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Verticillium Wilt in Spinach Seed Production

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ABSTRACT

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There are no previous reports of Verticillium wilt in fresh and processing spinach (*Spinacia oleracea*) crops in the United States. In 2002, a hybrid spinach seed crop in the Pacific Northwest developed late-season wilt symptoms. Assays of the harvested seed and stock seed of the male and female parents revealed 59.5, 44.0, and 1.5%, respectively, were infected with *Verticillium dahliae*. Assays of 13 stock or commercial seed lots grown in 2002 and 62 commercial lots harvested in 2003 in Denmark, Holland, New Zealand, and the United States revealed the prevalence of *Verticillium* spp. in commercial spinach seed. Sixty-eight lots (89%) were infected with *Verticillium* spp. at incidences ranging from 0.3 to 84.8%. Five spinach seed isolates of *V. dahliae* were pathogenic on each of three spinach cultivars by root-dip inoculation. *V. dahliae* was detected on 26.4% of the seed from 7 of 11 inoculated plants but on none of the seed from 6 control plants, demonstrating systemic movement of *V. dahliae*. Seed-to-seed transmission was also demonstrated by planting naturally infected seed lots. This is the first report of Verticillium wilt of spinach in the primary region of spinach seed production in the United States.

Additional keywords: *Fusarium oxysporum* f. sp. *spinaciae*, premature senescence

Spinach (*Spinacia oleracea* L.) seed crops grown on 1,200 to 1,600 ha in the Pacific Northwest produce up to 50% of the U.S. and world supply of spinach seed annually (9). Fusarium wilt of spinach, caused by *Fusarium oxysporum* Schlecht.:Fr. f. sp. *spinaciae* (Sherb.) W.C. Snyder & H.H. Hans (4), is endemic in this region and necessitates crop rotations of 6 to 15 years for seed production, depending on the susceptibility of the parent lines (9). In contrast, the only report of *Verticillium dahliae* Kleb. on spinach in the United States was by Snyder and Wilhelm in New York in 1962 (15), who documented seed transmission. In Holland, van der Spek (18,19) demonstrated that spinach and sugar beet seed sown in soil infested with *V. dahliae* and grown to flowering produced infected seed. Furthermore, van der Spek (19) showed that *V. dahliae* could be introduced into soil by planting infested spinach seed, and that soil microflora may influence seed transmission and subsequent development of wilt symptoms. In

Canada, Sackston and Sedun (13) achieved almost 100% infection of seed harvested from spinach plants inoculated with a *Verticillium* sp.

Despite these reports of *Verticillium* as a pathogen of spinach, Verticillium wilt has not been observed in fresh or processing spinach crops in the United States (4), even when spinach seed is planted into fields heavily infested with *V. dahliae* from previous crops (S. T. Koike, *personal communication*). In 2002, a hybrid seed crop grown in western Washington developed late-season wilt symptoms, despite a 20-year rotation out of spinach in that field. Samples of the male and female stock seed (seed of the parent lines used to grow a hybrid seed crop) and the harvested seed were assayed for *Fusarium*, but the predominant fungus detected was *Verticillium*. Similarly, a high incidence of *Verticillium* was detected on female stock seed of a hybrid seed crop grown in western Oregon in 2002 that developed late-season wilt symptoms (L. J. du Toit, *unpublished data*).

The objectives of this research were to assess: (i) the prevalence of *Verticillium* in commercial spinach seed lots grown in the primary regions of the world in which spinach seed is produced, (ii) the pathogenicity of spinach seed isolates of *V. dahliae* to spinach, and (iii) seed-to-seed transmission of *Verticillium* from naturally

infected spinach seed. Preliminary results have been presented (6,7).

MATERIALS AND METHODS

Detection of *Verticillium* in commercial spinach seed. Samples were collected from male and female stock seed lots and the harvested seed of a proprietary hybrid spinach seed crop grown in northwestern Washington in 2002 that displayed late-season wilt symptoms (Cultivar F; Table 1). Samples were collected from 11 additional stock or harvested seed lots grown in Washington, Oregon, or the European Union (EU) in 2001 or 2002 (Table 1).

Each seed lot was assayed for *Verticillium* using a general freeze-blotter assay modified from that described by Derie et al. (5) for detection of *Phoma lingam* on crucifer seed. Four hundred seed per lot were either rinsed under running deionized water for 60 min (non-surface-sterilized assay) or soaked in 1.2% NaOCl for 60 s and triple-rinsed in sterile deionized water (surface-sterilized assay). The seed were then dried in a laminar flow hood on sterile paper toweling. One sterile Steel blue germination blotter (8.25-cm-diameter; Anchor Paper Co., St. Paul, MN) was placed within each sterile 10-cm-diameter plastic petri plate and moistened with 4 ml of sterile deionized water. Using forceps that were flame-sterilized between seed, the seed were transferred onto the blotters (20 seed per plate) and the plates sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI). The plates were incubated in the dark at approximately 24°C for 24 h to allow the seed to imbibe. The imbibed seed were then placed at -20°C for 24 h to kill the embryo and then transferred to an incubator (Model I30BLL, Percival Scientific, Perry, IA) set at 24°C with a 12 h/12 h day/night cycle (near-UV and cool-white fluorescent light by day) for 12 days. The seed were examined microscopically (×8 to ×100 magnification) approximately 5, 10, and 14 days after plating (Fig. 1). *Verticillium* and *Fusarium* cultures were identified to genus based on the presence of microsclerotia and/or conidiophore and conidial characteristics. A majority of the *Verticillium* isolates (>90%) resembled *V. dahliae*, as the bases of the conidiophores did not darken in color with maturity and

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the isolates formed microsclerotia on pericarps of the seed and on blotters (14).

To determine whether *V. dahliae* might be eradicated from spinach seed by surface-sterilization with NaOCl, seed of one cultivar on which *Verticillium* was detected at an incidence of 8.25% (Cultivar I; Table 1) was sterilized with 1.2% NaOCl for 10, 20, 30, or 40 min and triple-rinsed in sterile deionized water. The seed was then dried and assayed for *Verticillium* using the procedure described above.

In the winter/spring of 2003–04, an additional 62 spinach seed samples produced by six commercial seed companies from seed crops grown in 2003 in Denmark, Holland, New Zealand, or the United States (Table 2) were assayed using the surface-sterilization procedure described above. Analyses of variance (ANOVAs) and means comparisons were calculated by country and seed company for incidence of *Verticillium*, using seed lots as replications (weighted to account for different numbers of seed lots per country and company). Log transformations were used to generate normal residuals and homogeneous variances for parametric statistical analyses.

Pathogenicity of spinach seed isolates of *V. dahliae*. In the winter of 2002–03, five isolates of *V. dahliae* obtained from five spinach seed lots were tested in the greenhouse for pathogenicity on each of three spinach cultivars: J (a smooth leaf, medium-long standing, proprietary female inbred susceptible to Fusarium wilt), E (a smooth leaf, medium standing, proprietary female inbred partially resistant to Fusarium wilt; the same cultivar listed in Table

1), and ‘Winter Bloomsdale’ (a true Savoy, medium-long standing, open-pollinated cultivar susceptible to Fusarium wilt) (Table 3). A sample of 400 seed of each cultivar was assayed for *Verticillium* using the freeze-blotter assay described above. Isolates 1 to 4 of *V. dahliae* were from seed lots harvested from seed crops grown in the United States in 2001 or 2002, and isolate 5 was from a stock seed lot grown in Holland in 2001. A randomized complete block design with four replications was used. Seed of each lot was planted into RediEarth potting mix (W.R. Grace & Co., Ajax, ON, Canada) in a 76-cell plastic flat. For each replication of each isolate, the root plug of one transplant of each cultivar was wounded by manually tearing the roots. The root plug was then immersed in a spore suspension (approximately 10^6 conidia per ml) of the isolate for 60 s, and transplanted into Sunshine Mix no. 1 potting mix (Sungro Horticulture, Seba Beach, AB, Canada). For the noninoculated control treatment of each cultivar, the root plug was dipped in sterile deionized water. At the time of inoculation, transplants of Cultivar J were 2 weeks old and those of Cultivar E and ‘Winter Bloomsdale’ were 9 weeks old. The plants were placed on a greenhouse bench with supplemental lighting (approximately 100 $\mu\text{mol}/\text{m}^2/\text{s}$) provided by overhead lights for 8 h/day and monitored for wilt symptoms. Five weeks after inoculation, the duration of supplemental lighting was increased to 14 h/day to initiate bolting (conversion from vegetative to reproductive growth). Based on preliminary investigations into

the pathogenicity of *V. dahliae* on spinach, severity of symptoms was rated 25 and 49 days after inoculation on a scale of 0 to 4, where: 0 = no symptoms, 1 = red discoloration of the stem base, 2 = interveinal chlorosis of the oldest leaves, 3 = stunting and progression of interveinal chlorosis and necrosis up the plant, and 4 = plant dead.

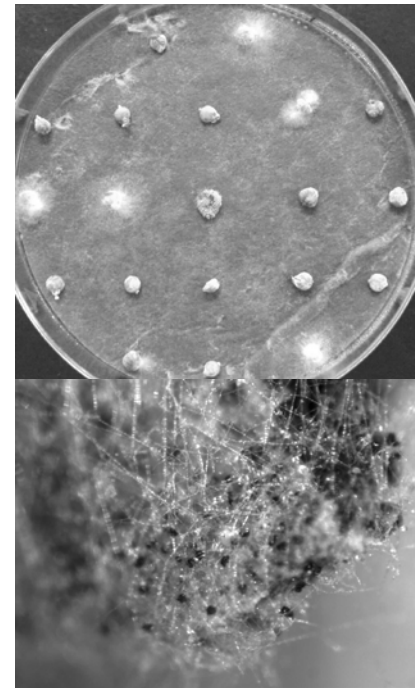


Fig. 1. Freeze-blotter assay for *Verticillium* on spinach seed (top), with microsclerotia and mycelium of *V. dahliae* on the pericarp (bottom).

Table 1. Incidence of *Verticillium* and *Fusarium* detected on spinach seed produced or planted in the United States (US) or the European Union (EU) in 2002

Cv. ^v	Seed company ^v	Fungicide seed treatment	Stock or harvested seed ^w	Region seed was grown ^x	Seed treatment ^y	% of 400 seed ^z	
						<i>Verticillium</i>	<i>Fusarium</i>
A	1	None	Harvested	EU	Rinsed	17.00	1.00
	2	Thiram	Harvested	EU	Rinsed	0.75	0.00
B	2	Unknown	Harvested	EU	Rinsed	6.25	0.00
			Harvested	EU	Rinsed	3.00	0.00
D	3	None	Harvested	US (WA)	Rinsed	0.00	0.00
E	3	None	Female stock	US (WA)	Rinsed	0.00	0.00
F	4	Unknown	Female stock	EU	Rinsed + sterilized	1.50	0.00
			Male stock	EU	Rinsed + sterilized	44.00	27.75
			Harvested	US (WA)	Rinsed	59.50	3.75
G	4	Unknown	Female stock	EU	Rinsed + sterilized	11.25	0.00
			Male stock	EU	Rinsed + sterilized	6.50	0.00
			Harvested	US (WA)	Rinsed	74.75	0.00
			Female stock	US (OR)	Rinsed	35.25	0.00
H	5	Unknown	Female stock	US (OR)	Rinsed	35.25	0.00
					Sterilized	26.25	0.00
					Rinsed	8.25	–
					Sterilized (10 min)	1.75	–
					Sterilized (20 min)	0.75	–
					Sterilized (30 min)	0.75	–
I	6	None	Harvested	US (WA)	Rinsed	8.25	–
					Sterilized (10 min)	1.75	–
					Sterilized (20 min)	0.75	–
					Sterilized (30 min)	0.75	–
					Sterilized (40 min)	0.50	–

^v Cultivar (cv.) and seed company identities are coded for proprietary purposes. All cultivars are smooth leaf hybrids, except ‘D’ which is a true Savoy, open pollinated cultivar. All cultivars are grown for fresh markets (baby leaf and bunching markets), except ‘D’ which is for home garden markets. Cultivars F and G are also grown for processing markets. All cultivars are medium to long standing.

^w Male and female stock seed = seed of male and female parent lines, respectively, used to produce a hybrid seed crop. Harvested seed = seed harvested from a seed crop for commercial markets.

^x Seed produced in the EU (Denmark or Holland) or US (western Washington [WA] or Oregon [OR]).

^y Rinsed = seed washed under running deionized water for 60 min, dried, and plated onto damp sterile blotters in petri plates. Surface-sterilized = seed soaked in 1.2% NaOCl for 60 s (unless stated otherwise), triple-rinsed in sterile deionized water, dried, and plated as described for the rinsed seed.

^z Seed examined microscopically ($\times 8$ to $\times 100$ magnification) approximately 5, 10, and 14 days after plating. – = incidence not recorded.

Forty-nine days after inoculation, the roots and stems of each plant in the first replication of the trial were washed, cut to assess vascular discoloration, incubated for 5 days on paper toweling moistened with 0.1% NaOCl, and examined microscopi-

cally for the presence of *V. dahliae*. Prior to placing the plants on damp paper toweling, root and stem sections (approximately 5 mm³) were cut from each plant, surface-sterilized for 5 min, triple-rinsed in sterile deionized water, dried on sterile paper

toweling, and plated onto Difco potato dextrose agar (PDA; Becton, Dickinson and Co., Sparks, MD) acidified with 4 drops of 85% lactic acid per liter of agar. Seed was harvested from female plants that survived and set seed. The harvested seed was assayed for *Verticillium* without surface-sterilization, as described above. Because incubation of plants in the moist chamber gave the same results as isolations onto PDA for the first replication, the moist chamber incubation procedure was repeated 56 days after inoculation for each plant in the second replication and 63 days after inoculation for each plant in the third and fourth replications.

The pathogenicity test was repeated in the winter of 2003–04 with slight modifications. The root plugs of 6-week-old seedlings of cultivars J, E, and ‘Winter Bloomsdale’ were inoculated with spore suspensions of the five isolates of *V. dahliae* used in the previous assay, or soaked in sterile deionized water (control treatment) as described above. Two weeks after inoculation, the duration of greenhouse lighting was increased from 8 to 14 h/day to initiate bolting. Plants were rated for incidence and severity of wilt symptoms 22 and 53 days after inoculation. The root system of each plant was washed 59, 67, and 72 days after inoculation for the first, second, and third and fourth replications, respectively. A new pair of latex gloves

Table 2. Incidence of *Verticillium* detected on 62 spinach seed samples produced by five seed companies in four countries in 2003

Country or seed company ^w	Seed lots infected/ lots assayed ^x	Incidence (%) of seedborne <i>Verticillium</i> ^y		
		Mean ^z	Range	Median
Country				
Denmark	25/27	8.78 a	0.00–84.75	3.25
Netherlands	6/6	26.33 ab	2.00–83.00	22.00
New Zealand	0/1	0.00 a	0.00	0.00
U.S.	26/28	26.88 b	0.00–75.25	25.38
Company				
1	22/23	28.66 b	0.00–75.25	24.50
2	4/5	18.05 a	0.00–84.75	0.25
3	22/24	5.89 a	0.00–19.00	3.13
4	4/4	34.25 b	2.00–83.00	26.00
5	2/3	9.25 a	0.00–25.75	2.00
6	3/3	30.75 b	28.00–36.00	28.25
Total	57/62	18.51	0.00–84.75	8.00

^w Identities of seed companies are coded for proprietary purposes and do not necessarily correspond to codes used for companies listed in Table 1.

^x All seed lots were harvested from seed crops grown in 2003. A total of 62 lots were assayed.

^y Four hundred seed assayed per seed lot. All seed was surface-sterilized in 1.2% NaOCl for 60 s, triple-rinsed in sterile deionized water, dried, plated onto damp sterile blotters in 10-cm-diameter petri plates, and examined microscopically approximately 5, 10, and 14 days after plating.

^z Means comparisons based on weighted analyses of variance by country and by company, with seed lots as replications. Log transformation used to generate normal residuals and homogeneous variances. Country means followed by the same letter are not significantly different based on Fisher’s protected least significant difference ($P < 0.05$); similarly for company means.

Table 3. Pathogenicity of five spinach seed isolates of *Verticillium dahliae* inoculated onto each of three spinach cultivars

Cultivar or isolate ^u	Trial 1 (winter 2002–03)							Trial 2 (winter 2003–04)						
	Symptomatic plants (%) ^y		Severity of wilt ^w		Root rot/ vasc. disc. (%) ^x	Reisolated or obsvd. ^x	Infested seed (%) ^y	Symptomatic plants (%) ^y		Severity of wilt ^w		Root rot/ vasc. disc. (%) ^x	Reisolated or obsvd. ^x	
	25	49	25	49				22	53	22	53			
Cultivar ‘Winter Bloomsdale’	85.0 a ^z	100.0 a	0.9 a	2.4 a	90.0 a	100.0 a	33.3 (6)	100.0 a	100.0 a	2.3 a	3.8 a	87.5 a	100.0 a	
E	45.0 b	100.0 a	0.5 b	2.5 a	100.0 a	100.0 a	28.0 (10)	70.0 b	90.0 a	1.6 a	3.0 b	79.2 a	100.0 a	
J	5.0 c	50.0 b	0.1 c	0.6 b	25.0 b	80.0 b	0.0 (1)	40.0 c	85.0 a	0.6 b	2.7 b	69.6 a	100.0 a	
Isolate														
1	66.7 a	75.0 a	0.7 a	2.1 a	75.0 ab	91.7 a	72.7 (2)	83.3 a	91.7 a	1.9 a	3.5 a	100.0 a	100.0 a	
2	41.7 a	75.0 a	0.4 ab	1.4 a	66.7 ab	83.3 a	2.9 (1)	75.0 a	100.0 a	1.6 a	3.3 a	92.0 a	100.0 a	
3	58.3 a	91.7 a	0.6 ab	1.9 a	75.0 ab	100.0 a	34.3 (3)	50.0 a	66.7 b	1.0 a	2.0 c	66.7 b	100.0 a	
4	33.3 a	83.3 a	0.3 abc	1.5 a	58.3 b	100.0 a	19.5 (4)	58.3 a	100.0 a	1.1 a	3.0 bc	100.0 a	100.0 a	
5	25.0 a	91.7 a	0.3 bc	2.1 a	83.3 a	91.7 a	0.0 (1)	83.3 a	100.0 a	1.8 a	3.8 a	100.0 a	100.0 a	
Control	0.0	0.0	0.0 c	0.0 b	0.0	16.7	0.0 (6)	0.0	41.7	0.0 b	0.4 d	17.0	8.3	

^u All isolates of *V. dahliae* were obtained from spinach seed. Root plugs of each of four seedlings (replications) per cultivar were soaked in a spore suspension of the appropriate isolate (or in water for the control treatment), transplanted, and monitored for wilt symptoms. Seedlings of cultivars J, E, and ‘Winter Bloomsdale’ were 2, 9, and 9 weeks old at time of inoculation for trial 1, and 6 weeks old at inoculation for trial 2.

^y Incidence (% of four plants) with wilt symptoms at different days after inoculation.

^w Severity of wilt symptoms at different days after inoculation rated on a scale of 0 to 4, where: 0 = no symptoms, 1 = red discoloration of the lower stem, 2 = interveinal chlorosis of the oldest leaves, 3 = stunting with interveinal chlorosis and necrosis progressing up the plant, and 4 = plant dead.

^x % of plants with root rot or vascular discoloration. Plants were washed, surface-sterilized in NaOCl, rinsed, cut lengthwise through the stem/root to assess vascular and root discoloration, incubated in a moist chamber, and examined for *V. dahliae*. Plants were processed 49, 56, and 63 days after inoculation for the first, second, and third and fourth replications in trial 1; and 59, 67, and 72 days after inoculation for the first, second, and third and fourth replications in trial 2, respectively.

^y Seed harvested from female plants that survived and set seed were assayed for *V. dahliae* using a modified freeze-blotter assay. Analyses of variance (ANOVAs) and means separations could not be calculated because of the limited number of plants that set seed and because plants did not set seed in each cultivar-by-isolate treatment combination. Seed had not developed on plants in Trial 2 at the time plants were washed and processed.

^z Numbers followed by the same letter within a column are not significantly different based on Fisher’s protected least significant difference ($P < 0.05$). Means comparisons were calculated separately for the main effects of spinach cultivar and isolate of *V. dahliae*. Except for wilt severity ratings, non-inoculated control treatments were not included in ANOVAs and means comparisons to avoid violating assumptions of parametric statistical analyses. Mean wilt severity ratings are presented, but means separations were based on Friedman’s nonparametric rank test because of the qualitative wilt severity scale.

was worn for washing each plant to avoid transferring *V. dahliae* propagules between plants. Each plant was assessed for root discoloration, soaked in 0.5% NaOCl for 5 min, rinsed under running deionized water, sliced lengthwise through the roots and stem using a knife sterilized with 70% ethyl alcohol between plants, and incubated for 5 days on paper toweling dampened with 0.1% NaOCl. Each plant was then examined microscopically ($\times 8$ to $\times 100$ magnification) for development of *V. dahliae* conidiophores and/or microsclerotia on the roots, crowns, and/or stems. Seed had not developed on any of the plants by the time the roots were washed, so seed assays were not carried out for the 2003–04 pathogenicity test.

ANOVAs and Fisher's protected least significant differences (LSDs) were calculated using SAS Version 8.0 (SAS Institute, Inc., Cary, NC). Except for wilt severity ratings, noninoculated control treatments were not included in the ANOVAs and means separations to avoid violating assumptions of parametric statistical analyses. Friedman's nonparametric rank ANOVAs and means separations were used for wilt severity ratings because of the qualitative severity scale.

Cultures of the five isolates of *V. dahliae* tested for pathogenicity were sent to Randy Rowe and Medani Omer at the Ohio State University to determine the vegetative compatibility group (VCG) of each isolate. Nitrate-nonutilizing mutants were generated on chlorate medium for the spinach isolates as well as each of two tester strains of VCG1, 2a, 2b, 3, 4a, and 4b (8,11). The phenotypes of the *nit* mutants were determined by growing the mutants on media amended with different nitrogen sources. *NitI* mutants (unable to utilize nitrate but able to utilize hypoxanthine as a nitrogen source) and *nitM* mutants (unable to utilize nitrate or hypoxanthine) of the spinach isolates and the tester strains were paired on minimal media to determine the vegetative compatibility groups of the spinach isolates. The five isolates were also sent to Jim

Correll at the University of Arkansas for VCG testing.

Seed transmission of *Verticillium*. In February 2003, seed of each of four spinach cultivars, 'Winter Bloomsdale' and proprietary cultivars H, F, and G, with 0.0, 35.3, 59.5, and 74.8% incidence of seedborne *Verticillium*, respectively (Tables 1 and 4), were planted into RediEarth potting mix in a 76-cell flat (2 seeds per cell). Approximately 1 month after planting, each seedling plug was transplanted into a 10-cm-diameter pot. A majority of the pots contained two plants, as both seeds germinated in most of the cells prior to transplanting. Latex gloves were worn by people handling the seedlings, and the gloves and bench surfaces were sterilized with 70% ethyl alcohol between plugs to avoid transferring *Verticillium* propagules between plants. The plants were maintained on a greenhouse bench with lights set at 14 h/day to promote bolting, and were monitored for wilt symptoms. Given the dioecious nature of spinach, male plants of 'Winter Bloomsdale' were used to pollinate female flowers of the cultivars F, G, and H for seed to develop. In May 2003, the roots, crown, and stem of three plants of each of cultivars F and G with symptoms suggestive of *Verticillium* wilt, one plant of cultivar G with symptoms typical of *Fusarium* wilt, and two healthy (non-wilting) plants of each of cultivars F, G, and H were washed thoroughly under running tap water. Each plant was then surface-sterilized for 5 min in 0.6% NaOCl, rinsed three times in sterile deionized water, and 5 mm³ sections of the roots, crown, and stem were plated onto Sorensen's NP-10 agar medium (16) and half-strength PDA amended with streptomycin (100 ppm). In June and July 2003, seed was harvested from each female plant that had survived and set seed. One hundred seed collected from each of 5, 18, 19, and 47 plants selected randomly from 'Winter Bloomsdale' and cultivars F, G, and H, respectively, were assayed for *Verticillium* using the non-surface-sterilized freeze-blotter assay (Table 4).

RESULTS

Detection of *Verticillium* in commercial spinach seed. The incidence of seedborne *Verticillium* in the male and female stock seed and the harvested seed of Cultivar F (the crop that developed late-season wilt symptoms in western Washington in 2002) was 44.0, 1.5, and 59.5%, respectively (Table 1). The isolates produced microsclerotia on the pericarps of the seed and were identified as *V. dahliae*. In contrast, the incidence of seedborne *Fusarium* was 27.8, 0.0, and 3.8%, respectively. Of 11 additional seed lots assayed in 2002, eight were infected with *Verticillium* at incidences ranging from 0.8 to 74.8%, and one was infected with *Fusarium* at an incidence of 1.0% (Table 1). Ten-minute surface-sterilization of the seed of Cultivar I in 1.2% NaOCl reduced the incidence of seedborne *Verticillium* detected to 1.8% compared with 8.3% on nonsterilized seed. Sterilization of the seed for up to 40 min did not eradicate *Verticillium* but reduced its incidence to 0.5% (Table 1). Microsclerotia characteristic of *V. dahliae* were observed on the pericarps of a majority of the infected seed (Fig. 1) and sometimes developed on the blue blotter around the infected seed. The bases of the conidiophores did not darken in color with maturity, indicating the isolates were not *V. albo-atrum* (14).

The incidence of *Verticillium* detected on 62 spinach seed lots harvested in 2003 ranged from 0.0 to 84.8% with a mean \pm standard deviation of $18.5 \pm 22.6\%$ infected seed per lot and a median of 8.0 infected seed per lot (Table 2). Although the highest incidence of *Verticillium* was detected on a seed lot grown in Denmark, the mean incidence of *Verticillium* was highest for seed lots grown in the United States (26.9%) and the Netherlands (26.3%), followed by Denmark (8.8%). Similarly, the median incidence of infected seed per lot was highest for the United States and the Netherlands (25.4 and 22.0%, respectively), and lowest for Denmark (3.25%). *Verticillium* was not detected in the single seed lot from New

Table 4. Incidence of wilt symptoms and seed-to-seed transmission of *Verticillium* for four spinach cultivars

Cultivar ^y	Incidence (%) of planted seed infested ^w		Incidence of wilt (% of plants) 78 days after transplanting			Incidence (%) of plants infested (no. tested) ^x		Mean incidence (%) of harvested seed infested (range)	
	<i>Verticillium</i>	<i>Fusarium</i>	Plants rated	<i>Verticillium</i>	<i>Fusarium</i>	<i>Verticillium</i>	<i>Fusarium</i>	<i>Verticillium</i> ^z	<i>Fusarium</i> ^z
'Winter Bloomsdale'	0.0	0.0	97	0.0	0.0	0.0 (5)	40.0 (5)	0.0 (0-0)	15.4 (1-76)
H	35.3	0.0	137	4.4	0.7	50.0 (18)	33.3 (18)	18.1 (0-95)	7.4 (0-61)
F	59.5	3.8	115	27.8	44.3	78.9 (19)	47.4 (19)	37.5 (0-100)	8.7 (0-61)
G	74.8	0.0	98	51.0	1.0	100.0 (47)	8.5 (47)	77.8 (1-100)	0.3 (0-7)

^y F, G, and H are proprietary cultivars, and 'Winter Bloomsdale' is an open pollinated cultivar (as described for Tables 1 and 2).

^w Incidence of planted seed naturally infested with *V. dahliae* and *Fusarium* spp., determined by a freeze-blotter assay.

^x Incidence of plants from which harvested seed tested positive for *Verticillium* or *Fusarium*.

^z Mean and range in incidence (% seed per plant) of harvested seed infested with *Verticillium*, based on the freeze-blotter assay without surface-sterilization. Most seed was infected with *V. dahliae*, based on sporulation characteristics and microsclerotia that formed on the pericarps.

^z Mean and range in incidence (% seed per plant) of harvested seed infested with *Fusarium*, based on the freeze-blotter assay without surface-sterilization. Isolates were not identified to species.

Zealand. Two seed lots from company 3; one seed lot from each of companies 1, 2, and 5; and none of the lots from companies 4 and 6 tested free of *Verticillium* (Table 2). The mean incidence of infected seed per lot was significantly lower for lots from companies 3, 5, and 2 (5.9, 9.3, and 18.1%, respectively), followed by lots from companies 1, 6, and 4 (28.7, 30.8, and 34.3%, respectively). The median incidence of infected seed per lot was below 10% for companies 2, 3, and 5 (0.3, 3.1, and 2.0%, respectively), and above 20% for companies 1, 4, and 6 (24.5, 26.0, and 28.3%, respectively).

Pathogenicity of seed isolates of *V. dahliae* on spinach. All five isolates of *V. dahliae* were pathogenic on the three spinach cultivars tested (Table 3). Results could not be pooled across the two trials because of significant interactions of the trial factor with the main effects of spinach cultivar and isolate of *V. dahliae* in the ANOVAs (*data not shown*). Wilt symptoms were observed primarily after bolting was initiated by increasing the duration of supplemental lighting to 14 h/day (i.e., when plants of cultivar J were 7 weeks old, and of cultivars E and ‘Winter Bloomsdale’ were 14 weeks old). The cultivar-by-isolate interaction term of the ANOVAs was not significant for any of the dependent variables except incidence of symptomatic plants rated 53 days after inoculation in trial 2 (*data not shown*). In the latter case, the incidence of plants with symptoms was 100% for all but 3 of the 18 cultivar-by-isolate treatment combinations, i.e., isolate 3 on cultivars J (25%) and E (75%), and isolate 1 on cultivar E (75%). Therefore, means separations are presented in Table 3 for the main effects of spinach cultivar and isolate of *V. dahliae*.

Incidence and severity of symptomatic plants increased with maturity of the plants (Table 3). In trial 1 (2002–03), the total incidence of symptomatic plants increased from 47% (28/60 plants) to 83% (50/60

plants) between 25 and 49 days after inoculation, respectively; similarly, the incidence of wilted plants in trial 2 (2003–04) increased from 50% (30/60 plants) to 87% (52/60 plants) between 22 and 53 days after inoculation, respectively. In each trial, the incidence and severity of symptomatic plants at the first rating was highest for ‘Winter Bloomsdale’ followed by cultivars E and J (Table 3). These differences among cultivars were less significant at the second rating in both trials. Although cultivar J had the lowest incidence of root rot and vascular discoloration, and the lowest incidence of *V. dahliae* reisolated or observed on plants in moist chamber, plants of this cultivar were 7 weeks younger than those of ‘Winter Bloomsdale’ and cultivar E in trial 1. In trial 2, in which plants of all three cultivars were the same age at the time of inoculation, cultivars E and J were similarly susceptible to *V. dahliae* at the second rating (Table 3).

The incidence of symptomatic plants was not significantly different among isolates of *V. dahliae* at 25 or 49 days after inoculation in trial 1, nor at 22 days after inoculation in trial 2 (Table 3). By 53 days

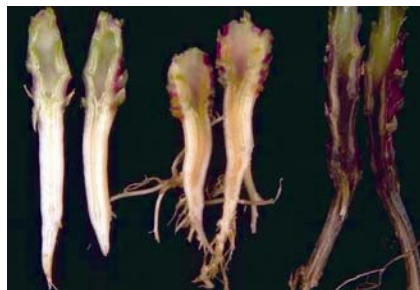


Fig. 3. Longitudinal section of the roots and crown of a healthy spinach plant (left), a spinach plant infected with *Verticillium dahliae* (center, showing pale brown discoloration), and a spinach plant infected with *Fusarium oxysporum* f. sp. *spinaciae* (right, showing dark brown to black discoloration).

after inoculation in trial 2, the incidence of symptomatic plants was significantly less for isolate 3 than for the other four isolates. Similarly, the incidence of plants with root rot or vascular discoloration was significantly less for isolate 3 than for the other isolates of *V. dahliae* at the time the plants were processed. In trial 1, small but significant differences in wilt severity ratings were observed among isolates of *V. dahliae* 25 days after inoculation, but not by 49 days after inoculation (Table 3). In contrast, 22 days after inoculation in trial 2, there were no significant differences in severity of wilt among isolates, but by 53 days after inoculation, isolates 1, 2, and 5 had caused significantly more severe wilting than isolates 3 and 4. Mild symptoms (severity rating of 1) were observed on a few control plants in trial 2 by 53 days after inoculation.

Root rot and pale brown discoloration of the vascular tissues in the roots, crown,



Fig. 4. Longitudinal section of the stem of a spinach plant not inoculated (left) or inoculated (right) with *Verticillium dahliae* by dipping the root plug in a spore suspension of the fungus prior to transplanting. Note abundant microsclerotia in the vascular tissue of the inoculated plant, observed 5 days after incubation of the inoculated plant in a moist chamber following surface-sterilization in 0.5% NaOCl for 5 min.



Fig. 2. Symptoms of *Verticillium* wilt of spinach after early initiation of bolting (left) and seed set (center: left plant noninoculated, right plant inoculated) resulting from seed transmission of *Verticillium dahliae*, and symptoms of *Fusarium* wilt of spinach (right) resulting from seed transmission of *Fusarium oxysporum* f. sp. *spinaciae*. Note the interveinal chlorosis and wilting of the oldest leaves on plants infected with *Verticillium* wilt versus general wilting of the entire plant and a lack of distinct interveinal chlorosis on the oldest leaves for the plant infected with *Fusarium* wilt.

and lower stem were observed on 88% (53/60) and 92% (55/60) of the inoculated plants in trials 1 and 2, respectively, but on none (0/12) of the noninoculated plants in trial 1, and on 17% (2/12) of the noninoculated plants in trial 2 (Table 3). *V. dahliae* was reisolated from the roots, crowns, and stems (or spores and microsclerotia were observed in moist chamber) on 95% (57/60) and 100% (60/60) of the inoculated plants in trials 1 and 2, respectively. *Verticillium* was also reisolated from 17% (2/12) and 8% (1/12) of the noninoculated plants in trials 1 and 2, respectively. This was probably the result of cross-contamination of *Verticillium* propagules during handling and washing of the plants, as none of the control plants displayed symptoms of *Verticillium* wilt in trial 1.

The first symptom observed on inoculated plants was reddening of the lower stem. This was followed by a general flaccid appearance of the lowest (oldest) leaves, which developed distinct interveinal chlorosis (Fig. 2A). Symptoms progressed up the stem of symptomatic plants, some of which were severely stunted compared with noninoculated control plants, and several died (Fig. 2B). Pale brown discoloration of the vascular cylinder in the roots and crown was observed on most inoculated plants that did not die, unlike the dark brown to black discoloration of spinach roots caused by *Fusarium* wilt (3) (Fig. 3). Incubation of inoculated plants in a moist chamber for 5 days led to formation of abundant microsclerotia, primarily in the vascular tissues of the stem and crown (Fig. 4), but also in the roots.

Freeze-blotter assays of 400 seed of each of cultivars J and E showed that only seed of cultivar J was infected with *Verticillium*, at a low incidence of 0.25% (1/400 seed). In contrast, assays of the seed harvested from inoculated plants showed *Verticillium* was present on 26% (47/178) of the seed from 7 of 11 inoculated plants that set seed in trial 1, but on none (0/205) of the seed from 6 control plants that set seed (Table 3). Seed harvested from plants in the third and fourth replications had the highest incidence of seedborne *Verticillium* per plant (up to 88%, *data not shown*), probably reflecting the maturity of seed harvested from these plants compared with seed harvested from plants in the first two replications. An ANOVA and means comparisons could not be calculated for incidence of harvested seed infected with *V. dahliae*, as there were too many cultivar-by-isolate treatment combinations in which plants did not set seed.

Vegetative compatibility grouping of the spinach seed isolates of *V. dahliae* tested for pathogenicity on spinach revealed that isolates 1 and 5 belonged to VCG 2b, and isolate 3 belonged to VCG 4b. The VCG of the remaining two isolates could not be determined by R. Rowe and M. Omer, as

one isolate did not form *nit* mutants and the other did not pair with any known tester strains (*data not shown*). Correll confirmed that isolates 1 and 5 belonged to one VCG and isolates 2, 3, and 4 belonged to a second VCG, although the particular VCGs were not determined by Correll (*data not shown*).

Seed transmission of *Verticillium*. Wilt symptoms were observed on transplanted seedlings only after stem elongation (initiation of bolting), except for a few plants of cultivar F that, prior to bolting, developed wilt symptoms characteristic of *Fusarium* wilt. This was the only cultivar for which the planted seed lot tested positive for *Fusarium* spp. (3.8%) (Table 4), but no attempt was made to identify the species to which these isolates belong. By 78 days after transplanting, 0.0, 5.1, 72.2, and 52.0% of the plants of 'Winter Bloomsdale' and cultivars H, F, and G had developed symptoms of wilting (Table 4). A majority of the symptomatic plants of cultivars G and H showed symptoms typical of *Verticillium* wilt, while 44.3% of the plants of cultivar F displayed symptoms typical of *Fusarium* wilt. Isolations from root, crown, and stem tissue of plants with either type of wilt symptom verified the association of *F. oxysporum* with general wilting symptoms and a lack of distinct interveinal chlorosis of the oldest leaves, and of *Verticillium* with symptoms initiated after bolting by wilting and interveinal chlorosis of the lowest leaves and acropetal progression of symptoms.

The highest incidence of plants from which *Verticillium* was detected on the harvested seed was for cultivar G (47/47 plants), followed by cultivars F (15/19 plants) and H (9/18 plants) (Table 4). *Verticillium* was not detected on any harvested seed of 'Winter Bloomsdale'. For cultivar G, *Verticillium* was detected on $\geq 90\%$ of the seed harvested from 53.2% (25/47) plants, and on $< 10\%$ of the seed harvested from 6.4% (3/47) plants. The incidence of plants of this cultivar on which harvested seed tested positive for *Verticillium* (100.0%) was greater than the incidence of *Verticillium* detected on planted seed of Cultivar D (74.8%). Similarly, the incidence of plants with infected harvested seed was greater than the incidence of plants with *Verticillium* wilt symptoms 78 days after transplanting (Table 4).

DISCUSSION

To our knowledge, this is the first report of *V. dahliae* as a pathogen of spinach seed crops in the Pacific Northwest, the primary region of spinach seed production in the United States. In addition, this is the first report of *Verticillium* wilt of spinach in the United States outside of New York since 1962, when the disease was reported by Snyder and Wilhelm (15). Despite the lack of reports of *Verticillium* wilt in fresh and processing spinach crops, *Verticillium* was

found to be prevalent on commercial spinach seed lots produced in both the EU and the United States. In the initial survey of seed lots, 11 of 13 lots (84.6%) were found to be infected with *Verticillium*. Subsequent assays of 62 lots harvested from spinach seed grown in 2003 revealed 57 (91.9%) were infected with *Verticillium*, at incidences ranging from 0.3 to 84.8%. Infected lots were detected from seed crops grown in Denmark, the Netherlands, and the United States, but not on the single lot from New Zealand. Both the number of infected seed lots and the incidence of infection of individual lots were greater than that detected by van der Spek (18), who assayed 34 spinach seed lots grown in the Netherlands in 1971 and found 20 (58.8%) were infected with *V. dahliae*, at incidences ranging from 0.5 to 4.0%.

In this study, symptoms of *Verticillium* wilt were not observed until after initiation of bolting, regardless of whether plants were infected by dipping the roots in a spore suspension of the pathogen or as a result of seed-to-seed transmission from naturally infected seed. The delay in development of symptoms until after bolting might explain why the disease has not been observed in fresh and processing spinach crops, which are harvested prior to bolting. To this extent, *Verticillium* wilt of spinach appears to be similar to *Verticillium* wilt of cauliflower, which is only observed in the field at the onset of "heading" (10), and *Verticillium* wilt of potatoes, which is commonly referred to as "early dying" (12).

Verticillium wilt symptoms followed a distinct pattern of progression on spinach plants. The first evidence of infection, observed after stem elongation, was a bright red external discoloration of the lower stem. As infection progressed, the oldest (lowest) leaves displayed interveinal chlorosis (distinct on some cultivars) which developed into interveinal necrosis. Symptoms gradually progressed up the stem, resembling premature senescence. Symptomatic plants cut lengthwise through the crown and roots typically displayed pale brown discoloration of the vascular tissue. Incubation of infected plants in a moist chamber led to formation of microsclerotia, primarily in the vascular tissues. In contrast, symptoms of infection by *F. oxysporum* f. sp. *spinaciae* can be observed as early as preemergence or postemergence damping-off (4). *Fusarium* wilt symptoms that develop after emergence include external reddening of the lower stem (similar to *Verticillium* wilt), followed by dark brown to black discoloration of the vascular tissue in the roots and lower stem, and general flaccid wilting of all leaves regardless of their maturity (dissimilar to *Verticillium* wilt). Infected plants of susceptible cultivars may die rapidly (4). Both *V. dahliae* and *F. oxysporum* f. sp. *spinaciae* can be seedborne

on spinach (2). External reddening of the lower stem of spinach plants appears to be a general stress response that is not indicative specifically of *Verticillium* wilt or *Fusarium* wilt, as similar symptoms have been observed on spinach plants in response to aphid infestations and heat stress (L. J. du Toit, *personal observation*).

V. dahliae is systemic in spinach and readily seed transmitted. The high rate of seed-to-seed transmission demonstrated in this study corroborates the reports of seed transmission by other researchers (13,15,19) and emphasizes the importance of using clean stock seed for spinach seed production. In the seed transmission trial, the incidence of plants from which the harvested seed was infected with *Verticillium* was higher than the incidence of *Verticillium* on planted seed. Given that two seeds were planted per pot, the higher incidence of plants with infected harvested seed may reflect movement of *Verticillium* between the roots of adjacent plants within a pot when only one of the planted seeds was infected. Seed-to-seed transmission was also observed on plants that did not develop obvious symptoms of *Verticillium* wilt, suggesting the possibility of asymptomatic infection of spinach by this pathogen. Alternatively, symptoms on these plants may have been masked as premature senescence. The resemblance of symptoms of *Verticillium* wilt to premature senescence may also explain why the disease has not previously been reported in spinach seed crops in the United States despite the prevalence of the pathogen in commercial seed lots.

There was no apparent relationship between the incidence of *Fusarium* spp. detected on the harvested seed and the incidence of plants with symptoms of *Fusarium* wilt (Table 4), as many of the isolates of *Fusarium* observed on the planted seed were species other than *F. oxysporum* (*data not shown*). The incidence of *Fusarium* wilt observed on cultivar F in the seed transmission trial was much greater than the incidence of *Fusarium* spp. detected on the planted seed (3.8%), which suggested the freeze-blotter assay may be less effective for detecting seedborne *Fusarium* spp. than seedborne *Verticillium* spp. on spinach.

The significance of seedborne *V. dahliae* on spinach remains to be determined. Although *Verticillium* wilt does not appear to cause losses in fresh and processing spinach crops, the potential for the pathogen to cause losses in seed crops has been demonstrated in this study and observed in commercial seed crops in the Pacific Northwest. Furthermore, van der Spek (19) demonstrated that planting spinach seed infected with *V. dahliae* can result in infestation of the soil, which may lead to *Verticillium* wilt of subsequent susceptible crops planted in infested fields. Therefore, it is important to determine the specificity

of the host range of isolates of *Verticillium* from spinach seed (3), particularly given the susceptibility of some crops (e.g., cauliflower) commonly grown in rotation with spinach in many areas of production (e.g., the Salinas Valley of California; 10).

Two of the spinach seed isolates of *V. dahliae* evaluated for pathogenicity on spinach belonged to VCG 2b, and one to VCG 4b. Spinach seed crops in the Pacific Northwest are commonly grown after potato crops because of the excellent weed control afforded by potato crops and the limited selection of herbicides that are not phytotoxic to spinach (9). Isolates of *V. dahliae* pathogenic to potatoes are primarily from VCG 4a or VCG 4b (12). If potato isolates of *V. dahliae* prove pathogenic on spinach, planting spinach seed crops following potatoes may increase disease pressure for *Verticillium* wilt in spinach seed crops. Therefore, the susceptibility of spinach cultivars to isolates of *V. dahliae* pathogenic on potatoes and other crops needs to be investigated. Preliminary results of ongoing research on the VCGs of a geographically diverse collection of spinach seed isolates of *Verticillium* indicate that all 50 isolates belong to one of two VCGs (*data not shown*).

Despite the lack of epidemiological knowledge on *Verticillium* wilt of spinach, seedborne *V. dahliae* can have a direct economic impact on the spinach seed industry because of the international nature of the seed industry and associated regulations affecting movement of seed across international borders. For example, Mexico has a phytosanitary certification requirement to prevent the import of spinach seed lots with $\geq 10\%$ *V. dahliae* (1). Of 62 commercial spinach seed lots grown in the United States and the EU in 2003 and assayed in this study, 30 could not have been exported to Mexico under this phytosanitary regulation. Such regulations emphasize the need for research to generate epidemiological evidence that supports, or justifies modifying, phytosanitary measures associated with seedborne *Verticillium*.

Research is also needed to evaluate the efficacy of physical, chemical, and biological seed treatments for control or eradication of seedborne *V. dahliae* on spinach. The thick, corky nature of the spinach pericarp, combined with the systemic infection of spinach plants by *V. dahliae*, suggests that complete eradication of *V. dahliae* from infected seed lots may be difficult. However, this study showed a reduction in incidence of seedborne *Verticillium* from 8.3% to $<1.0\%$ following a 20-min soak in 1.2% NaOCl, suggesting that seed treatments may effectively reduce viable seedborne inoculum below economic or phytosanitary thresholds. Increasing the duration of treatment from 20 to 40 min did not eradicate *Verticillium*, indicating that some infection was

internal, as demonstrated originally by van der Spek (18).

Little is known about the variation in resistance of spinach germplasm to *V. dahliae*. Preliminary evidence from this study suggests resistance in spinach germplasm to *F. oxysporum* f. sp. *spinaciae* may be independent of resistance to *V. dahliae*. The availability of resistance to *Verticillium* wilt in commercial cultivars of other species (e.g., 12) emphasizes the potential value of screening spinach germ plasm for resistance to *Verticillium* wilt.

As a soilborne and seedborne pathogen that is readily seed-transmitted and highly systemic, *V. dahliae* poses significant hurdles for development of an integrated management plan for *Verticillium* wilt in spinach seed production. However, effective management practices have been developed for *Verticillium* wilt of other crops such as cauliflower, cotton, peppermint, potato, strawberries, and tomato (17); and these practices provide examples of efficient strategies for research efforts to increase our understanding and management of this disease in spinach.

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