Pathogenicity, Virulence, and Vegetative Compatibility Grouping of *Verticillium* Isolates from Spinach Seed

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Abstract

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In 2005, *Verticillium dahliae* was first reported to be pathogenic to spinach seed crops in the Pacific Northwest, with symptoms only developing after initiation of the reproductive stage of plant growth, and to be prevalent on commercial spinach seed lots produced in Denmark, The Netherlands, and the United States. In this study, the genetic diversity, pathogenicity, and virulence were examined for a collection of isolates of *Verticillium* spp. from spinach as well as other hosts (alfalfa, cotton, lettuce, mint, peppermint, potato, radish, and tomato) from various countries and from different vegetative compatibility groups (VCGs). Of a total of 210 isolates of *V. dahliae* obtained from spinach seed produced in Denmark, the Netherlands, New Zealand, or the United States, 128 were assigned to VCG 4B (89% of 91 U.S. isolates, 86% of 42 isolates from the Netherlands, 19% of 43 Denmark isolates, and 8% of 13 New Zealand isolates), 65 to VCG 2B (92% of the New Zealand isolates, 79% of the Denmark isolates, 14% of the Netherlands isolates, and 9% of the U.S. isolates), and 3 to VCG 2A (2% of each of the Denmark and U.S. isolates, and 0% of the Netherlands and New Zealand isolates); 14 isolates could not be assigned to a VCG. Although little variation in the sequence of the internal transcribed spacer (ITS) region of ribosomal DNA was observed among isolates

within each *Verticillium* sp., the ITS region readily differentiated isolates of the species *V. dahliae*, *V. tricornis*, and *Gibellulopsis nigrescens* (formerly *V. nigrescens*) obtained from spinach seed. Greenhouse pathogenicity assays on spinach, cotton, lettuce, and tomato plants using isolates of *V. dahliae* ($n = 29$ to 34 isolates), *V. tricornis* ($n = 3$), *G. nigrescens* ($n = 2$), and *V. albo-atrum* ($n = 1$) originally obtained from these hosts as well as from alfalfa, mint, peppermint, potato, and radish, revealed a wide range in virulence among the isolates. Isolates of *V. tricornis* and *G. nigrescens* recovered from spinach seed and an isolate of *V. albo-atrum* from alfalfa were not pathogenic on spinach. In addition, isolates of *V. dahliae* from mint and peppermint were not pathogenic or only weakly virulent on the hosts evaluated. Although there was a wide range in virulence among the isolates of *V. dahliae* tested, all of the *V. dahliae* isolates caused Verticillium wilt symptoms on spinach, lettuce, tomato, and cotton. None of the isolates of *V. dahliae* showed host specificity. These results indicate that *Verticillium* and related species associated with spinach seed display substantial variability in virulence and pathogenicity to spinach and other plants but the *V. dahliae* isolates were restricted to three VCGs.

Verticillium dahliae Kleb., a soilborne pathogen, causes Verticillium wilt on more than 200 plant species, including economically important vegetable and field crops (57). The first report of *V. dahliae* on spinach (*Spinacia oleracea* L.) in the United States was by Snyder and Wilhelm (66) in New York, who reported seed transmission in spinach. Seed transmission in spinach has also been documented in the Netherlands (78,79) and Canada (64). However, it was not until 2005 that *V. dahliae* was first reported to be pathogenic to spinach seed crops in the Pacific Northwest, the main region of spinach seed production in the United States (28). In addition, the prevalence of *Verticillium* spp. in commercial spinach seed lots was demonstrated in that study. Despite these reports, Verticillium wilt has not been identified as occurring in fresh-market or processing spinach crops in the United States because these crops are harvested before bolting (reproductive stage of growth), and Verticillium wilt symptoms only develop in spinach after initiation of bolting (28). Seed transmission may be important in the epidemiology of Verticillium wilt of a number of crops, including spinach (26,27). Planting a spinach seed crop in an infested field could lead to systemic infection of the plants and, therefore, the production of infected spinach seed (28). Furthermore, planting infested spinach seed could potentially introduce the pathogen to other areas (6,7,28). As a result, efforts have been initiated to treat

infested spinach seed lots to reduce the risk of seed transmission (26,27,29).

Although isolates of *V. dahliae* from a given host generally are able to cause symptoms on a range of other plant species (61,70), there are a few exceptions where distinct virulence phenotypes occur, such as cotton (*Gossypium hirsutum* L.) defoliating and nondefoliating types (32), and tomato (*Solanum lycopersicum* L.) (10) and lettuce (*Lactuca sativa* L.) races of *V. dahliae* (76). In tomato and lettuce, race 1 isolates are controlled by host resistance, whereas race 2 isolates are virulent on race-1-resistant cultivars (10,76). Additionally, some degree of host specialization has been reported among isolates of *V. dahliae* from other hosts, including cocoa (*Theobroma cacao* L.) (62), tobacco (*Nicotiana tabacum* L.) (72), mint (*Mentha × gracilis* Sole) and peppermint (*Mentha × piperita* L.) (12,25,33), bell pepper (*Capsicum annuum* L.), cabbage (*Brassica oleracea* L. var. *capitata* L.), cauliflower (*B. oleracea* var. *botrytis* L.), cotton, and eggplant (*S. melongena* L.) (12). Isolates of *V. dahliae*, *V. tricornis* I. Isaac, and *Gibellulopsis nigrescens* (Pethybr.) Zare, W. Gams & Summerb. (formerly *V. nigrescens*) (85) have also been recovered from seed from spinach germplasm collections and from commercial spinach seed lots (L. J. du Toit, unpublished data; 81,82). Severe symptoms can be caused by *V. dahliae* on several plant species, whereas *V. tricornis* and *G. nigrescens* are generally reported as weak pathogens or saprotrophs (9,57). There are no published reports of isolates of these species being evaluated for pathogenicity on spinach.

Isolates of *V. dahliae* from diverse hosts have been divided into six main groups based on vegetative compatibility (11,39), with each of vegetative compatibility groups (VCGs) 1, 2, and 4 further divided into two subgroups, A and B, according to the frequency and vigor of complementation (41,42,46,63,68). Because *Verticil-*

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lium spp. are apparently asexually reproducing, isolates in the same VCG could be genetically distinct populations with similarity in a number of physiological, ecological, virulence, and host-range traits (15,46,48,59). Thus, genetic diversity of isolates of *V. dahliae* from various hosts has been associated with host preferences and virulence. For instance, isolates belonging to VCG 4A exhibited significantly greater virulence on potato (*S. tuberosum* L.) than isolates of other VCGs (42,68). Additionally, Zeise and von Tiedemann (86) found that the host range of isolates of *V. dahliae* and *V. longisporum* was more related to VCG than to host of origin. Nonetheless, the relevance and suitability of VCG analyses in genetic diversity studies of *V. dahliae* have recently been questioned because of the polyphyletic origin of some of the VCGs, based on analyses of sequences of the intergenic spacer (IGS) region of ribosomal DNA (rDNA) and six other polymorphic sequences (40). A preliminary study on VCGs of five isolates of *V. dahliae* from spinach seed revealed that isolates from spinach belonged to two distinct VCGs, VCGs 2B and 4B (28). However, a comprehensive evaluation of VCGs among isolates of *V. dahliae* from spinach seed has not been completed, nor has the relative virulence of spinach seed isolates been investigated.

In addition to VCGs, genetic diversity of *V. dahliae* isolates has been studied by analysis of DNA based on nuclear ribosomal RNA genes and various molecular markers (16,17,49,53). The internal transcribed spacer (ITS) region of rDNA has been used frequently in phylogenetic studies of *Verticillium* spp. (13,56,61). However, sequences of the ITS rDNA and mitochondrial genes have failed to differentiate isolates within a *Verticillium* spp. (4,61). Furthermore, analyses of the association between genetic diversity and characteristics of isolates of *V. dahliae* have often been contradictory. Whereas the relationships among isolates based on molecular markers have been associated with variables such as geographic origin of strains (55), original host (55), pathotype (45,58), and VCG (22), other genetic studies found no correlation between characteristics of isolates of *V. dahliae* and molecular data (5,12,23). Even though several studies have been completed on genetic diversity within the genus *Verticillium*, isolates of *Verticillium* from crops such as spinach have not been characterized extensively. Management of *Verticillium* wilt in general is difficult due to the broad host range of the pathogen and the persistence of microscerotia in soils (43,57,76). Additionally, economic, environmental, and regulatory constraints may hinder the application of effective soil fumigants (5,43). Because *Verticillium* wilt of spinach is a relatively newly recognized disease, little is known about the genetic diversity of *Verticillium* spp. found on spinach seed and the pathogenicity to spinach and related crops of *Verticillium* spp. recovered from spinach seed. Thus, the objectives of this study were to characterize VCG diversity, pathogenicity, and virulence of a collection of *Verticillium* and related species recovered from spinach seed as well as several other plant hosts. This may help with development of effective management practices for *Verticillium* wilt in spinach seed crops and other crops susceptible to these fungi.

Materials and Methods

Isolates of *Verticillium* spp. A collection of 261 isolates of *V. dahliae* and other *Verticillium* spp. from spinach and eight other plant species was used in this study. Monoconidial isolates were recovered from each culture prior to storage. Isolates were stored at -20°C on desiccated filter paper as described by Correll et al. (21). The isolates were selected from a larger collection of isolates to represent isolates of VCGs 2A, 2B, and 4B (following VCG determination as described below) and different countries of origin. The collection included 255 isolates of *V. dahliae*, of which 212 were recovered from spinach seed lots produced in the United States, Denmark, the Netherlands, and New Zealand (28); 3 isolates were recovered from cotton, 22 from lettuce, 3 from mint, 1 from peppermint, 8 from potato, 3 from radish (*Raphanus sativus* L.), and 3 from tomato. Additionally, three isolates of *V. tricorpus* (two from spinach seed from the United States Department of Agriculture

[USDA] spinach germplasm collection and one from peppermint), two isolates of *G. nigrescens* recovered from spinach seed, and an isolate of *V. albo-atrum* Reinke & Berthold from alfalfa (*Medicago sativa* L.) were included in this study.

A subset of 40 representative isolates of *Verticillium* and related species was examined microscopically (×40 to ×100 magnification) for the presence or absence of resting structures and verticillate conidiophores to determine the conformity with known morphological standards for species of *Verticillium* (57). This core group included 34 isolates of *V. dahliae* from VCGs 2A, 2B, and 4B, of which 15 were recovered from spinach seed lots produced in the United States, Denmark, and the Netherlands (28); 3 isolates from cotton; 5 from lettuce; 2 from mint; 1 from peppermint; 4 from potato; 1 from radish; and 3 from tomato (Table 1). Additionally, two isolates of *G. nigrescens* recovered from spinach seed, three isolates of *V. tricorpus* (two from spinach seed from the USDA spinach germplasm collection and one from peppermint), and an isolate of *V. albo-atrum* from alfalfa were included (Table 1). Isolates were grown on potato dextrose agar (PDA; Difco) and on NP-10 agar, a semiselective medium for *Verticillium* spp. (67), at 22°C for 14 to 21 days.

VCG analysis. A total of 250 isolates of *V. dahliae* was examined for VCG diversity. To generate chlorate-resistant mutants, mycelial plugs of each isolate were placed on 5% water agar chlorate medium (WAC) (20 g of agar, 0.2 g of glucose, and 50 g of KClO₃ per liter) (46), and the resultant chlorate-resistant sectors were transferred to a minimal agar medium (MM) (30 g of sucrose, 1 g of KH₂PO₄, 0.5 g of MgSO₄, 0.5 g of KCl, 0.01 g of FeSO₄, 0.2 ml of trace elements, 20 g of agar, and 2 g of NaNO₃ per liter) to confirm that they were nitrate nonutilizing (*nit*) mutants. VCG tests were conducted as described previously (20,41), with an incubation period of 10 to 21 days. Reference VCG testers of *V. dahliae* belonging to VCG 1 (T9 and V44), VCG 2A (PH and WM), VCG 2B (115, S92, VD1/13, and VD5/16), VCG 3 (70-21 and PCW), VCG 4A (BB and P-103), and VCG 4B (MT, S39, VD2/22, and VD4/36) were used in complementation tests (69). The *nit* mutants that formed heterokaryons, observed as wild-type growth where the two *nit* mutants came in contact, were assigned to the same VCG.

Amplification of the ITS rDNA region, and phylogenetic analysis. The core group of 40 isolates of *Verticillium* representing different hosts, countries of origin, and VCGs was examined after polymerase chain reaction (PCR) amplification of the ITS1-5.8S-ITS2 region of rDNA using the ITS primers ITS1 (5'-TCCGTA GGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (83). Total DNA was extracted from the isolates following a standard procedure (19). PCR amplifications were each performed in a 100-μl volume containing sterilized distilled water, 20 ng of fungal DNA, 0.2 μM primer, 2.0 mM MgCl₂, 100 μM dNTP, 1× Taq polymerase buffer, and 2.0 units of DNA Taq polymerase. For amplification, a thermal cycler (Eppendorf) was programmed for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C; and a final extension period at 72°C for 5 min. The amplified products were separated electrophoretically in 1.0% agarose gels in 0.5× Tris-acetate-EDTA (TAE) buffer. PCR products were then purified using a QIAquick PCR purification Kit (Qiagen) following the manufacturer's instructions. Purified DNA was sequenced at the University of Arkansas DNA Resource Center (Fayetteville). Sequences were visualized in Chromas (Technelysium) and aligned using Clustal W (47). Phylogenetic relationships among the sequences were calculated by neighbor-joining using MEGA 4 software (71). Bootstrap values were calculated with 1,000 resampling replicates.

Pathogenicity and virulence analyses. Pathogenicity and virulence of the core group of 40 isolates of *Verticillium* were determined using the inbred spinach line AC1, 'DP 161' cotton, 'Bradley' tomato, and lettuce line plant introduction (PI) 251246. AC1 is a smooth-leaf, strongly female line selected for partial resistance to Fusarium wilt that is susceptible to *V. dahliae* (28). PI 251246 is an early-flowering lettuce line previously used to evaluate *Verticillium* wilt symptoms in growth-chamber tests (44).

Seed were sown in 200-cell seedling plug trays (Hummert Int.) and maintained for approximately 3 weeks on benches in a greenhouse or in a growth chamber with 12 h of supplemental light by day and 23°C average temperature. Temperatures in the greenhouse were 6.7 to 21.8°C in winter and 19.9 to 37.7°C in summer. Plants were fertilized once per week in the seedling trays and after transplanting with Miracle-Gro (Stern's Miracle-Gro) at 1.5 g/liter of water.

To prepare inoculum for the greenhouse experiments, isolates of *Verticillium* were grown on PDA plates at room temperature for 2 weeks. Conidia were collected by washing the agar surface using cold, sterilized, deionized water. Conidial concentrations were quantified with a hemacytometer and adjusted to 1×10^7 conidia/ml. A standard root-dip inoculation protocol was used in the pathogenicity assays, similar to that described by du Toit et al. (28). The length of the root plug of each seedling was trimmed one-quarter with sterile scissors. Plants were then inoculated with each isolate by dipping the roots in 50 ml of conidial suspension per seedling for at least 1 h; then, each inoculated seedling was planted into Sunshine LC1 potting mix in a 15-cm-diameter pot. Water was used similarly for treating the noninoculated control plants, and care was taken to sterilize gloves between isolates to avoid cross-contamination. For all the pathogenicity assays, four pots (replications) were used per treatment, with three plants per pot. The pots were arranged in a randomized complete block design on greenhouse benches. Plants were monitored daily for symptom development and to determine when to induce bolting

(conversion from vegetative to reproductive growth) by increasing the duration of the greenhouse supplemental lighting, as described below.

Plants were monitored once per week for incidence and severity of *Verticillium* wilt from 9 to 28 days after inoculation. For the spinach inoculation experiments, disease severity was rated using a scale of 0 to 6 (28,81,82), in which 0 = no symptoms, 1 = >0 to 10% of the plant showing chlorosis or necrosis, 2 = >10 to 25% chlorosis or necrosis, 3 = >25 to 50% chlorosis or necrosis, 4 = >50 to 75% chlorosis or necrosis, 5 = >75 to <100% chlorosis or necrosis, and 6 = dead plant. The midpoints of each range (0.0, 5.0, 17.5, 37.5, 62.5, 87.5, and 100.0%) were used for statistical analysis. For the inoculation experiments on cotton, each plant was rated on a 0-to-3 scale, where 0 = no symptoms, 1 = mild chlorosis and necrosis (>0 to <50% chlorosis or necrosis), 2 = severe chlorosis and necrosis (>50% to <100% chlorosis or necrosis), and 3 = dead plant (100% chlorosis and necrosis). The midpoints of each category range (0.0, 25.0, 75.0, and 100.0%) were used for statistical analysis. For the assays on lettuce and tomato, disease severity was expressed as the percentage of leaves symptomatic (chlorotic or necrotic) per plant.

Disease scoring was performed for individual plants and then averaged per pot (experimental unit). For the analysis of variance (ANOVA), means for the last day of scoring were compared. ANOVA was conducted using JMP Software (SAS Institute Inc.), with 95% confidence intervals for detection of statistically significant differences among treatments. Comparisons of isolates were

Table 1. Core isolates of *Verticillium* species from spinach and non-spinach hosts used to evaluate variation in internal transcribed spacer (ITS) rDNA sequence, virulence, and pathogenicity on spinach and other plant hosts

Species ^a	Host	Isolate code	Origin	VCG ^b
<i>Verticillium dahliae</i>	Spinach	WSU 107	Denmark	2B
<i>V. dahliae</i>	Spinach	WSU 210	Denmark	2B
<i>V. dahliae</i>	Spinach	WSU 523	The Netherlands	2B
<i>V. dahliae</i>	Spinach	WSU 541	The Netherlands	2B
<i>V. dahliae</i>	Spinach	WSU 268	United States	2B
<i>V. dahliae</i>	Spinach	WSU 384	United States	2B
<i>V. dahliae</i>	Spinach	VD1	United States	2B
<i>V. dahliae</i>	Spinach	JD4ss2	United States	2B
<i>V. dahliae</i>	Spinach	WSU 311	Denmark	4B
<i>V. dahliae</i>	Spinach	WSU 359	Denmark	4B
<i>V. dahliae</i>	Spinach	WSU 511	The Netherlands	4B
<i>V. dahliae</i>	Spinach	WSU 509	The Netherlands	4B
<i>V. dahliae</i>	Spinach	WSU 139	United States	4B
<i>V. dahliae</i>	Spinach	WSU 49	United States	4B
<i>V. dahliae</i>	Spinach	VD4	United States	4B
<i>V. dahliae</i>	Cotton	Gh1011	United States	2A
<i>V. dahliae</i>	Cotton	Gh1018	United States	2A
<i>V. dahliae</i>	Cotton	CS1	United States	2B
<i>V. dahliae</i>	Lettuce	Ls847	United States	2A
<i>V. dahliae</i>	Lettuce	Ls636	United States	2A
<i>V. dahliae</i>	Lettuce	Ls822	United States	2A
<i>V. dahliae</i>	Lettuce	Ls842	United States	2A
<i>V. dahliae</i>	Lettuce	Ls17	United States	2B
<i>V. dahliae</i>	Mint	LD4	United States	2B
<i>V. dahliae</i>	Mint	LD2	United States	2B
<i>V. dahliae</i>	Peppermint	LD1	United States	2B
<i>V. dahliae</i>	Potato	P19	United States	2B
<i>V. dahliae</i>	Potato	JD1	United States	2B
<i>V. dahliae</i>	Potato	W140	United States	4B
<i>V. dahliae</i>	Potato	JD2	United States	4B
<i>V. dahliae</i>	Radish	RV1	United States	4B
<i>V. dahliae</i>	Tomato	Le1103	United States	4B
<i>V. dahliae</i>	Tomato	Le1112	United States	4B
<i>V. dahliae</i>	Tomato	Le1101	United States	4B
<i>V. tricorpus</i>	Spinach	USDA 11	United States	n.d.
<i>V. tricorpus</i>	Spinach	USDA 29	United States	n.d.
<i>V. tricorpus</i>	Peppermint	VB1aM	United States	n.d.
<i>V. albo-atrum</i>	Alfalfa	LD9	United States	n.d.
<i>Gibellulopsis nigrescens</i>	Spinach	LD10	United States	n.d.
<i>G. nigrescens</i>	Spinach	USDA 95	United States	n.d.

^a Species based on morphological evaluation and sequences of the ITS region of ribosomal DNA. *G. nigrescens* = *Verticillium nigrescens* (85).

^b VCG = vegetative compatibility group verified as described in the main text (20,41); n.d. = not determined.

made using Fisher's least significant difference (LSD) tests ($P \leq 0.05$). Correlation analyses of disease severity ratings among isolates used in the various tests were calculated using JMP Software with 95% confidence intervals.

In each experiment, two petiole pieces per experimental unit were cut from symptomatic and asymptomatic plants, surface-disinfested by immersing the tissue in 0.5% NaOCl for 1 min, and plated on water agar to test for the presence or absence of *Verticillium* spp. Each petiole piece was examined with a microscope ($\times 10$ to $\times 100$ magnification) approximately 5 days after plating for the presence of *Verticillium* microsclerotia or conidiophores. Plant height and dry weight per plant were also evaluated as a complement to disease evaluations for the cotton and tomato inoculation tests. The dry weight of plants was determined by cutting each plant approximately 1 cm below the soil line, placing the plant in a paper bag, and drying the plant for 96 h in a forced-draft air oven set at 55°C. Plant height and dry weight data were analyzed by ANOVA and LSD comparison of means, and the correlation coefficients of plant growth parameters and disease severity were calculated using JMP. Plant height and dry weight data were not collected for the inoculated spinach plants based on prior experience with *Verticillium* wilt ratings of spinach (82).

All inoculation tests were conducted at least twice. Plants in each test were evaluated multiple times but disease ratings were analyzed statistically for the last day of evaluation. The disease rating selected for statistical analysis for each host was based on when disease severity was at a maximum for at least one of the plants in the test. For the first spinach test, which was performed during the summer, seedlings were kept in a growth chamber and, after inoculation, were exposed to long photoperiods in a greenhouse 11 days after inoculation, which induced bolting. For the second spinach test, plants were induced to bolt 15 days after inoculation by increasing the duration of supplemental lighting to 24 h for 3 days. For both spinach tests, disease severity was assessed 27 days after inoculation. For the first and second cotton inoculation tests, severity of symptoms was assessed 24 and 22 days after inoculation, respectively. For tomato, disease ratings were assessed 27 days after inoculation for both tests. To induce bolting of lettuce, the duration of the greenhouse supplemental lighting was increased to 16 h for 1 day after inoculation in the first test, which was completed in the winter, and plants were observed to bolt 3 days after inoculation. For the second lettuce test, performed dur-

ing the summer, seedlings were kept in a growth chamber and, after inoculation, were exposed to long photoperiods in a greenhouse, which induced bolting 3 days after inoculation. Severity of symptoms was assessed 28 days after inoculation for both lettuce tests.

Results

Morphological analysis. Microscopic and cultural examination of the core set of 40 isolates of *Verticillium* spp. evaluated in this study revealed characteristics consistent with those of *V. albo-atrum*, *V. dahliae*, *V. tricorpus*, and *G. nigrescens* (24,57,85) (Table 1). The morphological examinations were completed based on the more broad taxonomic classification of *Verticillium* and did not include the more recently proposed taxa whereby the genus was divided into 10 species (35).

VCG analysis. VCG analysis of 250 isolates of *V. dahliae* indicated that isolates belonged to VCGs 2A, 2B, or 4B. Of 210 isolates of *V. dahliae* obtained from spinach seed, 128 isolates belonged to VCG 4B, 65 isolates to VCG 2B, and 3 isolates to VCG 2A (Table 2). For the remaining 14 isolates, *nit* mutants could not be recovered despite several attempts. Of the 128 isolates that belonged to VCG 4B, 81 (63.3%) originated from spinach seed lots produced in the United States, 8 (6.2%) from seed produced in Denmark, 36 (28.1%) from seed grown in the Netherlands, 1 (0.8%) from seed produced in New Zealand, and 2 (1.6%) from seed of unknown origin (Table 2). Of the 65 isolates in VCG 2B, 8 (12.3%) originated from spinach seed lots produced in the United States, while 34 (52.3%) isolates originated from Denmark, 6 (9.2%) from the Netherlands, 12 (18.5%) from seed produced in New Zealand, and 5 (7.7%) from seed of unknown origin (Table 2). Of the three spinach isolates that belonged to VCG 2A, two (66.7%) originated from seed produced in the United States and one (33.3%) from seed produced in Denmark. Interestingly, of the 91 isolates of *V. dahliae* obtained from U.S. spinach seed crops, only 8 (8.8%) belonged to VCG 2B, whereas 81 (89.0%) belonged to VCG 4B, and the remaining 2 (2.2%) belonged to VCG 2A. A similar ratio was observed for the 42 isolates obtained from spinach seed grown in the Netherlands; that is, six (14.3%) belonged to VCG 2B whereas 36 (85.7%) belonged to VCG 4B. In contrast, of the 43 isolates from spinach seed crops produced in Denmark, 34 (79.1%) belonged to VCG 2B, only 8 (18.6%) belonged to VCG 4B, and 1 (2.3%) belonged to VCG2A.

Table 2. Summary of vegetative compatibility group (VCG) analysis of 235 isolates of *Verticillium dahliae*

VCG ^a	Number of isolates ^b	Origin of isolates		Total ^c
		Host crop	Country, state	
4B	81	Spinach	United States, Washington	128
	8	Spinach	Denmark	
	36	Spinach	The Netherlands	
	1	Spinach	New Zealand	
	2	Spinach	Unknown	
	4	Potato	United States, Washington	4
	3	Radish	United States, Oregon	3
	3	Tomato	United States, California	3
	8	Spinach	United States, Washington	65
	34	Spinach	Denmark	
2B	6	Spinach	The Netherlands	
	12	Spinach	New Zealand	
	5	Spinach	Unknown	
	1	Potato	United States, Washington	1
	1	Peppermint	United States, Texas	1
	3	Mint	United States, Texas	3
	1	Lettuce	United States, California	1
	2	Spinach	United States, Washington	3
	1	Spinach	Denmark	
	21	Lettuce	United States, California	21
2A	2	Cotton	United States, California	2

^a VCG verified as described in the main text (20,41).

^b Number of isolates that belong to VCG 4B, VCG 2B, or VCG 2A, organized by host and geographic region of origin.

^c Total number of isolates by host crop that belong to VCG 4B, VCG 2B, and VCG 2A.

Of the 22 isolates of *V. dahliae* from lettuce, 21 belonged to VCG 2A and only 1 belonged to VCG 2B. Four of the six isolates of *V. dahliae* from potato plants belonged to VCG 4B, one to VCG 2B, and one (isolate LD6) failed to form a heterokaryon with the testers. All three radish isolates of *V. dahliae* (RV1, RV2, and RV3) and the three tomato isolates of *V. dahliae* (Le1101, Le1102, and Le1103) belonged to VCG 4B. The peppermint isolate and three mint isolates of *V. dahliae* belonged to VCG 2B. The two cotton isolates of *V. dahliae* (Gh1011 and Gh1018) belonged to VCG 2A (Table 2).

Comparison of DNA sequence variation in the ITS rDNA region of *Verticillium*. The ITS primers ITS1 and ITS4 amplified a 400- to 500-bp DNA fragment from all 40 isolates in the core collection. A 429-bp sequence of the ITS1-5.8S-ITS2 region of the isolates was used to generate a neighbor-joining tree that discriminated the isolates into two major clusters, representing the genera *Verticillium* and *Gibellulopsis* (Fig. 1). Based on alignment of the sequences, isolates of *V. dahliae*, *V. tricorpus*, *V. albo-atrum*, and *G. nigrescens* were discriminated, and the sequence identity was

Fig. 1. Neighbor-joining tree derived from sequences of the internal transcribed spacer (ITS)1-5.8s-ITS2 ribosomal DNA region of isolates of *Verticillium dahliae*, *V. albo-atrum*, *V. tricorpus*, and *Gibellulopsis nigrescens*. Bootstrap values (1,000 replicates) are indicated adjacent to the nodes.

confirmed by comparison with ITS sequences in GenBank (*data not shown*).

No variation occurred in this ITS region among 28 of the 34 isolates of *V. dahliae*. However, six isolates of *V. dahliae* in VCG 2A, representing four isolates from lettuce (Ls636, Ls822, Ls842, and Ls847) and two from cotton (Gh1011 and Gh1018), had a single nucleotide polymorphism (SNP) at base pair 296 in the ITS region. Thus, the isolates of *V. dahliae* clustered into two groups, one including isolates of VCG 2B and 4B and the other containing the six isolates with an SNP which belonged to VCG 2A. There was no ITS sequence variation among the three isolates of *V. tricorpus* examined. However, minor variation in ITS sequences was observed among the two isolates of *G. nigrescens* examined (4-bp differences).

Pathogenicity and virulence analyses. Analyses of data from pathogenicity experiments with isolates of *Verticillium* tested on spinach, cotton, tomato, and lettuce indicated differential pathogenicity among isolates of the species of *Verticillium* obtained from spinach and non-spinach hosts. Although the isolates of *V. dahliae* exhibited a broad host range, there were virulence differences among isolates of *V. dahliae* from the hosts examined. All inoculation tests were conducted at least twice and results on the same crop were similar, with no significant effect of experiment on results for any individual crop. Thus, for each of the plant species evaluated, results of just one of the repeat experiments are presented below.

Inoculation of spinach. Distinct symptoms of Verticillium wilt developed between 12 and 27 days after inoculation of spinach plants with the 40 isolates of *Verticillium*. There was a wide range in disease severity caused by the isolates. Of the 29 isolates of *V. dahliae* examined, 27 caused symptoms of Verticillium wilt on

spinach, with significant differences in incidence and severity of Verticillium wilt caused by the isolates ($P < 0.0001$ at $\alpha = 0.05$) (Fig. 2). Disease incidence and severity were 25.0 to 100.0 and 2.3 to 99.9%, respectively. Isolates of *V. dahliae* from spinach and in VCG 2B caused disease severity ratings of 32.5 to 86.5%, with a mean $\pm$ standard error of $74.5 \pm 5.9\%$, and isolates in VCG 4B caused severity ratings of 42.5 to 78.1%, with a mean of $64.1 \pm 6.1\%$ (Fig. 2). Isolates of *V. dahliae* from spinach, lettuce, potato, tomato, and radish were consistently pathogenic on spinach line AC1. Isolates Ls822 and Ls847 of *V. dahliae* from lettuce ranked consistently among the most virulent isolates examined. Isolates of *V. dahliae* from peppermint and mint (LD1 and LD4, respectively) as well as isolates of *V. tricorpus*, *V. albo-atrum*, and *G. nigrescens* were nonpathogenic on spinach under these conditions. *V. dahliae* was recovered from petiole sections (100% incidence) sampled from symptomatic plants inoculated with isolates of *V. dahliae*. In contrast, *V. tricorpus*, *V. albo-atrum*, and *G. nigrescens* were not recovered from petioles or stem sections of plants inoculated with isolates of these species, all of which remained asymptomatic. In general, more severe ratings were observed in the first experiment than in the second experiment. In the second experiment, isolates of *V. dahliae* in VCG 2B from spinach caused disease severity ratings of 23.0 to 72.5%, with a mean of $46.3 \pm 6.3\%$, and isolates in VCG 4B from spinach caused disease severity of 20.6 to 59.8%, with a mean of $38.7 \pm 5.1\%$. However, highly significant correlations existed between disease severity ratings for the two experiments ($R = 0.67$; $P < 0.0001$).

Inoculation of cotton. All isolates of *V. dahliae* caused Verticillium wilt symptoms on cotton DP 161, with a final Verticillium wilt incidence of 8.3 to 100% and severity of 2.1 to 66.7% ($P <$

Fig. 2. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on severity of Verticillium wilt on spinach inbred line AC1. Mean disease severity rating was converted to percentage (midpoint) values and averaged for three plants/pot and four pots/treatment. Disease severity ratings of 0 were not included in statistical analyses. LSD = least significant difference at $P < 0.05$. *V.t.* = *Verticillium tricorpus*, *V.a.* = *V. albo-atrum*, *G.n.* = *Gibellulopsis nigrescens*.

0.0001 at $\alpha = 0.05$) (Fig. 3). Isolates of *V. dahliae* in VCG 2B that were originally obtained from spinach caused disease severity on cotton of 25.0 to 52.1%, with a mean of $39.9 \pm 4.0\%$, and isolates in VCG 4B from spinach caused severity ratings on cotton of 16.7 to 50.0%, with a mean of $31.0 \pm 4.1\%$. The isolates of *V. dahliae* in VCG 2B and the two isolates in VCG 2A from cotton caused symptoms of 20.8 to 66.7% in severity and >94% incidence (Fig. 3). In both inoculation tests on cotton, isolates of *V. dahliae* from cotton, lettuce, potato, and tomato, as well as one isolate from radish, were pathogenic on cotton DP 161. Isolates of *V. dahliae* from peppermint and mint were weakly virulent and isolates from spinach and lettuce were virulent to highly virulent on cotton, with the lettuce isolate of Ls847 consistently ranking among the most virulent. All isolates of *V. tricornis*, *G. nigrescens*, and *V. albo-atrum* were nonpathogenic on DP 161 cotton.

There was significant variation in dry weight of cotton plants inoculated with the different isolates of *Verticillium*, ranging from 0.4 to 1.4 g/plant ($P < 0.0001$ at $\alpha = 0.05$) (Fig. 4). There also was a significant reduction in plant dry weight relative to that of the noninoculated cotton plants for all *Verticillium* isolates examined except for two isolates of *V. tricornis*, USDA11 and VB1aM, originally obtained from spinach and peppermint, respectively. *V. tricornis* isolate USDA29 and *G. nigrescens* isolates LD9 and LD10 caused very mild reductions in weight of cotton plants (Fig. 4). The five isolates that caused the greatest reduction in cotton plant weight were from spinach (WSU 523, WSU 359, and JD4ss2) and lettuce (Ls847 and Ls822). A highly significant negative correlation between disease severity and plant dry weight was calculated ($R = -0.62$; $P < 0.0001$). There was a significant difference in the height of the cotton plants inoculated with the different

isolates of *Verticillium* ($P < 0.0001$ at $\alpha = 0.05$) (Fig. 5), with a significant reduction in plant height caused by 27 of the 33 isolates of *V. dahliae* examined (Fig. 5). There was also a highly significant negative correlation between plant height and disease severity ratings ($R = -0.76$; $P < 0.0001$). *V. dahliae* was recovered from 100% of the petiole sections sampled from symptomatic cotton plants that had been inoculated with *V. dahliae*. In contrast, *V. tricornis*, *V. albo-atrum*, and *G. nigrescens* were not recovered from petiole sections of the plants inoculated with isolates of these species, with all of these plants remaining asymptomatic.

Inoculation of tomato. Of 30 isolates of *V. dahliae* inoculated on Bradley tomato, 22 caused Verticillium wilt symptoms, with significant differences in disease severity and incidence among the isolates ($P < 0.0001$ at $\alpha = 0.05$ for both variables). Disease incidence and severity were 8.3 to 100 and 1.4 to 100.0%, respectively (Fig. 6). Only 8 of the 13 isolates of *V. dahliae* from spinach caused Verticillium wilt symptoms on tomato, with a range in severity of 1.4 to 10.4%. The three isolates of *V. dahliae* from tomato (Le1101, Le1103, and Le1112) caused symptoms on Bradley of 65.0 to 100.0% severity, with 100% disease incidence. These three isolates of *V. dahliae* from tomato, as well as three isolates from lettuce (Ls847, Ls842, and Ls822), one isolate from cotton (Gh1011), and one from potato (P19), caused more severe symptoms of Verticillium wilt on Bradley than the other isolates of *V. dahliae* (Fig. 6). Tomato plant height also was affected significantly differently among isolates ($P < 0.0001$ at $\alpha = 0.05$) (Fig. 7), with a significant reduction in plant height caused by 9 of the 30 isolates of *V. dahliae* examined. There was a highly significant negative correlation between the height of tomato plants and disease severity rating ($R = -0.76$; $P < 0.0001$). *V. dahliae* was recov-

Fig. 3. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on severity of Verticillium wilt on the cotton 'DP 161'. Mean disease severity rating was converted to percentage (midpoint) values and averaged for three plants/pot and four pots/treatment. Disease severity ratings of 0 were not included in statistical analyses. LSD = least significant difference at $P < 0.05$. V.t. = *Verticillium tricornis*, V.a. = *V. albo-atrum*, G.n. = *Gibellulopsis nigrescens*.

ered from 100% of the petiole sections sampled from symptomatic plants inoculated with *V. dahliae*. In contrast, *V. tricornis* and *G. nigrescens* were not recovered from petiole sections of plants inoculated with isolates of these species, and all of these plants remained asymptomatic. Consistent results were observed in the repeat inoculation test.

In both inoculation tests on tomato, isolates of *V. dahliae* from tomato and lettuce were virulent to highly virulent on Bradley. There was variation in severity of symptoms among isolates of *V. dahliae* ranging from nonpathogenic to highly virulent. In general, isolates of *V. dahliae* from spinach, peppermint, and mint were nonpathogenic to weakly virulent on tomato, whereas isolates from lettuce, tomato, and a cotton isolate were moderately to highly virulent. Although tomato plants were smaller in the second test, in both experiments, the same nine isolates of *V. dahliae* reduced plant height significantly relative to the height of noninoculated control plants (*data not shown*).

Inoculation of lettuce. Of 31 isolates of *V. dahliae* inoculated onto lettuce plants, 30 were pathogenic, with significant differences in disease severity and incidence observed among the isolates ($P < 0.0001$ at $\alpha = 0.05$). Disease incidence and severity were 41.7 to 100.0 and 2.8 to 58.9%, respectively (Fig. 8). Isolates of *V. dahliae* in VCG 2B from spinach caused disease severity ratings on lettuce line P1 251246 of 8.9 to 26.7%, with a mean of $17.7 \pm 2.2\%$, and isolates in VCG 4B from spinach caused ratings of 13.9 to 27.8%, with a mean of $19.5 \pm 2.1\%$. An isolate of *V. dahliae* from cotton (CS1) was nonpathogenic on lettuce, as were the isolates of *V. tricornis* and *G. nigrescens*. Isolates of *V. dahliae* VCG 2A from lettuce caused symptoms on lettuce line P1 251246 ranging in severity from 31.7 to 58.9%. *V. dahliae* was recovered only from petiole sections (55% incidence) sampled from symptomatic plants inoculated with isolates of *V. dahliae*. In contrast, *V. tricornis* and *G. nigrescens* were not recovered from petiole sections of the plants inoculated with isolates of these species, which remained

asymptomatic. Overall, in both repeats of the lettuce inoculation experiment, isolates of *V. dahliae* from lettuce, potato, tomato, and radish were pathogenic. There was variation in severity of symptoms among isolates, ranging from weakly virulent to moderately to highly virulent for isolates of *V. dahliae* from spinach and cotton, and from weakly virulent to moderately virulent for isolates from mint and peppermint.

Discussion

V. dahliae was reported on commercial spinach seed lots in 2005, and has since been recognized as a concern for spinach seed production in the European Union and the United States (28). Furthermore, although *V. dahliae* does not appear to cause symptoms in vegetative, fresh-market, or processing spinach crops, the incidence of the pathogen on commercially produced spinach seed lots is a major concern due to the potential of introducing the pathogen when planting infested spinach seed into noninfested fields in which other Verticillium wilt-susceptible crops are grown (28). For instance, recent outbreaks of Verticillium wilt in lettuce have been associated, in part, with the presence of *V. dahliae* on spinach seed lots planted in rotation with lettuce crops in the Salinas Valley of California, as well as other susceptible plant species rotated with lettuce (e.g., strawberry [*Fragaria × ananassa*], pepper, and artichoke [*Cynara scolymus*]), based on molecular analyses of populations of the pathogen using microsatellite markers and population analyses (6,7). This has highlighted the need to investigate the pathogenicity and virulence on spinach and alternative crops of Verticillium isolates obtained from spinach seed. Such studies may be important for developing effective management strategies for Verticillium wilt, including appropriate crop rotation schemes. Results of this study help elucidate the degree of host specificity, diversity in virulence, and genetic diversity of isolates of Verticillium from spinach seed as well as isolates from crops that might be grown in rotation with spinach.

Fig. 4. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on plant dry weight of the cotton 'DP 161'. Mean plant dry weight (g) of four pots/isolate with three plants/pot. LSD = least significant difference at $P < 0.05$. V.t. = *Verticillium tricornis*, V.a. = *V. albo-atrum*, G.n. = *Gibellulopsis nigrescens*.

species recovered was *V. dahliae*. However, additional taxa, including *V. tricornutus* and *G. nigrescens*, were recovered from these spinach seed lots as well as from seed of PI lines from the USDA germplasm repository (L. J. du Toit et al., *unpublished data*;

Fig. 5. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on plant height of the cotton 'DP 161'. Mean of plant height (cm) of four pots/isolate with three plants/pot. LSD = least significant difference at $P < 0.05$. V.t. = *Verticillium tricorpus*, V.a. = *V. albo-atrum*, G.n. = *Gibellulopsis nigrescens*.

Fig. 6. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on *Verticillium* wilt severity on tomato 'Bradley'. Mean disease severity rating was converted to a percentage (midpoint) and averaged for three plants/pot and four pots/treatment. Disease severity ratings of 0.0 were not included in statistical analyses. LSD = least significant difference at $P < 0.05$. V.t. = *Verticillium tricorpus*, G.n. = *Gibellulopsis nigrescens*.

30,81,82). Examination of a subset of 210 isolates of *V. dahliae* from this collection of isolates demonstrated that the majority of spinach isolates belonged to one of two VCGs, VCG 2B or VCG 4B, and a very small proportion belonged to VCG 2A. The major-

ity of isolates from Denmark were grouped in VCG 2B, whereas the majority of isolates from the United States and the Netherlands were in VCG 4B. Differences in VCGs may be associated with crop rotation practices in regions where spinach seed is produced,

Fig. 7. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on height of 'Bradley' tomato plants. Each data point is the mean plant height (cm) in four replicate pots/isolate with three plants/pot. LSD = least significant difference at $P < 0.05$. V.t. = *Verticillium tricorpus*, G.n. = *Gibellulopsis nigrescens*.

Fig. 8. Effect of isolates of *Verticillium* spp. from spinach and non-spinach hosts on Verticillium wilt severity of the lettuce line PI 251246. Mean disease severity rating was converted to a percentage (midpoint) and averaged for three plants/pot and four pots/treatment. Disease severity ratings of 0 were not included in statistical analyses. LSD = least significant difference at $P < 0.05$. V.t. = *Verticillium tricorpus*, G.n. = *Gibellulopsis nigrescens*.

as well as susceptibility of these crops to isolates of *V. dahliae* in VCGs 2A, 2B, and 4B. In the United States, spinach seed is produced almost solely in the maritime Pacific Northwest because of the favorable climatic conditions necessary for producing high-quality spinach seed. In this maritime region of the United States, spinach seed crops are commonly grown in rotation with potato, with a significant increase in potato acreage in western Washington over the last 20 years (2,50). This may have contributed to increasing populations of *V. dahliae* isolates of VCG 4, because isolates in this VCG are predominant among those pathogenic on potato (42,68). In Denmark, spinach seed crops are most commonly rotated with cereals and grass seed, which are not susceptible to *V. dahliae*, and, to a minor degree, with crops such as sugar beet (*Beta vulgaris*) (73). Similarly, in the Netherlands, spinach seed crops are commonly rotated with potato crops. However, the association between cropping history and populations of *V. dahliae* in specific VCGs needs further examination.

In this study, a subset of 40 *Verticillium* isolates from different VCGs, host plants, and geographic origins was evaluated for VCG diversity, sequence variation in the ITS region of rDNA (83), and pathogenicity on spinach, cotton, tomato, and lettuce. Sequences of the ITS rDNA distinguished and confirmed that taxa associated with spinach seed include *V. dahliae*, *V. tricorpus*, and *G. nigrescens*. However, little to no variation was observed among isolates within each species for the ITS sequences. Because of morphological similarities among the *Verticillium* spp. recovered from spinach seed, identification of these species should be completed carefully, particularly when the incidence of *V. dahliae* in seed lots may have economic implications. For example, spinach seed exported to Mexico currently must meet a phytosanitary certification requirement of <10% contamination by *V. dahliae* (1), whereas there are no regulations for the presence of other species of *Verticillium* on spinach seed.

Although caution should be exercised to avoid extrapolating from limited collections of isolates, the lack of differentiation among isolates of *V. dahliae* from different hosts examined in this study, and the tendency for isolates to cluster within a limited number of VCGs in the phylogenetic analysis, supports studies by Attallah et al. (6,7). After analyzing 22 microsatellite markers and a collection of 243 *V. dahliae* isolates from different plant hosts, Attallah et al. (6) found evidence of significant gene flow among isolates from host plants they evaluated (artichoke, lettuce, pepper, spinach, strawberry, and some weed species). They suggested that recombination within *V. dahliae* populations might explain recent outbreaks of *Verticillium* wilt in crops that formerly were not affected by *Verticillium*, specifically lettuce, by the introduction of novel pathogen genotypes to major areas of production of these "immune" plant species.

Lack of a significant correlation between molecular or genetic diversity and virulence characteristics of *V. dahliae* was observed in this study, as documented by other authors (8,11,12,23,52,54). Furthermore, the incongruence among VCG characterization, virulence, and genetic characteristics of the isolates would confirm recent concerns about the suitability and relevance of vegetative compatibility as a genetic marker in diversity studies (5,40). Therefore, although the majority of lettuce isolates used in this study belonged to VCG 2A, a VCG different from the predominant groups detected in spinach isolates, no conclusions can be drawn, and the need remains for further evaluation of populations of *V. dahliae* to assess the potential origin and impact of isolates of the pathogen that might affect both spinach seed and lettuce production.

Among the fungal taxa recovered from spinach seed and examined for pathogenicity on spinach, tomato, cotton, and lettuce, isolates of *V. tricorpus* and *G. nigrescens* were nonpathogenic on each of these hosts. Furthermore, none of the isolates could be recovered from the petioles of plants inoculated with these isolates. Although *V. tricorpus* and *G. nigrescens* have generally been reported as weak pathogens on some plant species (31,38,51,60, 65,80,84) or as plant pathogens, especially under conducive

environmental conditions (34,36–38,74,75), pathogenicity of isolates of these species was not observed in this study. Nonpathogenic isolates of *V. tricorpus* on Chinese cabbage, cotton, eggplant, green pepper, larkspur, parsley, snapdragon, soybean, tobacco, and tomato (31,34,65) have been documented, as well as nonpathogenic isolates of *G. nigrescens* on cantaloupe, eggplant, peppermint, and tomato (65). Differential pathogenicity among isolates of *Verticillium* may reflect the distinct genetic background of some of the isolates used in previous studies, which recently have led to taxonomic revisions of the genus (35,85). In this study, isolates of *V. tricorpus* and *G. nigrescens* were recovered originally from spinach seed; therefore, factors leading to colonization of spinach seed by these isolates remain to be determined. Vascular colonization was not evident in these studies; therefore, one hypothesis is the possibility of aerial infection of spinach flowers and developing seed, similar to what has been observed by *V. dahliae* on flowering lettuce plants (77).

Inoculation of 40 *Verticillium* and related species onto spinach, cotton, tomato, and lettuce indicated differential pathogenicity and virulence among the isolates. None of the isolates of *V. dahliae* examined was pathogenic specifically to a single host; however, the isolates did exhibit different degrees of virulence on the host plants evaluated. Correspondingly, previous reports have characterized *V. dahliae* as a host-adapted pathogen that, despite the capacity to infect a wide range of hosts, is more virulent on certain crops (25,70). All isolates of *V. dahliae* from spinach, cotton, tomato, and lettuce evaluated in this study were pathogenic on the host plants from which they were originally recovered. In general, isolates of *V. dahliae* from spinach were more virulent on cotton and lettuce than on tomato, whereas isolates of *V. dahliae* from lettuce, potato, and tomato were pathogenic on spinach, cotton, tomato, and lettuce; and isolates from peppermint and mint were nonpathogenic or very weakly virulent on those crops. The lack of virulence of mint and peppermint isolates might be related to the pronounced host specialization previously reported for isolates from these plant species (12,25,33). These isolates had previously been confirmed as pathogenic on mint and peppermint cultivars by D. Johnson at Washington State University (*personal communication*). Isolates of *V. dahliae* from cotton that were inoculated on spinach, lettuce, and tomato ranged from nonpathogenic to pathogenic, with a wide range in virulence among the pathogenic isolates. These data support the results of previous studies in which the virulence of isolates from cotton on cotton were quite variable (3). Although a single isolate of *V. albo-atrum* from alfalfa was included in the inoculation experiments, the results indicated differential pathogenicity of this isolate, as documented previously (12,18). However, additional isolates of *V. albo-atrum* should be evaluated on various plant hosts for more definitive conclusions.

Verticillium spp. were recovered only from symptomatic petiole pieces sampled from plants inoculated with isolates of *V. dahliae*, which is consistent with previous reports of isolations of *Verticillium* from various plant hosts (12). However, in some other studies, *V. dahliae* was recovered from asymptomatic roots (14). Isolation from roots of inoculated plants was not assessed in this study. Nevertheless, asymptomatic infection of spinach by *V. dahliae* has been indicated by du Toit et al. (28), because seed-to-seed transmission occurred on some spinach plants without definitive symptoms evident, possibly confounded by *Verticillium* wilt symptoms resembling natural senescence of spinach plants after flowering and pollination. In the absence of wilt symptoms, measurement of plant growth (dry weight and plant height) can be used in conjunction with the assessment of severity of *Verticillium* wilt for some crops (e.g. spinach, cotton, and tomato), as demonstrated in this study. The fact that severity of symptoms on spinach has been correlated significantly with reduced plant weight suggests that *V. dahliae* can have a significant effect on plant growth even in the absence of symptoms, as sometimes is the case in spinach seed crops (L. J. du Toit and J. C. Correll, *unpublished data*).

Results of the pathogenicity experiments confirmed that *V. dahliae* isolates recovered from spinach seed are pathogenic on spin-

ach, as previously reported (28,66). However, a continuum of virulence was found among the spinach seed isolates, from weakly to highly virulent. This has been observed on other hosts such as cotton (3), lettuce (76), and potato (42). This range of virulence among isolates affects assessment of the potential impact of a population of the pathogen on a spinach seed crop. The relationship between inoculum density and disease severity might differ among fields or among regions within a field, and could be associated with the relative incidence of different isolates in specific locations.

Isolates of *V. dahliae* from lettuce, potato, radish, and tomato were as virulent on spinach as isolates recovered from spinach. The susceptibility of spinach to isolates of *V. dahliae* known to be pathogenic on other species of vegetables is highly significant because some of these crops are grown in the same areas of production as spinach (e.g., potato is commonly grown in rotation with spinach seed crops in the Pacific Northwest United States [28]). The current hypothesis that isolates from spinach seed may be associated, at least in part, with outbreaks of Verticillium wilt on lettuce in the Salinas and Pajaro Valleys of California, two major areas of vegetable production (6,7), is supported by results of this study, because isolates of *V. dahliae* from spinach were pathogenic on lettuce and vice versa. However, some of the most virulent isolates on spinach were lettuce isolates in VCG 2A. It is not known what proportion of isolates causing Verticillium wilt of lettuce in California belongs to VCG 2A but isolates of VCG 2A were not prevalent among isolates from spinach seed evaluated in this study. VCG analysis may not provide a full assessment of genetic diversity (40) and, because of the lack of significant correlation between genetic markers and virulence phenotypes of isolates of *Verticillium* (8), there is a need for further genetic analyses of populations of *V. dahliae* recovered from spinach and lettuce.

Identification of rotation crops that might influence infection of spinach by *V. dahliae*, and vice versa, as well as a greater understanding of the genetic diversity of isolates of *Verticillium*, is expected to help with development of effective management strategies for Verticillium wilt. The results of this study demonstrate that *Verticillium* and related species associated with spinach seed display genetic diversity as well as variability in virulence and pathogenicity on spinach and other plant species.

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