Screening for Resistance to Verticillium Wilt in Spinach and Isolation of *Verticillium dahliae* from Seed of Spinach Accessions

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Abstract. *Verticillium dahliae* is a pathogen of spinach (*Spinacia oleracea*) during spinach seed crop production but not in vegetative leafy spinach crops, because plants remain asymptomatic until bolting has been initiated (conversion from vegetative to reproductive growth). The objectives of this research were to evaluate a set of USDA spinach germplasm accessions for resistance to Verticillium wilt and to determine the range in incidence of natural seed infection/infestation in a subset of the USDA spinach accessions screened for resistance. A total of 120 *Spinacia* spp. accessions from the USDA North Central Regional Plant Introduction Station spinach germplasm collection and 10 commercial *S. oleracea* hybrids were screened for resistance to *V. dahliae* in Trials 1, 2, and 3 in 2006, 2007, and 2008, respectively, in greenhouse evaluations. Each accession was grown in either *V. dahliae*-infested or non-infested potting mix and rated weekly on a seven-step scale from 1 week before bolting to 4 weeks after bolting. A wide range of disease severity ratings was observed among the accessions evaluated. Most of the accessions were highly susceptible. There was no evidence of qualitative resistance but some showed greater levels of quantitative resistance than others. Plants in soil infested with *V. dahliae* senesced faster and had significantly reduced biomass compared with plants in non-infested soil of the same accession. In addition, in Trial 2 (2007), 34% (20 of 59) of the seed samples assayed of the accessions were infested or infected with *V. dahliae*; and in Trial 3, (2008) 16% (21 of 130) of the seed samples of the USDA accessions evaluated were infested or infected with *V. dahliae*, *V. tricornutum*, or *Gibellulopsis nigrescens* (formerly known as *V. nigrescens*). These results are valuable for characterizing potential genetic variability within spinach germplasm for resistance to *V. dahliae*.

Spinach (*Spinacia oleracea* L.) is an economically valuable crop grown worldwide (Morelock and Correll, 2008) and is affected by a number of biotic and abiotic disorders that can impact yield and quality (Correll

et al., 1994). Several soilborne pathogens affect the production of spinach leaf and seed crops (Correll et al., 1994). Although *Verticillium dahliae* causes Verticillium wilt of more than 200 species of vegetables, field crops, and trees, the fungus was not, until recently, recognized as a pathogen of spinach (du Toit et al., 2005). However, *V. dahliae* was recovered from spinach seed from New York in 1962 (Snyder and Wilhelm, 1962), and seed transmission of *V. dahliae* was reported in The Netherlands (van der Spek, 1972, 1973) and Canada (Sackston and Sedun, 1982). Verticillium wilt is not recognized as a disease in the commercial fresh market or processing spinach crops in the United States or elsewhere (Correll et al., 1994). However, *V. dahliae* was recently recovered from spinach plants grown for seed production and demonstrated to be pathogenic on spinach (du Toit et al., 2005). Interestingly, Verticillium wilt symptoms did not appear on inoculated

plants until after the reproductive phase of the plant life cycle had been initiated (bolted) by increasing the duration of daylength (du Toit et al., 2005). Symptoms of Verticillium wilt of spinach include leaf chlorosis, sometimes interveinal chlorosis, and wilting, starting with the lower (older) leaves and moving up the plant, stunting, a very light brown vascular discoloration of the roots and crown, and accelerated plant death (du Toit et al., 2005; Koike et al., 2007).

Spinach seed production is limited to areas where environmental conditions include long summer days to induce flowering and moderate summer temperatures (Metzger and Zeevaert, 1985). Denmark produces ≈75% of the world's spinach seed supply followed by the Pacific Northwest region of the United States (20%) (Correll et al., 2011; Daehnfeldt, 2007; Foss and Jones, 2005). In the Pacific Northwest, ≈600 to 1600 ha of spinach seed crops are grown annually, representing 25% to 50% of the U.S. spinach seed supply (Correll et al., 2011; Daehnfeldt, 2007). *V. dahliae* was recovered from 89% of 75 spinach seed lots produced in Washington, The Netherlands, and Denmark (du Toit et al., 2005) as well as seed lots produced in Chile and New Zealand (du Toit, unpublished data). Seed lots were found with incidences of *V. dahliae* infection up to 84.8% per lot, and seed-to-seed transmission was demonstrated by planting infected seed lots and taking the plants that grew out to flowering and seed set (du Toit et al., 2005). Although symptoms of Verticillium wilt have not been observed in fresh market and processing spinach crops, the seedborne nature of the pathogen is a significant concern because infested or infected spinach seed could potentially introduce *V. dahliae* into production fields and result in disease problems in subsequent crops (e.g., spinach and potato crops grown in rotation in the Pacific Northwest) (Atallah et al., 2010), or planting a spinach seed crop into soil infested from a previous crop could potentially lead to infection of the harvested seed (du Toit et al., 2005).

Verticillium wilt is particularly difficult to manage because microsclerotia of the pathogen can survive in the soil for long periods of time, and even a few microsclerotia per gram of soil can result in significant disease incidence in some crops under favorable environmental conditions (Grogan et al., 1979; Nicot and Rouse, 1987). Although a wide range of management options are available to help control Verticillium wilt, perhaps the most effective and economical practice to control *V. dahliae* is the use of plant resistance (Atibalentja and Eastburn, 1998; Diwan et al., 1999; Harrison and Beckman, 1982; Vallad and Subbarao, 2008; Wilhelm, 1955). Resistance to *V. dahliae* has been identified in various crops, including tomato (Diwan et al., 1999), sunflower (Klisiewicz, 1981), cotton (Harrison and Beckman, 1982), cacao (Resende et al., 1996), artichoke (Cirulli et al., 1994), horseradish (Atibalentja and Eastburn, 1998), eggplant (Bejarano-Alcazar et al., 1999), lettuce (Hayes et al., 2011), and potato (Jansky

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et al., 2004). Both single dominant gene and quantitative resistance have been identified in numerous crop hosts (Hayes et al., 2011; Schaible et al., 1951; Simko et al., 2004). Screening the USDA spinach germplasm collection has been valuable for identifying resistance to several diseases including downy mildew (Brandenberger et al., 1991), Fusarium wilt (Laguna, 2000), and leaf spot (Mou et al., 2008). However, there have been no published studies on resistance screening to *Verticillium* wilt in spinach to date. Therefore, the objectives of this study were to: 1) quantify the reactions of a subset of the USDA spinach germplasm collection to inoculation with *V. dahliae*; and 2) evaluate the incidence of natural infestation/infection by *V. dahliae* and other *Verticillium* species on spinach seed lots of the USDA germplasm collection. Preliminary results have been presented (Villarroel-Zeballos et al., 2006, 2007, 2008).

Materials and Methods

Screening for resistance to *V. dahliae*

Plant material and plant production. A total of 120 accessions of *Spinacia* spp. from the U.S. Department of Agriculture–North Central Regional Plant Introduction Station (USDA-NCRPIS) spinach germplasm collection, selected from a total of 360 accessions to represent diverse geographical origins, were screened for resistance to *V. dahliae* in 2006 (Trial 1), 2007 (Trial 2), and 2008 (Trial 3). Additionally, 10 commercial spinach hybrids commonly used as differentials for race identification of strains of the downy mildew pathogen, *Peronospora farinosa* f. sp. *spinaceae*, were included in the 2007 and 2008 resistance screening trials. Seed of each spinach genotype were planted and incubated in a greenhouse with 12-h daylength at 25 °C average temperature. The plants were watered daily and fertilized with Peters 20N–20P–20K every 8 to 10 d at a rate of $\approx 2 \text{ g L}^{-1}$. The tests were planted on 17 Apr. 2006, 14 Mar. 2007, and 28 Jan. 2008 for Trials 1, 2, and 3, respectively. Spinach is a long daylength plant whereby flowering is initiated as daylength increases (Metzger and Zeevaert, 1985). Although there is a lot of genetic variability in the number of hours required to induce flowering in spinach, the 2006 and 2007 experiments were planted in early spring to allow the natural increase in daylength that time of year to induce bolting ≈ 2 to 6 weeks after planting. In 2008, seeds were planted during the winter to let the plants grow for 30 d and then induced to bolt by increasing the photoperiod in the greenhouse to 20 h·d⁻¹ for 8 d using supplemental lighting. This resulted in all of the genotypes bolting at a similar number of days after planting to avoid confounding daylength sensitivity of the genotypes with the timing of symptom development.

Isolate and inoculum production. An isolate of *V. dahliae* (VD1), demonstrated to be a pathogen of spinach (du Toit et al., 2005) and belonging to VCG 2B (Iglesias-Garcia, 2011), was used to infest soil for the resistance screening test. A 2-mm² piece of colonized

filter paper on which isolate VD1 was stored was plated on potato dextrose agar (PDA). After 2 weeks, colonies were transferred to new plates of PDA and incubated for 12 d. Erlenmeyer flasks (1 L) containing sterile vermiculite amended with minimal medium nutrient solution (Korolev and Katan, 1997) were each inoculated with 10 mycelial plugs taken from the actively growing edge of a colony of VD1. Non-infested, sterile vermiculite amended with minimal medium nutrient solution was prepared for the non-inoculated control treatment of each spinach accession. The inoculated vermiculite was incubated in the dark at room temperature (22 to 24 °C) for 30 d, by which time microsclerotia of *V. dahliae* had developed in the vermiculite. Scott's RediEarth potting mix was steamed for 30 min at 71 °C and then amended at a rate of 1.0 g vermiculite inoculum/kg potting mix. This potting mix inoculum was mixed and watered periodically for 1 week before planting.

Experimental design and disease severity evaluations. The resistance screening experiment was set up as a split plot design with soil treatment (infested or non-infested) as the whole plot and 120 spinach accessions in 2006 or 130 lines in each of 2007 and 2008 as the subplots with six replications (pots)/treatment combination and three plants/pot. Plants were observed every 7 d to determine the timing of onset of bolting and development of symptoms of *Verticillium* wilt. Plant gender (spinach is dioecious) was recorded at the same time as the disease ratings. Each plant was rated for disease severity 35 or 36 d after planting (≈ 1 week before bolting) and weekly thereafter for ≈ 3 weeks after bolting (35, 43, 51, and 62 d after planting in Trial 1; 38, 50, 57, and 64 d in Trial 2; and 36, 47, 51, and 70 d in Trial 3) using the disease severity scale described by du Toit et al. (2005) with modifications. Briefly, each plant was rated on a seven-step scale, where: 0 = no symptoms (0% disease severity), 1 = 1% to 10% of the plant showing chlorosis and necrosis, 2 = 11% to 25% chlorosis and necrosis, 3 = 26% to 50% chlorosis and necrosis, 4 = 51% to 75% chlorosis and necrosis, 5 = 76% to 99% chlorosis and necrosis, and 6 = 100% chlorosis and necrosis. The scoring was performed on each plant, and the results were converted to percentage values (midpoints). Midpoints of the severity ratings were then averaged per pot. The disease rating 50 to 51 d after planting was determined to be optimum for disease development, before potential confounding with natural senescence. Thus, analysis of variance (ANOVA) was performed for the disease rating done 50 to 51 d after planting using PROC MIXED (SAS Institute Inc., Cary, NC) to obtain LSMeans estimates for each entry. However, because there was very little variability in the replication means of the non-infested data, they were excluded from the final analysis, and an arithmetic average was obtained instead. As a result, the ANOVA was based on a randomized block design with random blocks and entry as the fixed factor. The spinach accessions were ranked for disease severity in all

three trials and identified as highly susceptible or displaying some degree of resistance to *V. dahliae*. Accessions with less than 25% mean disease severity were considered partially resistant, whereas accessions with a mean disease severity greater than 75% were considered highly susceptible.

Re-isolation of the pathogen. All plants that grew in infested potting mix senesced after bolting was initiated. In addition, some plants in the non-infested potting mix senesced after bolting was triggered. Consequently, in 2006, isolations were made from plants in each of six replications of three spinach lines (three plants/replication) selected at random from both the infested and non-infested treatments. In 2007, isolations were completed for 50% of the plants of each genotype in the non-infested soil (nine plants/genotype) and from plants of 10 accessions selected randomly from the infested potting mix. The nine plants represented three replications (pots) of the most severely symptomatic plants of each genotype. Isolations were carried out using five petiole pieces/plant. In 2008, isolations were conducted on all 130 accessions by selecting the most symptomatic plant of each accession in the non-infested treatment and from 10 accessions selected randomly from the infested potting mix treatment. The isolations were performed by surface-disinfecting each leaf petiole for 1 min with 0.5% NaOCl, rinsing the petiole with deionized water, and then plating petiole sections on water agar. Each petiole was then examined microscopically (40 × to 500 × magnification) for development of conidiophores and/or microsclerotia of *V. dahliae*.

In the 2007 trial, aboveground biomass of the plants of each line from the infested and non-infested potting mix treatments was also measured. At 64 d after planting (16 May 2007), all plants were cut at the crown and the aboveground tissues dried in a forced-air oven at 60 °C. Dry biomass/plant was analyzed by ANOVA on SAS using PROC MIXED, and least square means were generated from the model. Additionally, the reduction in biomass of plants in infested soil vs. biomass of plants in non-infested soil was calculated/plant as follows:

$$\frac{[(\text{Dry plant biomass in non} - \text{infested soil}) - (\text{Dry plant biomass in infested soil})]}{(\text{Dry plant biomass in non} - \text{infested soil})}.$$

Pearson's correlation coefficient between aboveground biomass reduction and severity of *Verticillium* wilt was calculated using JMP software.

Freeze-blotter seed health assay

To assess the potential that seedborne inoculum may have contributed inoculum of *Verticillium* to infection of plants growing in non-infested potting mix, a freeze-blotter seed health assay for *V. dahliae* (du Toit et al., 2005) was performed on a small sample of seed from the original seed lots obtained from the USDA-NCRPIS for all accessions that had a disease severity rating greater than 0%

when evaluated 50 to 51 d after planting in Trial 2. Approximately 10 to 20 seeds (depending on how much seed was available) from each of the 59 genotypes were examined in 2007. In 2008, 20 seeds/accession of each of the 130 accessions were evaluated for the presence/absence of *V. dahliae*. Seed from three different lots with known *V. dahliae* infection levels of 84%, 45%, and 25% (du Toit et al., 2005) were included as control samples in the freeze-blotter seed health tests in 2007 and 2008.

Isolates recovered from seed were examined for colony morphology, colony pigmentation, conidiophore length and appearance, and microsclerotial production (Domsch et al., 1993; Smith, 1965). In addition, representative isolates of the different species putatively identified based on morphology were examined based on the internally transcribed spacer (ITS) region of ribosomal DNA (rDNA) (White et al., 1990). The region was amplified using the ITS primers ITS1 (5'-TCCGTAGGTGA ACCTGCGG-3') and ITS4 (5'-TCCTCCGC

TTATTGATATGC-3'). The amplified product was subsequently sequenced and, existing sequences in Genbank of various species of *Verticillium* were examined for similarities to the ITS rDNA sequences.

Results

Screening for resistance to *Verticillium* wilt. Symptoms of *Verticillium* wilt were initially observed 4 to 5 weeks after planting, after the plants had started to bolt. Symptoms started on the oldest leaves as interveinal chlorosis and progressed as the plants aged. Severity of interveinal chlorosis increased and spread to most leaves of each inoculated plant, and many leaves became necrotic and died. A wide range in disease severity values was observed 51, 50, and 51 d after planting when the plants were rated for disease severity in Trials 1, 2, and 3, respectively. *Verticillium* wilt severity ranged from 5.7% to 91.1%, 5.7% to 99.3%, and 5.5% to 93.8% in Trials 1, 2, and 3, respectively. No accession

appeared immune in any of the trials (data not shown; for complete data set, see Villarreal-Zeballos, 2007). In the non-infested soil treatment, most of the accessions (104 of 120 in Trial 1, 109 of 130 in Trial 2, and 126 of 130 in Trial 3) had low disease severity ratings (less than 20%) (data not shown).

ANOVAs for Trial 1 (2006) indicated statistically significant effects of spinach accession ($P < 0.0001$). Accessions with the lowest disease severity ratings were PI 193618, Ames 26243, PI 604789, PI 179591, PI 261789, PI 319220, and PI 286435 (Table 1). Seven accessions were rated as highly susceptible to *V. dahliae* (disease severity 75% or greater), including NSL 28217, PI 358251, PI 165560, PI 361127, PI 173972, NSL 101759, and PI 200882 (Table 1).

ANOVA for Trial 2 (2007) indicated statistically significant effects for spinach accession ($P < 0.0001$). Twenty-seven accessions were rated as resistant to *Verticillium* wilt (disease severity 25% or less). The lowest disease severity ratings were observed for PI

Table 1. Mean *Verticillium* wilt severity ratings (%) for a subset of the most resistant and most susceptible of 130 spinach accessions from the USDA-NCRPIS germplasm collection that were planted into potting medium infested or not infested with *Verticillium dahliae* in each of three greenhouse trials.^z

Spinach entry ^y	USDA accession no. ^x	Origin	Trial 1 (2006)			Trial 2 (2007)			Trial 3 (2008)		
			Infested ^w	Rank ^u	Non-infested ^t	Infested ^w	Rank ^u	Non-infested ^t	Infested ^w	Rank ^u	Non-infested ^t
45	PI 175931	Turkey	21.9	30	5.0	5.7	1	0.0	8.3	6	0.3
4	Ames 26243	China	7.1	2	5.0	10.6	2	0.0	9.0	8	0.0
25	PI 163309	India	16.7	12	6.4	11.7	3	0.3	20.2	34	0.0
75	PI 261789	France	8.9	5	6.2	12.3	4	0.0	14.3	16	0.0
104	PI 494751	Uzbekistan	18.5	17	5.0	14.4	5	0.0	33.3	83	0.0
19	NSL 6090	USA	46.4	93	5.0	14.9	7	0.0	13.2	12	0.0
47	PI 176371	Italy	27.6	44	5.0	15.3	8	7.0	16.6	21	0.9
74	PI 254565	Afghanistan	43.0	87	5.6	16.3	9	6.4	18.3	24	0.0
33	PI 169689	Turkey	37.1	70	5.0	17.0	10	0.8	28.1	61	0.0
22	NSL 6692	USA	56.6	107	5.0	18.5	11	0.0	19.1	29	1.5
93	PI 368825	Yugoslavia	19.3	19	5.0	18.6	12	0.0	32.7	81	0.0
32	PI 169688	Turkey	33.3	61	5.0	18.8	13	0.3	22.1	40	0.0
112	PI 604777	Japan	14.3	9	5.0	19.6	14	8.3	33.2	82	4.0
44	PI 175928	Turkey	22.6	34	5.0	19.6	15	0.3	20.1	33	0.0
48	PI 176372	Italy	50.1	100	5.0	20.0	17	1.3	15.3	17	0.6
81	PI 274056		47.7	98	15.6	61.7	115	13.0	18.4	26	6.7
63	PI 200882	Afghanistan	91.1	120	5.0	63.6	116	20.0	30.3	72	0.3
12	NSL 32678	USA	31.1	53	41.7	63.6	117	5.6	32.5	80	0.0
31	PI 169685	Turkey	35.7	67	5.0	65.8	118	10.7	22.5	42	0.3
39	PI 173972	India	88.6	118	47.2	66.5	119	40.3	80.9	127	8.0
8	NSL 101759	USA	90.6	119	43.0	66.7	120	74.7	61.9	120	47.5
54	PI 179508	Iraq	51.8	104	5.0	69.1	121	57.0	64.8	123	13.1
59	PI 181964	Syria	17.8	14	36.7	70.0	122	0.0	45.3	108	0.8
99	PI 419218	Hong Kong	22.2	32	5.0	71.6	123	9.3	53.8	115	0.3
71	PI 229731	Iran	57.8	108	5.0	74.8	125	52.7	40.4	100	4.2
107	PI 508504	S. Korea	63.1	110	15.2	75.8	126	7.5	33.9	84	0.7
65	PI 209645	Iran	36.5	68	28.7	78.0	127	79.5	64.7	122	3.9
103	PI 491261	Greece	18.1	15	5.0	84.0	128	44.0	43.8	104	0.3
120	PI 608762	Thailand	67.7	111	12.2	92.7	129	41.5	89.9	128	1.7
84	PI 274311	Pakistan	70.4	112	33.3	99.3	130	75.3	93.8	130	24.3
Tukey's ^v			71.0			58.3			47.4		

^zUSDA North Central Regional Plant Introduction Station (NCRPIS) accessions are sorted by ranking of severity ratings from Trial 2 (2007). Severity of *Verticillium* wilt was evaluated 50 to 52 d after planting using the following scale: 0 = no symptoms (0% disease severity), 1 = 1% to 10% of the plant showing chlorosis and necrosis, 2 = 11% to 25% chlorosis and necrosis, 3 = 26% to 50% chlorosis and necrosis, 4 = 51% to 75% chlorosis and necrosis, 5 = 76% to 99% chlorosis and necrosis, and 6 = plant dead. Disease scoring was based on each plant and the results converted to percentage values. The values were then averaged for all plants/pot (experimental unit). Treatments included spinach accessions (120, 130, and 130 evaluated in 2006, 2007, and 2008, respectively) and *V. dahliae* inoculation treatments (infested with 1 g colonized vermiculite/kg potting medium vs. non-infested soil with 1 g non-infested vermiculite/kg potting medium). The plants were incubated in a greenhouse at 25 °C under a 12-h photoperiod.

^yNumber assigned to each accession as received from the USDA-NCRPIS.

^xPI 494751, Ames 23666, PI 608713, and PI 604792 are *Spinacia turkestanica* accessions. All other accessions are *Spinacia oleracea*.

^wLSMeans were estimated for the infested treatment in the indicated year using a randomized complete block design.

^tTukey's test for significant difference to compare entries within the infested soil treatment ($P = 0.1$) in the indicated year.

^uRank: accession ranking based on severity of *Verticillium* wilt.

^vBecause the majority of the non-inoculated entries showed no measurable variability among the replications, they were not included in the statistical analysis of the data. An arithmetic average for each of the non-infested entries is presented in the indicated year instead.

175931, Ames 26243, PI 163309, PI 261789, and PI 494751 (Table 1). In contrast, nine accessions were rated as highly susceptible (75% or greater), including PI 181964, PI 181808, PI 419218, PI 229731, PI 508504, PI 209645, PI 491261, PI 608762, and PI 274311 (Table 1).

ANOVA for Trial 3 (2008) also indicated statistically significant effects of spinach accession ($P < 0.0001$). The lowest disease severity ratings (25% or less) were observed for Ames 26243, NSL 68263, PI 175595, PI 176770, PI 204736, PI 175931, and PI 175932 (Table 1). In contrast, four accessions were rated as highly susceptible (75% or greater), including PI 173962, PI 608762, PI 165560, and PI 274311 (Table 1).

Based on the rankings of the spinach accessions for disease severity across all three trials, four spinach accessions were consistently rated as partially resistant to *Verticillium* wilt (disease severity 25% or less): PI 175931, Ames 26243, PI 163309, and PI 261789 (Table 1; Fig. 1A). In contrast, 15 accessions were consistently located in the upper quartile for disease severity, i.e., highly susceptible to *Verticillium* wilt (Fig. 1B). When mean disease severity ratings from infested and non-infested treatments were analyzed for correlation between pairs of

years, there was a significant positive correlation in severity ratings between each pair of years: $r = 0.37$ ($P = 0.0418$) between Trials 1 and 2, $r = 0.35$ ($P = 0.0001$) between Trials 1 and 3, and $r = 0.63$ ($P = 0.0001$) between Trials 2 and 3 (Fig. 2).

In Trial 2 (2007), Lazio had the least severe *Verticillium* wilt symptoms of the 10 commercial spinach hybrids evaluated for resistance to *Verticillium* wilt (14.7%) followed by Viroflay (19.7%), Lion (20.9%), Avenger (22.2%), Polka (30.4%), Campania (31.0%), Califlay (34.5%), Dolphin (41.6%), Bolero (47.9%), and Resistoflay (55.0%). In Trial 3 (2008), Lazio again had the least severe *Verticillium* wilt of the commercial hybrids (12.9%) followed by Polka (16.0%), Avenger (21.0%), Viroflay (21.4%), Bolero (34.7%), Campania (35.4%), Lion (41.5%), Califlay (41.7%), Dolphin (44.4%), and Resistoflay (51.3%) (Table 2). Although male plants tended to senesce faster than female plants after initiation of bolting in the resistance screening trials, there was no apparent difference in overall disease severity ratings between male and female plants of the accessions and hybrids (data not shown).

In Trial 2 (2007), plants in infested potting mix appeared more stunted and more severely symptomatic than plants in non-infested potting mix. Thus, the impact of planting seed into infested vs. non-infested potting mix on spinach biomass was determined. Over 90% of the accessions showed a significant reduction in dry aerial biomass compared with the corresponding non-infested treatment (data not shown) with 95 of 130 accessions showing a biomass reduction of greater than 30% and 65 of the accessions showing a biomass reduction of greater than 50%. The mean plant biomass for plants in infested and non-infested potting mix was 0.65 and 1.26 g/plant, respectively. Additionally, there were significant differences among accessions for biomass of plants grown in infested potting mix ($P < 0.0001$). A significant positive correlation between biomass reduction and disease severity ($r = 0.31$; $P = 0.0078$) was observed (Fig. 3).

Conidiophores and/or microsclerotia of *V. dahliae* were observed on 100% of the petioles sampled from plants grown in infested potting medium. For accessions planted in non-infested medium, conidiophores and/or microsclerotia of *V. dahliae* were observed on 45% of the accessions (59 of 130) in Trial 2 and 7% of the accessions (nine of 130) in Trial 3. The incidences of infection among the 59 accessions varied from 11% to 100% (i.e., one to nine out of nine plants/entry from which isolations were carried out developed conidiophores and/or microsclerotia of *V. dahliae*). A majority of the infected accessions had low incidences of *V. dahliae* infection in the petioles (5% to 55% of the petioles/accession). However, five accessions had high incidences of *V. dahliae* infection in the petioles (60% to 100% of the petioles/accession).

Freeze-blotter seed health assay. The seed health assay demonstrated that *V. dahliae* was present on seeds of 34% (20 of 59) of the accessions for which subsamples of seed were assayed in Trial 2 (data not shown). In Trial 3, when seed assays were performed for all 130 accessions, 16% (21 of 130) of the USDA seed lots were infested or infected with *Verticillium* spp. Although a *Verticillium* sp. was recovered from 34% of the accessions in Trial 2, the infested or infected seed appear to have a small effect on disease severity ratings. For example, of the 20 accessions with detectable *V. dahliae* on the seed assayed, mean disease severity ratings for plants in the non-infested potting mix were low (less than 10%) for 17 accessions and moderate (10% to 30%) for three accessions. In Trial 3, only three accessions (PI163309, PI229731, and PI604785) of 21 on which conidiophores/microsclerotia of *verticillium* were observed on the seed health assay had fungal isolates identified specifically as *V. dahliae* based on ITS sequence comparisons. All three of these accessions had very low (less than 5.0%) disease severity ratings in non-infested potting mix.

Isolates of *V. dahliae*, *V. tricornis*, and *G. nigrescens* were recovered from USDA

Fig. 1. USDA *Spinacia oleracea* accessions Ames 26243 (A), showing a resistant reaction to infection by *Verticillium dahliae* (mean disease severity rating 10.6% in Trial 2, completed in 2007), and NSL 32678 (B), showing a susceptible reaction to infection by *Verticillium dahliae* (mean disease severity 63.6% or less in Trial 2). Plants were inoculated by sowing seed into potting medium infested with *V. dahliae*, as described in the text.

Fig. 2. Correlation and density ellipsis using a 95% confidence interval between mean severity ratings for *Verticillium* wilt of 130 USDA spinach accessions screened for resistance to *Verticillium dahliae* in Trials 2 and 3 completed in 2007 and 2008, respectively ($r = 0.63$; $P < 0.0001$).

Table 2. Mean Verticillium wilt severity ratings (%) for 10 commercial spinach hybrids planted into potting medium that was infested or not infested with *Verticillium dahliae* in each of two greenhouse trials.^z

Spinach entry	Commercial hybrid ^y	Seed company	Trial 2 (2007)		Trial 3 (2008)	
			Infested ^x	Non-infested ^y	Infested ^x	Non-infested ^y
121	Viroflay	Chriseed	19.7	0.3	21.4	1.7
122	Resistoflay	Chriseed	55.0	0.0	51.3	2.4
123	Califlay	Chriseed	34.5	0.6	41.7	0.3
124	Polka	Seminis	30.4	0.0	16.0	0.8
125	Bolero	Seminis	47.9	0.0	34.7	1.1
126	Campania	Pop Vriend	31.0	0.6	35.4	2.8
127	Dolphin	Rijk Zwaan	41.6	0.0	44.4	2.7
128	Avenger	Seminis	22.2	5.8	21.0	0.3
129	Lion	Rijk Zwaan	20.9	0.0	41.5	0.3
130	Lazio	Pop Vriend	14.7	0.0	12.9	0.0
	Tukey's ^w		58.3		47.4	

^zSeverity of Verticillium wilt was evaluated 50 to 52 d after planting using the following scale: 0 = no symptoms (0% disease severity), 1 = 1% to 10% of the plant showing chlorosis and necrosis, 2 = 11% to 25% chlorosis and necrosis, 3 = 26% to 50% chlorosis and necrosis, 4 = 51% to 75% chlorosis and necrosis, 5 = 76% to 99% chlorosis and necrosis, and 6 = plant dead. Disease scoring was based on each plant and the results converted to percentage values. The values were then averaged for all plants/pot (experimental unit). Treatments included spinach accessions (120, 130, and 130 evaluated in 2006, 2007, and 2008, respectively) and *V. dahliae* inoculation treatments (infested with 1 g colonized vermiculite/kg potting medium vs. non-infested soil with 1 g non-infested vermiculite/kg potting medium). The plants were incubated in a greenhouse at 25 °C under a 12-h photoperiod.

^yTen commercial spinach hybrids, that serve as a set of differentials for race identification of *Peronospora farinosa* f. sp. *spinaciae*, were included in Trial 2 (2007) and Trial 3 (2008), respectively.

^xLSMeans were estimated for the infested treatment in the indicated year using a randomized complete block design.

^wTukey's minimum significant difference ($P = 0.10$).

^yBecause the majority of the non-inoculated entries showed no measurable variability among the replications, they were not included in the formal statistical analysis of the data. An arithmetic average for each of the non-infested entries is presented for the indicated year instead.

Fig. 3. Correlation and density ellipsis using a 95% confidence interval between mean severity ratings for Verticillium wilt and reduction in aerial biomass of plants inoculated with *Verticillium dahliae* compared with non-inoculated plants of 130 spinach accessions screened for resistance in 2007 ($r = 0.31$; $P = 0.0078$).

spinach accessions based on morphological examination of the cultures. Representative isolates of each of the species were subsequently examined for species identification based on the DNA sequence of the ITS region. The representative isolates of *V. dahliae* (isolate VD4), *V. tricorpus* (isolate JD6), and *G. nigrescens* (isolate LD10) (Zare et al., 2007) were confirmed as the respective species based on a BLAST match of greater than

99% with ITS sequences available for these species in Genbank. Subsequent tests on spinach and several other hosts indicated that only the isolates of *V. dahliae* were pathogenic on spinach plants (Iglesias-Garcia, 2011).

Discussion

The screening of 120 to 130 USDA spinach accessions in three greenhouse trials indi-

cated that no accession appeared immune to infection by *V. dahliae*, and it is unlikely that qualitative or major resistance to *V. dahliae* was present in any of the accessions tested based on the wide range in severity of symptoms observed. However, substantial differences in disease severity ratings were observed among the accessions. Low disease severity ratings were observed consistently on five entries, namely PI 175931, Ames 26243, PI 163309, PI 261789, and PI 494751. To our knowledge, no previous studies have screened for resistance to Verticillium wilt in spinach. However, Laguna (2000) screened *Spinacia* accessions for reactions to the soilborne pathogen *Fusarium oxysporum* f. sp. *spinaciae*. When comparing the accessions putatively identified as partially resistant to Verticillium wilt in this study with accessions identified as resistant to Fusarium wilt by Laguna (2000) in a greenhouse screening effort, there was no correlation between disease reactions to the two vascular wilt pathogens (data not shown).

In this study, evaluation of resistance to Verticillium wilt was based on visual observation of severity of disease symptoms on plants and recovery of the pathogen from infected plant (petioles). Symptoms appeared ≈ 4 to 5 weeks after planting, when the plants started to bolt or had already bolted. These results coincided with those of du Toit et al. (2005) who reported that symptoms of Verticillium wilt on spinach were not observed until after the initiation of bolting, and Koike et al. (1994) who mentioned that symptoms on cauliflower were only observed after initiation of flowering. Approximately 50 d after planting, expression of symptoms of Verticillium wilt peaked, whereas natural senescence was minimal for most of the spinach accessions evaluated. By 57 to 64 d after planting, senescence had occurred for most accessions, when yellowing of the lowest leaves associated with natural senescence was difficult to separate from symptoms caused by *V. dahliae*. Additionally, differences were observed in severity of Verticillium wilt symptoms among plants within pots. This may have been the result of genetic variability within the accessions, the phenological stage of each plant at the time of rating, and/or variation in pathogen colonization of the inoculum substrate.

Two hypotheses might explain why *V. dahliae* was isolated from plants growing in non-infested potting medium. One is that *V. dahliae* infection occurred as a result of seed transmission from infested seed obtained from the USDA-NCRPIS. The other is that cross contamination may have occurred during the trials. Seed transmission of *V. dahliae* in spinach is now well documented (du Toit et al., 2005). To test for the possibility of infested seed serving as the source of inoculum for seed planted into non-infested potting medium, a freeze-blotter seed health assay was conducted using the limited samples of seed available for 59 of the spinach accessions. Results showed that seed samples of 34% of the accessions (20 of 59 assayed) were infested with *Verticillium* spp., i.e.,

seedborne inoculum and seed transmission were most likely involved in generating symptoms of Verticillium wilt in plants growing in non-infested potting medium. For some accessions in which plants developed symptoms of Verticillium wilt in the non-infested treatment, *V. dahliae* was not detected on the limited number of seed assayed. This suggests that either cross contamination may have occurred or the very limited number of seeds available to assay for each accession was too minimal for accurate assessment of the percentage of seed infected or infested for each accession, because infection levels as low as 0.25% have been detected on seed lots (du Toit et al., 2005).

Screening for resistance to *V. dahliae* in spinach may help develop more effective strategies to manage Verticillium wilt. Based on this study in which spinach accessions with putative partial resistance to *V. dahliae* were identified, the mechanism(s) of resistance could be in the form of reduced infection rates, reduced systemic movement of the pathogen in plants, reduced impact of infection on plant biomass, and/or reduced rate of seed infection and/or transmission. In this effort to quantify resistance to Verticillium wilt in spinach, disease severity ratings were confounded by several factors: 1) natural senescence, which occurred when the plants had flowered and set seed, mimicking the development of Verticillium wilt symptoms; 2) the spinach accessions differed in the minimum daylength required to induce bolting, which affected the timing of the onset of Verticillium wilt symptom development; 3) the presence of *V. dahliae* on seeds of some of the accessions highlighted the importance of including both inoculated and non-inoculated treatments in resistance evaluations to accommodate the fact that this pathogen is prevalent in commercial and Genebank seed collections; and 4) there was significant variation in severity of Verticillium wilt symptoms among plants of a given accession. Regarding disease evaluations, all genotypes were bolting by the time disease severity ratings were initiated, which was 1 week after the first genotypes to bolt were observed. Bolting had thus been initiated in most genotypes within a few days and all genotypes within a 1-week period. Thus, the disease ratings at the four rating periods were done and all disease severity ratings used in the analysis presented were 50 to 51 d after planting.

Regardless of the complicating variables outlined, *V. dahliae* clearly accelerated the rate of senescence of spinach plants as determined by comparing disease ratings in infested vs. non-infested potting medium. Furthermore, overall plant vigor, measured as biomass, was significantly reduced on plants growing in *V. dahliae*-infested vs. non-infested medium. This biomass reduction of infected plants is in agreement with what has been observed for other crops (Koike et al., 1994; Vallad and Subbarao, 2008; Xiao and Subbarao, 1996). A statistically significant positive correlation between reduction in biomass and

severity of Verticillium wilt symptoms was observed; however, this correlation was not strong enough to predict the resistance response of any particular accession to Verticillium wilt based on biomass reduction of plants growing in infested potting medium.

Conclusions

In this study, we reported the identification of accessions with different levels of quantitative resistance to Verticillium wilt, although we did not observe evidence of qualitative resistance in the screened accessions from the USDA germplasm collection or among 10 commercial spinach hybrids tested. *V. dahliae* infections significantly reduced plant biomass, and biomass reduction was significantly positively correlated with severity of Verticillium wilt among the accessions tested. These results are valuable for characterizing potential genetic variability within spinach germplasm for the reaction to *V. dahliae* inoculation. Furthermore, these findings are critical for the identification and potential deployment of resistant sources of spinach to Verticillium wilt.

Literature Cited

- Atallah, Z.K., K. Maruthachalam, L.J. du Toit, S.T. Koike, R.M. Davis, S.J. Klosterman, R.J. Hayes, and K.V. Subbarao. 2010. Population analyses of the vascular plant pathogen *Verticillium dahliae* detect recombination and transcontinental gene flow. *Fungal Genet. Biol.* 47:416–422.
- Atibalentja, N. and D.M. Eastburn. 1998. *Verticillium dahliae* resistance in horseradish germplasm from the University of Illinois collection. *Plant Dis.* 82:176–180.
- Bejarano-Alcazar, J., A.J. Termorshuizen, and R.M. Jimenez-Diaz. 1999. Single-site root inoculations on eggplant with microsclerotia of *Verticillium dahliae*. *Phytoparasitica* 27: 279–289.
- Brandenberger, L.P., J.C. Correll, and T.E. Morelock. 1991. Identification and cultivar reactions to a new race (race 4) of *Peronospora farinosa* f. sp. *spinaciae* on spinach in the United States. *Plant Dis.* 75:630–634.
- Cirulli, M., F. Ciccarese, and M. Amenduni. 1994. Evaluation of Italian clones of artichoke for resistance to *Verticillium dahliae*. *Plant Dis.* 78:680–682.
- Correll, J.C., B.H. Bluhm, C. Feng, K. Lamour, L.J. du Toit, and S.T. Koike. 2011. Spinach: Better management of downy mildew and white rust through genomics. *Eur. J. Plant Pathol.* 129: 193–205.
- Correll, J.C., T.E. Morelock, M.C. Black, S. Koike, L.P. Brandenberger, and F.J. Dainello. 1994. Economically important diseases of spinach. *Plant Dis.* 78:653–660.
- Daehnfeltd, L. 2007. Danish production of vegetable seed—Is it only spinach? 18 June 2007. <<http://www.daehnfeltd.com/index.asp?NyhedsId=38>>.
- Diwan, N., R. Fluhr, Y. Eshed, D. Zamir, and S.D. Tanksley. 1999. Mapping of *Ve* in tomato: A gene conferring resistance to the broad-spectrum pathogen, *Verticillium dahliae* race 1. *Theor. Appl. Genet.* 98:315–319.
- Domsch, K.J., W. Gams, and T.H. Anderson. 1993. *Verticillium* Nees ex Link 1824, p. 828–845. In:

- Compendium of soil fungi. Vol. 1. IHW-Verlag, Eching, Germany.
- du Toit, L.J., M.L. Derie, and P. Hernandez-Perez. 2005. Verticillium wilt in spinach seed production. *Plant Dis.* 89:4–11.
- Foss, C.R. and L.J. Jones. 2005. Crop profile for spinach seed in Washington. U.S. Dept. Agric. National Pest Management Centers.
- Grogan, R.G., N. Ioannou, R.W. Schneider, M.A. Sall, and K.A. Kimble. 1979. Verticillium wilt on resistant tomato cultivars in California: Virulence of isolates from plants and soil and relationship of inoculum density to disease incidence. *Phytopathology* 69:1176–1180.
- Harrison, N.A. and C.H. Beckman. 1982. Time/space relationships of colonizations and host response in wilt-resistant and wilt-susceptible cotton (*Gossypium*) cultivars inoculated with *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*. *Physiol. Plant Pathol.* 21:193–207.
- Hayes, R.J., L.K. McHale, G.E. Vallad, M.J. Truco, R.W. Michelmore, S.J. Klosterman, K. Maruthachalam, and K.V. Subbarao. 2011. The inheritance of resistance to Verticillium wilt caused by race 1 isolates of *Verticillium dahliae* in the lettuce cultivar La Brillante. *Theor. Appl. Genet.* 123:509–517.
- Iglesias-Garcia, A.M. 2011. Pathogenicity, virulence, and molecular characterization of *Verticillium* species from spinach and other hosts. MS thesis, University of Arkansas, Fayetteville, AR.
- Jansky, S., D.I. Rouse, and P.J. Kauth. 2004. Inheritance of resistance to *Verticillium dahliae* in diploid interspecific hybrids. *Plant Dis.* 88: 1075–1078.
- Klisiewicz, J.M. 1981. Reaction of sunflower and safflower germplasm to *Verticillium dahliae*. *Plant Dis.* 65:237–239.
- Koike, S.T., P. Gladders, and A.O. Paulus. 2007. Vegetable diseases. A color handbook. Academic Press, San Diego, CA.
- Koike, S.T., K.V. Subbarao, R.M. Davis, T.R. Gordon, and J.C. Hubbard. 1994. Verticillium wilt of cauliflower in California. *Plant Dis.* 78:1116–1121.
- Korolev, N. and T. Katan. 1997. Improved medium for selecting nitrate-nonutilizing (*nit*) mutants of *Verticillium dahliae*. *Phytopathology* 87:1067–1070.
- Laguna, R. 2000. Screening for disease resistance to Fusarium wilt of spinach. MS thesis, University of Arkansas, Fayetteville, AR.
- Metzger, J.D. and J.A.D. Zeveaart. 1985. *Spinacia oleracea*, p. 384–392. In: Halevy, A.H. (ed.). CRC handbook of flowering. Vol. IV. CRC Press, Boca Raton, FL.
- Morelock, T.E. and J.C. Correll. 2008. Spinach breeding, p. 183–212. In: Prohens, J. and F. Nuez (eds.). Vegetables I. J Springer, New York, NY.
- Mou, B., S.T. Koike, and L.J. du Toit. 2008. Screening for resistance to leaf spot diseases of spinach. *HortScience* 43:1706–1710.
- Nicot, P. C. and D. I. Rouse. 1987. Relationship between soil inoculum density of *Verticillium dahliae* and systemic colonization of potato stems in commercial fields over time. *Phytopathology* 77:1346–1355.
- Resende, M.L.V., J. Flood, J.D. Ramsden, M.G. Rowan, M.H. Beale, and R.M. Cooper. 1996. Novel phytoalexins including elemental sulphur in the resistance of cocoa (*Theobroma cacao* L.) to Verticillium wilt (*Verticillium dahliae* Kleb.). *Physiol. Mol. Plant Pathol.* 48:347–359.
- Sackston, W.E. and F.S. Sedun. 1982. Verticillium wilt of spinach: A useful experimental system. *Can. J. Plant Pathol.* 4:310.

- Schaible, L., O.S. Cannon, and V. Waddoups. 1951. Inheritance of resistance to *Verticillium* wilt in a tomato cross. *Phytopathology* 41:986–990.
- Simko, I., S. Costanzo, K.G. Haynes, B.J. Christ, and R.W. Jones. 2004. Linkage disequilibrium mapping of a *Verticillium dahliae* resistance quantitative trait locus in tetraploid potato (*Solanum tuberosum*) through a candidate gene approach. *Theor. Appl. Genet.* 108:217–224.
- Smith, H.C. 1965. The morphology of *Verticillium albo-atrum*, *V. dahliae*, and *V. tricornutum*. *N. Z. J. Agr. Res.* 8:450–478.
- Snyder, W.C. and S. Wilhelm. 1962. Seed transmission of *Verticillium* wilt of spinach. *Phytopathology* 52:365.
- Vallad, G.E. and K.V. Subbarao. 2008. Colonization of resistant and susceptible lettuce cultivars by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. *Phytopathology* 98:871–885.
- van der Spek, J. 1972. Internal carriage of *Verticillium dahliae* by seeds and its consequence. *Meded. Fac. Landbouwwet. Rijksuniv. Gent.* 37:567–573.
- van der Spek, J. 1973. Seed transmission of *Verticillium dahliae*. *Meded. Fac. Landbouwwet. Rijksuniv. Gent.* 38:1427–1434.
- Villarroel-Zeballos, M.I. 2007. Genetic, molecular, and virulence diversity of *Verticillium dahliae* and screening for disease resistance in spinach germplasm. MS thesis, University of Arkansas, Fayetteville, AR.
- Villarroel-Zeballos, M.I., L.J. du Toit, and J.C. Correll. 2006. Genetic and molecular characterization of *Verticillium dahliae* from spinach and screening for disease resistance. *Phytopathology* 96:S118.
- Villarroel-Zeballos, M.I., L.J. du Toit, and J.C. Correll. 2007. Screening for resistance to *Verticillium dahliae* in spinach. *Phytopathology* 97:S118.
- Villarroel-Zeballos, M.I., L.J. du Toit, and J.C. Correll. 2008. Screening for resistance to *Verticillium dahliae* in spinach. *Phytopathology* 98:S163–S164.
- White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. In: Innes, M.A., D.H. Gelfand, J.S. Sninsky, and T.J. White (eds.). *PCR protocols*. Academic Press, London, UK.
- Wilhelm, S. 1955. *Verticillium* wilt of strawberry with special references to resistance. *Phytopathology* 45:387–391.
- Xiao, C.L. and K.V. Subbarao. 1996. Relationships between *Verticillium dahliae* inoculum density, wilt incidence and severity, and growth of cauliflower. *Phytopathology* 86:S86.
- Zare, R., W. Gams, M. Starink-Willemse, and R.C. Summerbell. 2007. *Gibellulopsis*, a suitable genus for *Verticillium nigrescens*, and *Muscardinus*, a new genus for *V. theobromae*. *Nova Hedwigia* 85:463–489.