial production, and presence of microsclerotia. Isolates were maintained on potato dextrose agar (PDA) in vials at room temperature or as conidia suspended in a 35% (vol/vol) glycerol solution at –80°C. For inoculum preparation, isolates were grown in the dark on PDA plates for 4 to 5 days at either 20 or 25°C. Conidia were suspended gently from PDA plates in sterile, distilled water and adjusted to a final concentration of 2.0×10^6 conidia·ml^{-1}.

Seedling production and inoculation. Seedlings of lettuce cvs. Salinas, Sniper, La Brillante, and Little Gem were grown in an autoclaved sand/potting soil mixture (3:1) for 4 weeks in a greenhouse. Seedling roots were rinsed free of soil and dipped for at least 30 min in a conidial suspension containing 2×10^6 conidia·ml^{-1}. A separate set of seedlings also were dipped in sterile, distilled water to serve as noninoculated controls. All seedlings then were transplanted to 0.5-liter foam-insulated cups filled with a pasteurized sand/potting soil mixture (3:1) and maintained for an additional 8 to 10 weeks in a greenhouse with supplemental lighting (16-h photoperiod) provided from late fall (mid-October) to spring (mid-March) when the natural photoperiod is less than 12 h. Greenhouse temperatures, on average, were maintained at 24°C (±5°C) during the day and 12°C (±5°C) at night. For each isolate, 12 plants of each cultivar were inoculated per experiment and the whole experiment was conducted three times. In two of the three experiments, the fresh weight of foliar tissues was recorded (expressed in grams) using a laboratory scale. Fresh foliar weight data were not collected for Little Gem because of the excessive manual defoliation required to minimize plant loss to infection by *Botrytis cinerea*.

Disease assessment. After 8 to 10 weeks of incubation, plants were uprooted, rinsed free of soil, and cut longitudinally from the crown through the tap root to assess the severity of Verticillium wilt symptoms based on the extent of vascular discoloration and the presence of foliar symptoms, including wilting and angular regions of chlorosis and necrosis along leaf margins. Disease severity was rated on a scale of 0 to 5, in which 0 = no vascular discoloration; 1 = 1 to 25, 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% of vascular tissues in the tap root exhibited discoloration in the absence of foliar symptoms; and 5 = 100% of vascular tissues in the tap root exhibited discoloration extending into the crown and the presence of foliar symptoms typical of Verticillium wilt. Tap root and crown tissues of inoculated and noninoculated plants were sampled randomly and placed on a PDA medium to confirm the presence or absence of the pathogen.

Statistical analysis. Experimental units comprising groups of three to four plants for each isolate–cultivar combination were replicated three times and arranged in a randomized block design. Recorded values were averaged across plants within each experimental unit for further data analysis. In the case of disease severity, results across replicated experiments were consistent. Therefore, disease severity data from all experiments were pooled and the combined dataset analyzed using a nonparametric procedure for the analysis of ordinal data in factorial experiments (5,22). The overall effect of *Verticillium* isolates on the severity of disease on each lettuce cultivar was analyzed by the analysis of variance type statistic of ranked data using the PROC Mixed procedure in SAS (2004, version 9.1) to generate relative marginal effects (RME), and the LD_CI macro to generate 95% confidence intervals (5,22). The RME values are generated by the equation: $\text{RME} = (R - 0.5)/N$; where R is the mean treatment ranking and N is the total number of experimental units in the analysis. Replications of experiment and blocks within experiments were considered random effects in the analysis. Because of its susceptibility to *B. cinerea*, data for lettuce cv. Little Gem was collected from only two experiments and analyzed separately. Linear contrasts were performed to test specific interactions with-

in models. An isolate–cultivar interaction was considered pathogenic if a significant difference was observed in the mean treatment ranking compared with the corresponding noninoculated control. Data for fresh foliar weight were similarly analyzed for a two-way analysis of variance using PROC mixed; blocks within experiments were considered random effects.

RESULTS

Reaction of lettuce differentials to *V. dahliae* isolates. Across three independent experiments, the average incidence of Verticillium wilt caused by 12 isolates of *V. dahliae* from lettuce ranged from 20 to 48% on the susceptible cvs. Salinas and Sniper and 5 to 10% on the resistant cvs. La Brillante and Little Gem. There were also noticeable differences in the overall level of disease between experiments, with the average disease incidence in the fall experiment (22 September to 29 December) twice that observed in the winter (19 January to 4 May) and spring (15 March to 6 July) experiments. However, these seasonal differences did not influence the rankings of cultivar–isolate interactions even though differences in plant growth were quite apparent based on the fresh foliar weight data collected in the fall for experiment 1 and in the winter for experiment 2 (Fig. 1). Plants grown in the fall in experiment 1 were smaller than those grown in the winter in experiment 2, which were quite etiolated due to the supplemental light and increase in natural light that occurred by the end of the experiment. Overall, inoculated plants in both experiments generally were larger, with greater fresh foliar weight, than their corresponding uninoculated control plants; with the exception that inoculated plants of Sniper in experiment 2 generally were smaller than the respective noninoculated plants. Although the trends in fresh foliar weight were persistent in both experiments, few statistically significant differences were observed between inoculated and noninoculated treatments.

Significant differences in the mean disease severity rankings were detected between lettuce differentials La Brillante, Salinas, and Sniper ($P < 0.0001$), between isolates of *Verticillium* spp. ($P < 0.0001$), and significant two-way interactions of differentials and isolates ($P < 0.0001$) (Table 2). Significant differences among isolates in the mean disease severity rankings on Little Gem also were detected ($P < 0.0001$) (Table 2). Across all the isolates tested, Salinas and Sniper significantly differed from La Brillante ($P < 0.0001$) in mean disease severity rankings, whereas the difference between the two susceptible differentials, Salinas and Sniper, was not as apparent ($P = 0.0933$). The two isolates of *V. albo-atrum* (VaaMs103 and VaaMs107) and six isolates of *V. dahliae* from cruciferous hosts (VdAr139, VdBno188, VdBob127, VdBob70, VdBob71, and VdBoc74) were characterized as nonpathogenic on lettuce, because the mean disease severity rankings did not differ significantly ($P \geq 0.05$) from the noninoculated controls on any lettuce genotype tested (Table 3; Fig. 2). Of the 23 isolates of *V. dahliae* tested from noncruciferous hosts such as lettuce, watermelon, strawberry, artichoke, and chile pepper, 21 exhibited mean disease severity rankings significantly greater than the noninoculated controls ($P < 0.05$) for the two susceptible cultivars and, therefore, were characterized as pathogenic and exhibited varying degrees of virulence on lettuce (Table 3; Fig. 2). Isolates VdSt91 from potato and VdCa63 from bell pepper did not differ significantly from the noninoculated controls ($P = 0.4096$ and 0.0947, respectively) and were considered nonpathogenic on lettuce. Several pathogenic isolates of *V. dahliae* that originated from diseased watermelon (VdCv85), chile pepper (VdCf40), and

TABLE 1. Isolates of *Verticillium dahliae* and *V. albo-atrum* used in pathogenicity assays

Isolate[a]	Host	Origin	Collected	Conidia length × width (μm)[b]	Group[c]
VaaMs103	Alfalfa (*Medicago sativus* L.)	Pennsylvania	1986	3.8 × 1.7	*Vaa*
VaaMs107	…	Pennsylvania	1986	5.6 × 2.6	*Vaa*
VdCs80	Artichoke (*Cynara scolymus* L.)	California	1991	4.3 × 2.2	*Vd*NC1
VdCa63	Bell pepper (*Capsicum annuum* L.)	California	1996	4.0 × 2.0	*Vd*NC2
VdBoc74	Cabbage (*Brassica oleracea* var. *capitata* L.)	California	1991	8.2 × 2.7	*Vd*Cr
VdBob127	Cauliflower (*Brassica oleracea* var. *botrytis* L.)	California	1997	7.3 × 3.1	*Vd*Cr
VdBob70	…	California	1990	8.1 × 3.2	*Vd*Cr
VdBob71	…	California	1990	8.1 × 3.2	*Vd*Cr
VdCf40	Chile pepper (*Capsicum annuum* L.)	California	1996	4.7 × 2.3	*Vd*NC3
VdCf45	…	California	1996	3.6 × 1.3	*Vd*NC2
VdAr139	Horseradish (*Armoracia rusticana* Gaertn., Mey., Scherb.)	Illinois	1989	8.7 × 2.6	*Vd*Cr
VdLs1	Lettuce (*Lactuca sativa* L.)	California	1995	5.3 × 2.2	*Vd*NC1
VdLs7	…	California	1995	4.6 × 2.2	*Vd*NC1
VdLs14	…	California	1996	5.4 × 2.2	*Vd*NC1
VdLs16	…	California	1996	5.5 × 2.3	*Vd*NC1
VdLs17	…	California	1996	5.0 × 2.4	*Vd*NC3
VdLs331	…	California	2000	4.6 × 2.2	*Vd*NC1
VdLs336	…	California	2000	4.5 × 2.3	*Vd*NC1
VdLs429	…	California	2001	4.5 × 2.3	*Vd*NC1
VdLs439	…	California	2001	4.6 × 2.3	*Vd*NC3
VdLs472	…	California	2001	4.8 × 2.4	*Vd*NC1
VdLs474	…	California	2001	4.9 × 2.3	*Vd*NC1
VdLs476	…	California	2001	4.6 × 2.3	*Vd*NC1
VdLs3A	…	California	2004	ND	*Vd*NC
VdLsWats3	…	California	2004	ND	*Vd*NC
VdBno188	Oilseed rape (*Brassica napus* L.)	Germany	1989	8.0 × 3.3	*Vd*Cr
VdSt91	Potato (*Solanum tuberosum* L.)	Oregon	1996	4.7 × 2.5	*Vd*NC3
VdFca23	Strawberry (*Fragaria* × *ananassa* Duchesne)	California	1996	4.5 × 2.6	*Vd*NC1
VdFca29	…	California	1996	5.1 × 2.6	*Vd*NC1
VdCv85	Watermelon (*Citrullus vulgaris* Schrader)	California	1994	4.4 × 2.5	*Vd*NC1
VdCv111	…	California	1993	5.3 × 1.7	*Vd*NC1

[a] First letters of isolate name, Vd and Vaa, represent *V. dahliae* and *V. albo-atrum*, respectively. Isolates VaaMs103 and VaaMs107 provided by B. W. Pennypacker, The Pennsylvania State University, College Town; VdAr139 provided by D. M. Eastburn, University of Illinois, Urbana; VdBno188 provided by K. Zeise, University of Rostock, Germany; and VdSt91 provided by M. L. Powelson, Oregon State University, Corvallis.

[b] ND = not determined.

[c] Grouping based on similarity between sequences of the ribosomal intergenic spacer region (19). *Vaa* = *V. albo-atrum*, *Vd*Cr = *V. dahliae* isolates pathogenic on cruciferous hosts, and *Vd*NC = *V. dahliae* isolates pathogenic on noncruciferous hosts (subgroups 1 to 3; those without a number are undetermined).

strawberry (VdFca23) were as virulent on the two susceptible lettuce cultivars as isolates that originated from diseased lettuce (Table 3; Fig. 2). Of the 21 isolates of *V. dahliae* pathogenic on Salinas and Sniper, only isolates VdCf40, VdLs439, and VdLs17 caused significant levels of disease on the resistant differential La Brillante ($P = 0.0163$, $P = 0.0124$, and $P < 0.0001$, respectively) relative to the noninoculated controls; whereas the other 18 isolates failed to cause levels of disease that differed significantly from the noninoculated controls and, therefore, were considered avirulent (Table 3; Fig. 2). Similarly, on the resistant differential Little Gem, only VdLs439 and VdLs17 caused significant levels of disease ($P = 0.0254$ and 0.0266, respectively).

Based on observed sequence variation within the nuclear ribosomal IGS region among isolates of various phytopathogenic *Verticillium* spp., *V. dahliae* isolates from noncruciferous hosts previously were categorized into four distinct subgroups, designated as *Vd*NC1, *Vd*NC2, *Vd*NC3, and *Vd*NC4 (Table 1) (19). All isolates of *V. dahliae* that exhibited pathogenicity on lettuce in this study belonged to subgroups *Vd*NC1 and *Vd*NC3. Of the isolates in subgroup *Vd*NC1 that exhibited virulence on differentials Salinas and Sniper, all were avirulent on La Brillante and Little Gem. Only isolates VdLs17, VdLs439, and VdCf40 within subgroup *Vd*NC3 exhibited virulence toward all four lettuce differentials (Table 3; Fig. 1). Contrasts of mean rankings found no statistical differences between subgroups *Vd*NC1 and *Vd*NC3 in disease severity on Salinas ($P = 0.3958$) and Sniper ($P = 0.3026$), but significant differences on the two resistant differentials, La Brillante ($P = < 0.0001$) and Little Gem ($P < 0.0001$). Isolates VdCf45 and VdCa63 in *V. dahliae* subgroup *Vd*NC2 were characterized as weak pathogens of lettuce at best, and exhibited mean disease severity rankings across lettuce cultivars that differed significantly ($P = 0.0197$) or not at all ($P = 0.0951$), respectively,

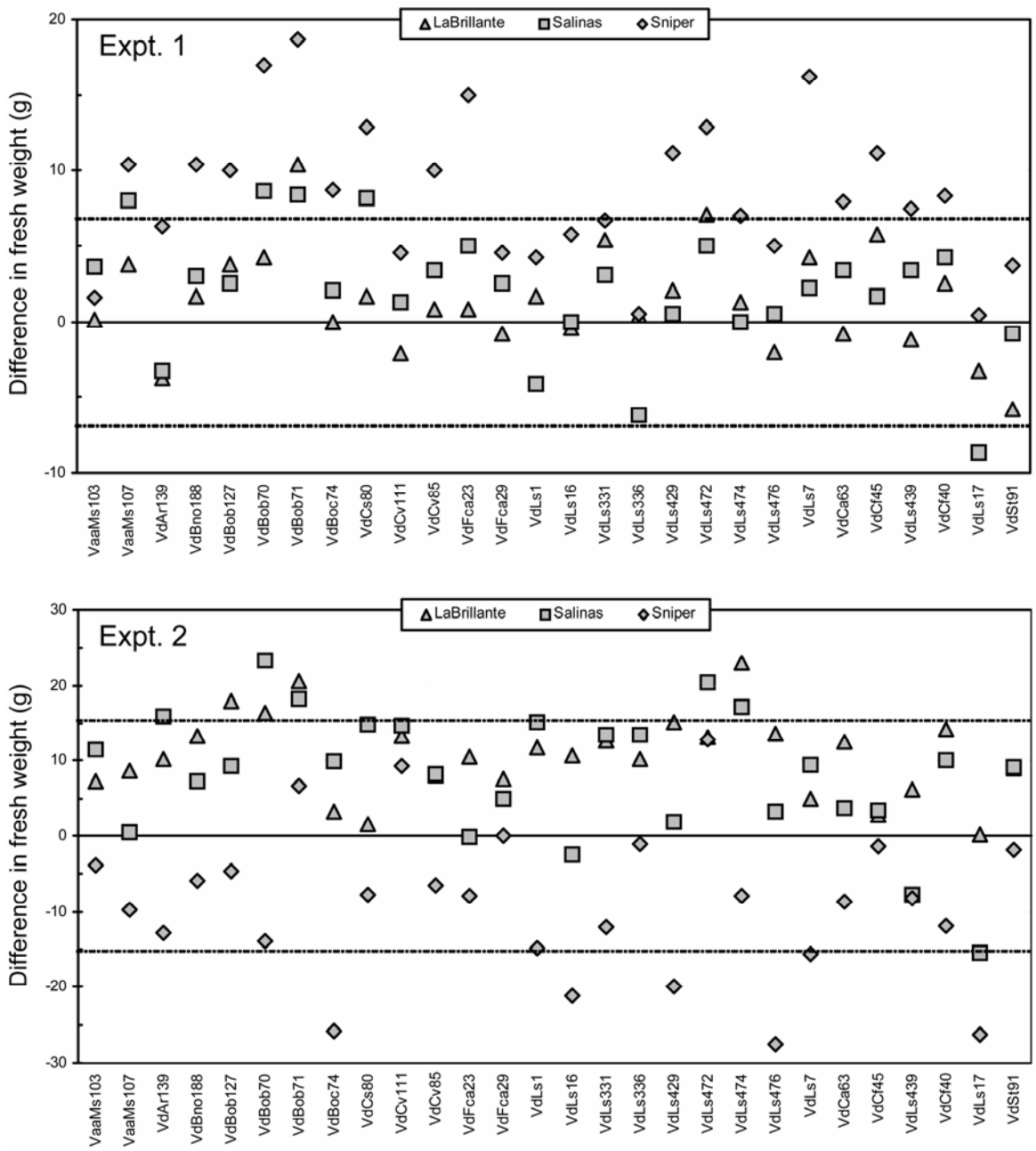

Fig. 1. Differences in least-significant means (LSM) for fresh foliar weight between individual lettuce cultivar–Verticillium isolate interactions and the corresponding noninoculated control. The LSM for the noninoculated control of La Brillante, Salinas, and Sniper was 17.5, 20.8, and 18.8 g, respectively, in experiment 1 and 25.0, 36.8, and 60.6 g, respectively, in experiment 2. Dashed lines represent the 95% confidence interval for the noninoculated control; ±7.0 g in experiment 1 and ±15.2 g in experiment 2.

from the noninoculated controls. Subgroups VdNC1 and VdNC2 differed statistically in mean disease severity rankings on Salinas ($P < 0.0001$) and Sniper ($P = 0.0043$) but not on La Brillante ($P = 0.5538$) and Little Gem ($P = 0.4248$). No isolates belonging to *V. dahliae* subgroup *Vd*NC4 were tested in this study. Two uncharacterized isolates, VdLsWats and VdLs3A, collected from separate lettuce fields afflicted with Verticillium wilt in 2004 also exhibited differential virulence patterns on resistant and susceptible cultivars, similar to those of isolates of subgroup *Vd*NC1 (Table 3; Fig. 1).

TABLE 2. Statistical analysis of variance (ANOVA) based on the ranked means of Verticillium wilt severity on lettuce caused by isolates of *Verticillium dahliae* and *V. albo-atrum* combined across three independent experiments

	ANOVA-type statistic (ATS)[a]							
	Salinas, Sniper, and La Brillante				Little Gem			
Effect	df_{Num}	df_{Den}	ATS	*P* value	df_{Num}	df_{Den}	ATS	*P* value
Cultivar	1.90	421	160.9	<0.0001	…	…	…	…
Isolate	26.40	∞	12.8	<0.0001	5.59	26.8	7.7	<0.0001
Cultivar × isolate	44.30	∞	2.8	<0.0001	…	…	…	…

[a] Experiments were sown, inoculated, transplanted, and rated for disease as follows: 22 September, 26 October, and 29 December 2004 for experiment 1; 19 January, 17 February, and 4 May 2005 for experiment 2; and 15 March, 19 April, and 6 July 2005 for experiment 3. Because similar trends were observed in each experiment, data from lettuce cvs. Salinas, Sniper, and La Brillante were combined across all experiments for statistical analysis. Data from lettuce cv. Little Gem was analyzed separately from experiments 1 and 3 because of unacceptable losses to *Botrytis cinerea* in experiment 2. df_{Num} = Numerator degrees of freedom and df_{Den} = denominator degrees of freedom.

TABLE 3. Median (Med) and mean rankings (*R*) calculated for the severity of Verticillium wilt on the lettuce cvs. Little Gem, La Brillante, Salinas, and Sniper caused by isolates of *Verticillium dahliae* and *V. albo-atrum*[a]

	Little Gem		La Brillante		Salinas		Sniper	
Group, isolate[b]	Med	*R*	Med	*R*	Med	*R*	Med	*R*
Control	0.0	75	0.0	239	0.0	239	0.0	329
Vaa								
VaaMs103	0.0	75	0.0	239	0.0	239	0.0	302
VaaMs107	0.0	75	0.0	239	0.0	367	0.0	342
*Vd*Cr								
VdAr139	0.0	75	0.0	267	0.0	354	0.0	397
VdBno188	0.0	75	0.0	299	0.0	320	0.0	315
VdBob127	0.0	75	0.0	239	0.0	309	0.0	342
VdBob70	0.0	75	0.0	239	0.0	316	0.0	299
VdBob71	0.0	87	0.0	269	0.0	338	0.0	388
VdBoc74	0.0	75	0.0	274	0.0	267	0.0	365
*Vd*NC								
VdLs3A	0.0	75	0.0	281	2.9	752	1.1	539
VdLsWats3	…	…	0.0	281	1.7	608	2.5	738
*Vd*NC1								
VdCs80	0.0	75	0.0	239	0.3	450	0.3	478
VdCv111	0.0	75	0.0	239	1.3	542	0.5	468
VdCv85	0.0	75	0.0	239	1.7	648	1.5	658
VdFca23	0.0	75	0.0	239	2.5	723	1.3	595
VdFca29	0.0	75	0.0	312	0.3	418	0.0	377
VdLs1	0.0	87	0.0	304	1.3	598	2.5	705
VdLs14	0.0	75	0.0	239	0.6	452	1.3	560
VdLs16	0.0	75	0.0	336	2.5	634	3.5	739
VdLs331	0.0	75	0.0	239	0.8	504	1.0	521
VdLs336	0.0	75	0.0	269	2.3	664	2.3	718
VdLs429	0.0	75	0.0	269	0.5	499	0.5	432
VdLs472	0.0	75	0.0	239	0.8	511	1.3	581
VdLs474	0.0	75	0.0	291	1.3	522	1.7	572
VdLs476	0.0	75	0.0	287	1.5	586	1.3	661
VdLs7	0.0	75	0.0	239	0.0	449	1.1	552
*Vd*NC2								
VdCa63	0.0	90	0.0	239	0.0	355	0.0	397
VdCf45	0.0	75	0.0	331	0.0	316	0.3	414
*Vd*NC3								
VdCf40	0.0	102	0.0	426	0.0	406	0.3	497
VdLs17	3.1	158	2.5	652	3.8	729	2.7	753
VdLs439	1.5	130	0.0	453	2.0	677	1.1	547
VdSt91	0.0	87	0.0	239	0.0	325	0.0	320

[a] Results represent data from three independent experiments, except for Little Gem, which represent data from only two experiments. *R* = mean rankings of Verticillium wilt severity for each cultivar–isolate interaction used to calculate relative marginal effects (RME); RME = $(R - 0.5)/N$, where *N* = total experimental units in the analysis ($N = 162$ for calculations of isolate interactions with lettuce cv. Little Gem and $N = 831$ for calculations of isolate interactions with cvs. La Brillante, Salinas, and Sniper). Disease severity was rated using an ordinal scale of 0 to 5, in which 0 = no vascular discoloration in the taproot; 1 = 1 to 25, 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% of vascular tissues in the taproot exhibited discoloration in the absence of foliar symptoms; and 5 = 100% of vascular tissues exhibited discoloration in the taproot and the presence of foliar symptoms typical of Verticillium wilt.

[b] Grouping of isolates based on sequence similarity within the ribosomal intergenic spacer region, Qin et al. 2006 (19). *Vaa* = *V. albo-atrum*, *Vd*Cr = *V. dahliae* isolates pathogenic on cruciferous hosts and *Vd*NC = *V. dahliae* isolates pathogenic on noncruciferous hosts in subgroups 1 to 3 (those without a number have not been sequenced). First two letters of isolate name, Vd and Va, represent *V. dahliae* and *V. albo-atrum*, respectively.

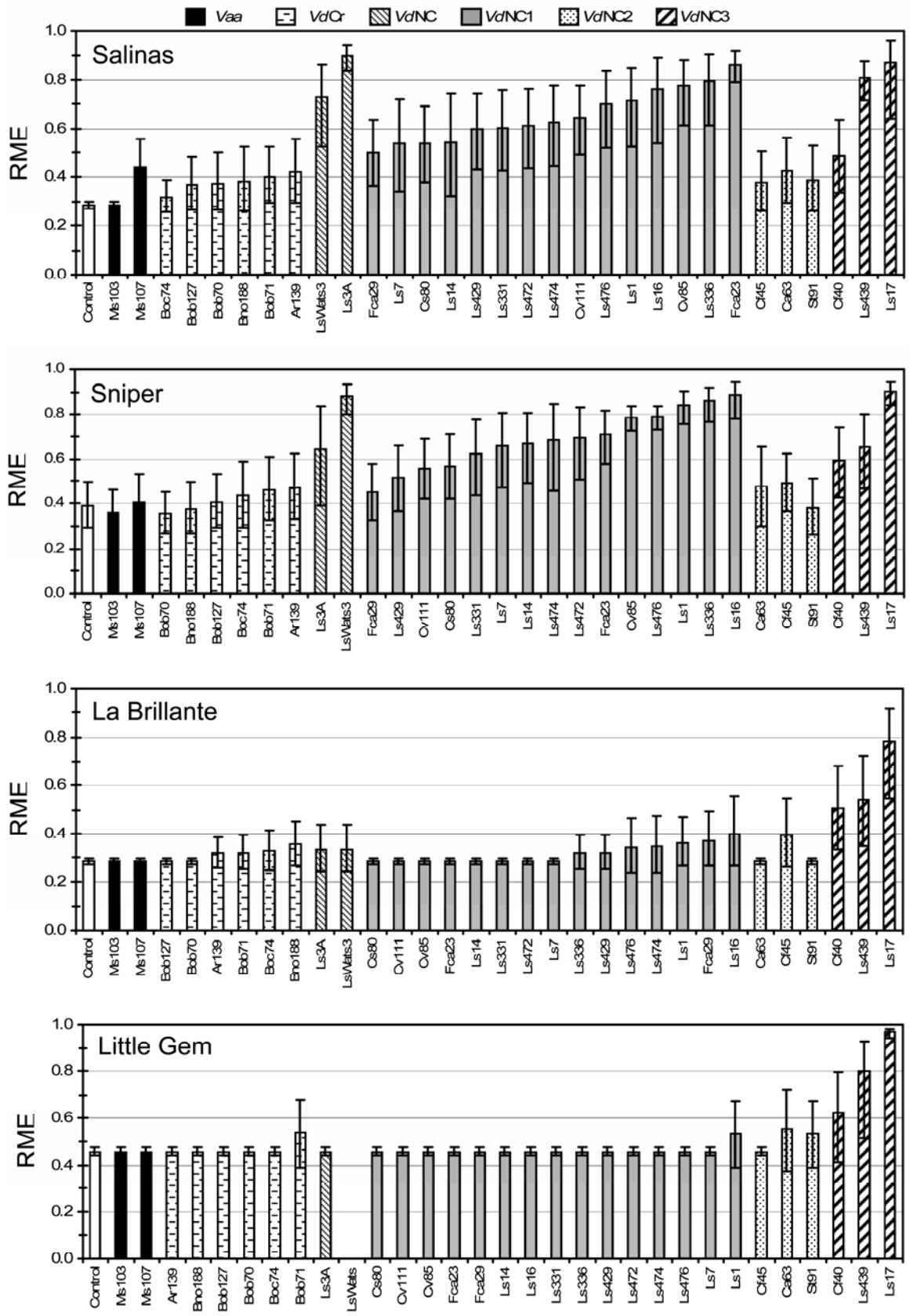

Fig. 2. Estimated relative marginal effects (RME) based on the analysis of variance-type statistics of ranked data for the severity of Verticillium wilt on the lettuce cvs. Salinas, Sniper, La Brillante, and Little Gem caused by individual isolates of *Verticillium dahliae* (*Vd*) and *V. albo-atrum* (*Vaa*). Confidence intervals (95%) for relative marginal effects are represented by vertical lines. Results represent data from three independent experiments; except those for Little Gem, which represent data from only two independent experiments. Bars are textured according to the grouping of isolates based on sequence similarity within the ribosomal intergenic spacer region (19); *V. dahliae* isolates pathogenic on cruciferous (Cr) host, and *V. dahliae* isolates pathogenic on noncruciferous (NC) hosts in subgroups NC1, NC2, NC3, and uncharacterized isolates, NC.

DISCUSSION

Our findings demonstrate that among those isolates of *V. dahliae* pathogenic on lettuce, at least two distinct virulence phenotypes exist based on their ability to cause Verticillium wilt on the resistant differentials La Brillante and Little Gem. These differential responses were consistent in three independent greenhouse experiments that differed in their environmental attributes due to seasonal fluctuations in photoperiod and temperature, as reflected by fresh foliar biomass and disease severity. In replicated field trials, Little Gem and La Brillante, as well as several other lettuce cultivars, also exhibited few or no symptoms of Verticillium wilt (R. J. Hayes, G. E. Vallad, Q.-M. Qin, R. Grube, and K. V. Subbarao, *unpublished data*). Isolates of *V. dahliae* exhibiting virulence on Salinas and Sniper, but avirulent on Little Gem and La Brillante, were designated as race 1, whereas those isolates exhibiting virulence on all four differentials, including the resistant Little Gem and La Brillante, were designated as race 2. Salinas and Sniper are both modern crisphead cultivars and share a common pedigree with most crisphead cultivars grown commercially, whereas Little Gem is a latin-type cultivar that differs from crisphead-type cultivars in regards to many horticultural traits and ancestry (12,20). La Brillante is a Batavia-type lettuce cultivar, a horticultural subtype that shares many horticultural traits and ancestry with modern crisphead cultivars (20). Unfortunately, much in the way of pedigree history is unknown for many of the non-crisphead-type cultivars; therefore, the exact relationship between the two susceptible and two resistant cultivars cannot be ascertained easily. Further studies are in progress to verify the resistance of additional lettuce cultivars identified in field trials, and to assess the inheritance of resistance in several populations developed from crosses between resistant and susceptible lettuce cultivars.

This is only the second example of race-specific resistance toward *V. dahliae* among the bewildering array of hosts of this pathogen, the first being the resistance conferred by the *Ve* resistance genes of tomato (2,21). Although resistance toward *V. dahliae* has been described for many crops, such as alfalfa (*Medicago sativa* L.), cauliflower (*Brassica oleraceae* var. *botrytis* L.), cotton (*Gossypium barbadense* L.), eggplant (*Solanum melongena* L.), mint (*Mentha* × *piperita* L.), and potato (*S. tuberosum* L.), most of these sources offer only partial resistance against the pathogen (6,7,17). Jansky et al. (13) recently characterized resistance to *V. dahliae* in diploid interspecific potato hybrids that was introgressed from wild *Solanum* spp., finding that a two-gene model best described the segregation of resistance among the progeny derived from crosses. This resistance, as evaluated in the field, seems to offer a level of protection to potato similar to that conferred by the *Ve* genes to tomato; however, it has not been assessed against individual isolates of *V. dahliae* and *V. albo-atrum* in controlled inoculations.

Variation in virulence toward susceptible lettuce cultivars was apparent among isolates of *V. dahliae* pathogenic on lettuce. Similar variation previously has been observed for isolates of *V. dahliae* pathogenic on tomato, potato, and cotton (3,10,14,24). Grogan et al. (10) found that isolates of *V. dahliae* obtained from commercial tomato fields varied in virulence for both races, with race 1 isolates typically exhibiting greater virulence compared with race 2 on a tomato cultivar lacking the *Ve* genes. Although it appears that similar variation for virulence exists among race 2 isolates on lettuce based on three tested isolates, the race 2 isolate VdLs17 was as virulent on susceptible lettuce cultivars as several race 1 isolates. These data are in agreement with previous observations suggesting that the genetic control of virulence may differ from that controlling race specificity (10,17,24).

The concern over the durability of resistance to *V. dahliae* in commercial lettuce cultivars is important. A similar scenario occurred with the release of commercial tomato cultivars with the *Ve* resistance genes and the subsequent identification of resistance-compromising race 2 isolates (2,17). The rapid spread of race 2 isolates of *V. dahliae* throughout tomato production areas worldwide has remained a mystery. Whether race 2 isolates already were endemic to production areas or whether these isolates were disseminated through infected plant materials has not been resolved. Interestingly, DNA fingerprinting and vegetative compatibility analysis of several isolates of *V. dahliae* collected in Ontario, Canada, indicated at least two independent origins for race 2 isolates pathogenic on tomato, and suggested that greater genetic diversity exists among isolates of *V. dahliae* pathogenic on tomato than was previously considered (9).

Based on sequencing of the nuclear ribosomal IGS region, Qin et al. (19) also found substantial genetic diversity among isolates of *V. dahliae* collected from susceptible crops grown in the Salinas Valley and neighboring areas. Isolates of *V. dahliae* from noncruciferous hosts could be subdivided, based on the sequence analysis of the IGS region, into four subgroups: *Vd*NC1, *Vd*NC2, *Vd*NC3, and *Vd*NC4 (19). Three isolates, VdLs17, VdLs439, and VdCf40, compromised the resistance in cvs. La Brillante and Little Gem, and clustered phylogenetically to *V. dahliae* subgroup *Vd*NC3, whereas the race 1 isolates clustered to subgroup *Vd*NC1. Isolates from lettuce in *Vd*NC1 and *Vd*NC3 exhibited higher sequence identity to isolates from other crops than to each other (19), suggesting separate origins for the two subgroups and the two races of *V. dahliae* pathogenic on lettuce. The status of virulence phenotypes within *V. dahliae* subgroups *Vd*NC2 and *Vd*NC4 remains unclear, and more isolates from these two subgroups need to be tested on lettuce to clarify their virulence phenotypes on lettuce. The two isolates tested from subgroup *Vd*NC2, VdCa63 and VdCf45, were considered weak pathogens at best on lettuce, while no isolates from subgroup *Vd*NC4 were tested for pathogenicity.

Two of the characterized race 2 isolates in this study were collected from lettuce fields in 1996 (VdLs17) and 2001 (VdLs439) from the primary focal point in Watsonville, CA where the disease first was observed in 1995. The third race 2 isolate (VdCf40) was collected from chile pepper near Morgan Hill, CA in 1996, and exhibited low virulence toward all four lettuce cultivars. Even though Morgan Hill lies nearly 25 miles to the northeast of Watsonville, local precipitation and runoff from fields feeds into the Pajaro River watershed that flows southwest through the principal lettuce- and strawberry-production areas surrounding Watsonville. Therefore, it is feasible that the race 2 isolates from lettuce actually were derived from a chile pepper isolate, such as VdCf40, that was carried by runoff to the Watsonville area. It also is interesting to note that the initial outbreak of Verticillium wilt occurred in low-lying fields adjacent to a series of sloughs that were dredged following severe seasonal flooding of the Pajaro River and surrounding areas in 1995, coinciding with the first outbreak of Verticillium wilt later that year (K. V. Subbarao, *unpublished data*).

It is clear that, when Verticillium wilt first appeared on lettuce and the initial isolates of *V. dahliae* were collected from these locations, the resistance-compromising race 2 isolates already were present in these fields. It also is clear that these race 2 isolates emerged in the absence of any resistance attributable to lettuce, because all widely grown commercial lettuce cultivars have tested susceptible to both races (R. J. Hayes, G. E. Vallad, Q.-M. Qin, R. Grube, and K. V. Subbarao, *unpublished data*). Although the appearance of Verticillium wilt on lettuce following the flooding in 1995 may be anecdotal, this scenario indeed fits the phylogenetic data on race 2 isolates. Race 1 isolates from lettuce, in contrast, share greater sequence identity at the IGS region with several isolates collected from strawberry, artichoke, watermelon, and tomato (19). Qin et al. (19) concluded that lettuce isolates of *Vd*NC1 probably were derived from strawberry isolates through selection pressure and host adaptation, because

lettuce often is grown in rotation with strawberry. If there are separate origins for the two races of *V. dahliae* from lettuce, it suggests that two independent mutational events occurred, which seems highly improbable. Only with further phenotyping and genotyping will the relationship between race 1 and race 2 isolates of *V. dahliae* from lettuce, and their relationship with isolates from other hosts, become apparent.

In summary, the data demonstrates the existence of two virulence phenotypes of *V. dahliae* on lettuce that differed in their ability to cause wilt on two diverse, resistant differentials. All isolates of *V. dahliae* pathogenic on lettuce varied in virulence on the susceptible cvs. Salinas and Sniper, whereas only a few isolates exhibited any virulence on cvs. La Brillante and Little Gem; isolates were categorized accordingly as race 1 and race 2. Based on previous sequencing of the IGS region (19), race 1 and race 2 isolates corresponded with the phylogenetic subgroups *Vd*NC1 and *Vd*NC3, respectively, implying that the two groups are genetically distinct and perhaps of different origins. These findings are significant for the characterization and introgression of resistance to *V. dahliae* into commercially acceptable lettuce cultivars, and in efforts to characterize and monitor the spread of lettuce-adapted isolates of *V. dahliae*. Although long recognized as an important soilborne pathogen of diverse plant species (17), little is known about the genetics behind local and global *V. dahliae* populations. Additional studies to better characterize the level of genetic variation within the local *V. dahliae* populations and the origins of new host-adapted isolates, such as those affecting lettuce, are required.

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