Seedborne *Cladosporium variabile* and *Stemphylium botryosum* in Spinach

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ABSTRACT

Hernandez-Perez, P., and du Toit, L. J. 2006. Seedborne *Cladosporium variabile* and *Stemphylium botryosum* in spinach. Plant Dis. 90:137-145.

Assays of 77 spinach (*Spinacia oleracea*) seed lots produced in the United States, Denmark, the Netherlands, or New Zealand in 2000 to 2003 showed that *Stemphylium botryosum*, causal agent of Stemphylium leaf spot, was present in every lot, at a mean incidence of 29.1% per lot. Either *Cladosporium variabile*, causal agent of Cladosporium leaf spot, or the morphologically similar species *C. macrocarpum*, was present in 37 of the 77 lots, at a mean incidence of 1.8% per lot. Some seed isolates of *S. botryosum* and *C. variabile* proved pathogenic on spinach. Nonpathogenic isolates resembling *C. variabile* were identified as *C. macrocarpum* by the absence of torulose aerial hyphae. Pathogenic isolates of *S. botryosum* were also detected in each of 12 seed lots stored for up to 11 years at 4.4°C and 60% relative humidity. *C. variabile* or *C. macrocarpum* was detected in only 2 of the 11 lots, which had been stored for 3 and 8 years. Component seed assays demonstrated that *S. botryosum* and *C. variabile* (or *C. macrocarpum*) were internal and external in spinach seed. *S. botryosum* was detected in 5 to 76% of the embryos of five seed lots, but the two *Cladosporium* species were detected in only 0 to 1% of the embryos of these lots. This suggests greater potential difficulty at eradicating *S. botryosum* than *C. variabile* from infected spinach seed using seed treatments.

A total of 600 to 1,600 ha of spinach (*Spinacia oleracea* L.) seed crops are grown in western Oregon and western Washington annually, providing up to 50% of the U.S. supply and up to 20% of the world supply of spinach seed (14). *Cladosporium variabile* (Cooke) de Vries was demonstrated to be the major leaf spot pathogen of spinach seed crops in the Pacific Northwest, causing significant economic losses when cool and moist conditions prevailed (15,21). Stemphylium leaf spot of spinach, caused by *Stemphylium botryosum* Wallr., was first reported in the United States in 2002 by Koike et al. (22), who documented outbreaks of this disease in fresh market spinach crops in the Salinas Valley of California. In 2002, du Toit and Derie (7) reported that *S. botryosum* caused outbreaks of Stemphylium leaf spot in spinach seed crops in western Washington. The presence of this disease also has been documented recently in Arizona (23), Delaware and Maryland (13), and Florida (34).

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Accepted for publication 3 September 2005.

DOI: 10.1094/PD-90-0137

Cladosporium and Stemphylium leaf spots may reduce the quality of processing spinach crops and may necessitate additional hand sorting for fresh market spinach crops (13,15,22). du Toit and Derie (8) demonstrated that the severity of these leaf spots is greater in the presence of spinach pollen, and that enhancement of disease in the presence of pollen is significantly greater for *S. botryosum* than for *C. variabile*. The abundance of pollen in spinach seed crops may exacerbate outbreaks of leaf spot in regions of spinach seed production (8,21).

Fuentes-Davila (15) reported *C. variabile* to be seedborne in spinach. du Toit and Derie (9) detected *S. botryosum* and *C. variabile* in each of 11 spinach seed lots produced in the European Union (EU) and the United States, but otherwise the prevalence of *S. botryosum* and *C. variabile* in the commercial spinach seed industry had not been determined prior to the present study. What is commonly called spinach "seed" is actually a fruit with a seed enclosed by a thick, corky pericarp (31,38). The true seed consists of a seed coat, embryo, and nonliving perisperm (maternal storage tissue). For this study, the entire spinach fruit consisting of the pericarp, testa, embryo, and perisperm is referred to as a seed. The location of *C. variabile* and *S. botryosum* on, or within, the complex spinach seed structure has not been investigated. Understanding the nature of spinach seed infection by these pathogens may help determine the potential for seed transmission, as well as the potential efficacy of chlorine, hot water, or fungicide seed treatments for eradication of the fungi from infected seed and/or prevention of seed transmission.

The objectives of this study were to assess: (i) prevalence of *C. variabile* and *S. botryosum* in commercial spinach seed lots from the primary countries of the world where spinach seed is produced, (ii) pathogenicity to spinach of isolates of *C. variabile* and *S. botryosum* obtained from spinach seed, and (iii) location of *C. variabile* and *S. botryosum* within spinach seed. This research formed part of the M.S. thesis of the first author (19), and preliminary results have been presented (9,20).

MATERIALS AND METHODS

Spinach seed lots. A total of 89 spinach seed lots were assayed, including 11 lots in a preliminary screen (Table 1), 66 lots produced in six countries in 2003 by six seed companies (Table 2), and 12 lots produced in Washington from 1993 to 2001 (Table 3). For the preliminary screen of seed lots (Table 1), samples were collected from seed of the parent lines (stock seed) used to grow each of two hybrid seed crops in western Washington, as well as the harvested seed from these crops. The stock seed lots were produced in the European Union (EU). Each crop had displayed approximately 5 to 20% leaf spot severity within a month of harvest. In addition, samples of four commercial spinach seed lots produced in the EU in 2000 or 2001 (EU-5 to EU-8, Table 1) were sampled to test for *C. variabile* and *S. botryosum*. Seed were also collected from a hybrid seed crop research trial (lot US-3, Table 1) at the Washington State University Northwestern Washington Research & Extension Center (WSU-NWREC). The crop had been inoculated with both *C. variabile* and *S. botryosum* as described by du Toit et al. (10). The US-3 seed were harvested in August 2001 from plots that displayed 70 to 85% leaf spot severity.

For comprehensive evaluation of the prevalence of *C. variabile* and *S. botryosum* in commercial spinach seed lots, 66 seed lots harvested in 2003 were collected

from six commercial seed companies. The seed lots were produced in Denmark, the Netherlands, New Zealand, or the United States (western Washington or western Oregon), with some companies producing seed in multiple countries (Table 2). Seed company names have been coded for proprietary reasons.

Duration of survival in spinach seed. Twelve spinach stock seed lots produced in the United States from 1993 to 2001 were assayed in 2004 for *C. variabile* and *S. botryosum* (Table 3), to determine the potential duration of survival of these fungi in spinach seed. The seed lots were

Table 1. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* or *C. macrocarpum* in spinach seed lots produced in 2000, 2001, or 2002 in the European Union (EU) or the United States (US)

			Incidence (%) of seed infected (mean ± SD)		No. pathogenic isolates/no. tested[a]	
Seed lot[b]	Stock or harvested seed[c]	Seed rinsed in water and/or soaked in 1.2% NaOCl[d]	*S. botryosum*	*C. variabile* and/or *C. macrocarpum*[e]	*S. botryosum*	*C. variabile* and/or *C. macrocarpum*
EU-1	Female stock	Rinsed + 1 min in NaOCl	0.8 ± 0.5	7.5 ± 4.5	…	…
EU-2	Male stock	Rinsed + 1 min in NaOCl	18.0 ± 2.6	0.8 ± 0.5	…	…
EU-3	Female stock	Rinsed + 1 min in NaOCl	4.0 ± 2.6	23.3 ± 6.8	…	…
EU-4	Male stock	Rinsed + 1 min in NaOCl	0.3 ± 0.5	27.3 ± 3.8	…	…
EU-5	Harvested	Rinsed	55.5 ± 13.4	5.3 ± 3.4	…	…
EU-6	Harvested	Rinsed	35.3 ± 6.2	0.8 ± 1.0	3/3	…
EU-7	Harvested	Rinsed	86.0 ± 4.1	0.3 ± 0.5	3/3	…
EU-8	Harvested	Rinsed	18.8 ± 3.8	8.5 ± 2.4	2/2	…
US-1	Harvested	Rinsed	3.0 ± 0.8	4.0 ± 2.2	1/1	1/1
US-2	Harvested	Rinsed	27.3 ± 5.1	3.3 ± 0.5	2/2	1/1
US-3	Harvested	Rinsed	54.8 ± 5.1	49.0 ± 3.7	3/3	1/1
		10.0 min in NaOCl	23.3 ± 6.4	0.3 ± 0.5		
		20.0 min in NaOCl	16.8 ± 3.9	0.0		
		30.0 min in NaOCl	19.0 ± 4.7	0.0		
		40.0 min in NaOCl	18.3 ± 2.9	0.0		

[a] For each isolate, three plants were inoculated with 20 ml of inoculum (10^5 conidia/ml) as described in the text.
[b] EU seed lots were produced in Denmark or the Netherlands in 2000 or 2001. US seed lots were produced in western Washington; US-3 was harvested in 2001, and US-1 and US-2 were harvested in 2002.
[c] Stock seed = seed of male or female inbred lines used to grow a hybrid seed crop. Harvested seed = seed harvested from the female inbred line and sold for fresh market or processing spinach production.
[d] Seed were rinsed under running deionized water for 60 min, and/or soaked in 1.2% NaOCl for the stated duration followed by a triple-rinse in sterile deionized water. Seed were then dried and subjected to a freeze-blotter seed health assay as described in the text. Seed assays were completed in the winter of 2002–2003.
[e] At the time seed assays were completed, isolates of *C. variabile* were not differentiated morphologically from *C. macrocarpum* (6,12).

Table 2. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* or *C. macrocarpum* in 66 spinach seed lots produced in 2003 in Denmark, the Netherlands, New Zealand, and the United States by six seed companies

		Incidence (%)[a]			
Company or country[b]	No. of seed lots infected/no. assayed[c]	Mean ± SD	Minimum	Maximum	No. of pathogenic isolates/no. tested[d]
Company			*S. botryosum*		
A	27/27	6.9 ± 2.2	0.5	19.0	0/5
B	5/5	61.3 ± 5.0	8.0	97.5	4/5
C	24/24	53.3 ± 3.1	0.3	96.0	0/5
D	4/4	12.6 ± 2.4	1.0	19.8	4/5
E	3/3	35.9 ± 4.6	17.5	58.5	3/4
F	3/3	3.1 ± 1.8	1.0	6.3	…
Country					
Denmark	27/27	58.4 ± 3.5	8.0	97.5	5/9
The Netherlands	6/6	18.2 ± 3.0	1.0	31.8	4/7
New Zealand	1/1	58.5 ± 7.5	8.5	58.5	2/2
United States	32/32	6.1 ± 2.0	0.3	19.0	0/6
Total	66/66	29.4 ± 2.8	0.3	97.5	11/24
Company			*C. variabile* and/or *C. macrocarpum*[e]		
A	17/27	0.5 ± 0.4	0.0	2.5	0/8
B	0/5	0.0	0.0	0.0	…
C	6/24	0.1 ± 0.1	0.0	1.0	5/7
D	0/4	0.0	0.0	0.0	…
E	0/3	0.0	0.0	0.0	…
F	3/3	2.9 ± 1.8	1.5	4.0	…
Country					
Denmark	3/27	0.1 ± 0.1	0.0	1.0	5/6
The Netherlands	1/6	0.1 ± 0.1	0.0	0.3	…
New Zealand	0/1	0.0	0.0	0.0	…
United States	22/32	0.7 ± 0.6	0.0	4.0	0/9
Total	26/66	0.4 ± 0.3	0.0	4.0	5/15

[a] For each lot, four samples of 100 seed were surface-sterilized in 1.2% NaOCl for 60 s, triple-rinsed, dried, and subjected to a freeze-blotter seed health assay as described in the text.
[b] Seed companies are coded for proprietary purposes.
[c] All seed lots were harvested from commercial seed crops. None of the lots was treated with fungicides.
[d] For each isolate, three plants were inoculated with 20 ml of inoculum (10^5 conidia/ml) as described in the text.
[e] At the time seed assays were completed, isolates of *C. variabile* were not differentiated morphologically from *C. macrocarpum* (6,12).

stored in a seed vault at 4.4°C and 60% relative humidity, according to seed company records. In addition, samples of seed lot US-3 (Table 1) were assayed again in June 2003 (21 months after harvest) and January 2004 (28 months after harvest) using the 60-s surface-sterilization protocol. This seed lot was stored from fall 2001 to July 2004 at the WSU-NWREC at 18.8 ± 2.9°C and 62 ± 5% relative humidity.

Freeze-blotter seed assays. A sample of each seed lot was subjected to a freeze-blotter seed health assay following the method described by du Toit et al. (11). Seed lots listed in Tables 1, 2, and 3 were assayed between October 2002 and March 2003, October 2003 and March 2004, and March to May 2004, respectively. Each seed assay consisted of four replications of 100 seed, unless indicated otherwise. The seed for each replication was placed into a mesh tea strainer (Model 101, Venalicia Tea, Neuss, Germany). The tea strainer was placed in 150 ml of 1.2% NaOCl and shaken manually for 60 s (or other durations as specified). The seed were then triple-rinsed in sterile deionized water and dried on sterile paper towel in a laminar flow hood. Using flame-sterilized forceps, 20 seed were plated onto a sterile steel blue germination blotter (8.25 cm diameter; Anchor Paper Co., St. Paul, MN) moistened with 4 ml of sterile deionized water in a 10-cm-diameter petri dish. Each petri dish was sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI). The seed were incubated in the dark at 24°C for 24 h to imbibe moisture, frozen at –20°C for 22 to 24 h to prevent further germination, and placed in an incubator (Model I30BLL, Percival Scientific, Perry, IA) set at 24°C for 12 to 14 days with a 12 h/12 h day/night cycle, with cool-white fluorescent light and near-ultraviolet light by day. The seed were examined approximately 4, 7, and 14 days after plating, at ×8 to ×100 magnification using a dissecting microscope, for development of *C. variabile*, *S. botryosum*, and other fungi. For each seed lot, the mean and standard deviation of seedborne infection was calculated for each fungus. In addition, the mean, standard deviation, and range of seedborne infection was calculated by company and country for the 2003 seed lots.

In October 2001 (1 month after harvest), seed lot US-3 was disinfested and assayed for *C. variabile* and *S. botryosum* as described above. To determine whether spinach seed were infected internally by *S. botryosum* and/or *C. variabile*, additional samples of 400 seed of this lot were soaked in 100 ml of 1.2% NaOCl in a glass beaker on a gyratory shaker for 10, 20, 30, or 40 min. The seed were then triple-rinsed in sterile deionized water, dried, and assayed as described above.

Pathogenicity of seed isolates of *S. botryosum* and *C. variabile*. A total of 52 seed isolates of *S. botryosum* were tested for pathogenicity on spinach: 14 isolates from the preliminary survey of spinach seed lots (Table 1); 24 isolates from seed lots produced in 2003 in Denmark, the Netherlands, New Zealand, and the United States (Table 2); and 14 isolates from seed lots produced in Washington from 1993 to 2001 (Table 3). In addition, 18 putative isolates of *C. variabile* were tested for pathogenicity on spinach: 3 from the preliminary seed survey (Table 1), and 15 from seed lots produced in 2003 in Denmark and the United States (Table 2). Cultures resembling *S. botryosum*, *C. variabile*, and other *Cladosporium* spp. were isolated from the spinach seed lots during the freeze-blotter seed assays described above, by transferring spores from the seed onto nonclarified V8 juice agar medium (300 ml of V8 juice [Campbell Soup Company, Camden, NJ], 4.5 g of calcium carbonate [Mallinckrodt Baker, Inc., Phillipsburg, NJ], and 15 g of Bacto agar [Becton, Dickson and Co., Sparks, MD] per liter of deionized water) for isolating *S. botryosum*, and Difco potato dextrose agar (PDA) (Becton, Dickinson and Co.) for *C. variabile* and other *Cladosporium* spp. Approximately 1 week after isolation, spores from the culture of each isolate were streaked onto PDA or V8 agar and incubated under ambient laboratory conditions (approximately 12 h/12 h day/night cycle and 24 ± 4°C) for generation of single-spore cultures, which were transferred to the appropriate agar medium for each isolate. Four 1.5-cm-diameter sterile filter disks (VWR Scientific Products, West Chester, PA) were placed on the medium for colonization by the fungus. For *Cladosporium* cultures, 1.5 ml of sterile deionized water was added to each plate 7 days after single-spore transfer, and the plates were shaken to disperse conidia over the medium and filter disks. After the fungus had colonized the filter disks, the disks were removed and dried overnight in a laminar flow hood in sterilized coin envelopes (5.7 × 8.9 cm) (Westvaco Envelope Division, Springfield, MA). The dried, colonized disks were stored with desiccant at –20°C following the protocol of Peever et al. (30).

For the 2003 seed lots assayed, 2-week-old cultures of *S. botryosum* on V8 agar were tested for pathogenicity on spinach plants in the greenhouse as described below. The isolates included nine from Denmark, seven from the Netherlands, six from the United States, and two from New Zealand (Table 2). Similarly, 15 putative isolates of *C. variabile* were selected arbitrarily to test for pathogenicity on spinach plants in the greenhouse, including nine from the United States and six from Denmark (Table 2). In addition, five isolates of other *Cladosporium* spp. from seed lots from Denmark, the Netherlands, and New Zealand were tested for pathogenicity (Table 2), as were isolates of *S. botryosum* from seed lots produced in 1993 (three isolates), 1994 (five isolates), 1996 (three isolates), and 2001 (three isolates) (Table 3). The latter isolates were stored at –20°C for 4 to 5 months before pathogenicity tests were carried out, compared with 10 to 14 months of storage at –20°C for isolates from the 2003 seed lots.

A randomized complete block design with three single-plant replications per isolate was used for each pathogenicity test. Using an atomizer (Badger Air-Brush Co., Franklin Park, IL), 20 ml of approximately 10^5 conidia per ml of the appropriate isolate was inoculated onto 6- to 10-week-old plants of a proprietary, smooth leaf, medium-long standing, female spinach inbred line. Control plants were atomized with sterile deionized water. A known pathogenic isolate of *S. botryosum*, Chee-

Table 3. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* or *C. macrocarpum* in 12 spinach seed lots produced in 1993 to 2001 in western Washington, and assayed in 2004

	Incidence (%)[a]		
	S. botryosum		*C. variabile* and/or *C. macrocarpum*[b]
Year[c]	Mean ± SD	No. pathogenic isolates/no. tested[d]	Mean ± SD
1993	7.0 ± 2.6	2/2	0.0
1993	7.5 ± 3.1	1/1	0.0
1994	8.5 ± 2.4	2/2	0.0
1994	0.8 ± 0.5	2/2	0.0
1994	0.8 ± 0.5	1/1	0.0
1996	27.8 ± 2.2	1/1	0.5 ± 1.0
1996	17.3 ± 5.4	2/2	0.0
1997	54.8 ± 8.3	…	0.0
1998	36.5 ± 5.2	…	0.0
1998	20.3 ± 4.1	…	0.0
2001	54.0 ± 5.0	1/1	0.5 ± 0.6
2001	19.5 ± 1.9	2/2	0.0

[a] Four subsamples of 100 seed were surface-sterilized in 1.2% NaOCl for 60 s, triple-rinsed, dried, and subjected to a freeze-blotter seed health assay as described in the text.
[b] At the time seed assays were completed, isolates of *C. variabile* were not differentiated morphologically from *C. macrocarpum* (6,12).
[c] Year the seed was grown and harvested.
[d] For each isolate of *S. botryosum*, three plants were inoculated with a conidial suspension.

tah (obtained from lesions on a fresh market crop of the spinach cultivar Cheetah growing in Yuma, AZ in 2003), was used as the control isolate for *S. botryosum*. A known pathogenic isolate of *C. variabile*, 00-304 (obtained from an infected spinach seed crop in western Washington in 2000), served as the control isolate for *C. variabile*. Plants were assessed visually 1, 2, and 3 weeks after inoculation for symptoms of Stemphylium and Cladosporium leaf spots.

Approximately 3 weeks after inoculation, a leaf was removed from each plant showing symptoms of leaf spot. A 2 mm^2 section was cut from the margin of several spots on each leaf, surface-sterilized in 0.6% NaOCl for 60 to 90 s, triple-rinsed in sterile deionized water, dried on sterile paper towel in a laminar flow hood, and placed onto V8 agar (for isolation of *S. botryosum*) or PDA (for isolation of *Cladosporium* spp.). Leaf sections were also cut from asymptomatic leaves of the control plants and plated onto PDA. Plates were kept at room temperature and exposed to a 12 h/12 h day/night cycle. Fungal isolates were examined microscopically for identification. A symptomatic leaf from each plant was also placed in a moist chamber. The plates and leaves were examined microscopically after 3 days for the development of conidiophores and conidia of *S. botryosum* and *C. variabile*. The pathogenicity on spinach of other fungi observed on the seed was not tested, except as specified below for other *Cladosporium* sp.

For seed lots EU-1 to EU-8 and US-1 to US-3 (Table 1), isolates of fungi resembling *C. variabile* and *S. botryosum* on the seed were transferred to PDA and V8 agar, respectively. Spore suspensions (4×10^5 spores per ml) of isolates of both fungi, and one isolate of an unidentified *Cladosporium* sp. from seed lot US-3, were inoculated onto spinach plants in the greenhouse as described above. Control plants were inoculated with water. For each isolate, an inoculated leaf was sampled 15 days later. Sections (5 mm^2) from each leaf were surface-sterilized in 0.6% NaOCl for 60 to 90 s, rinsed in sterile deionized water, dried, and plated onto PDA or V8 agar. The plates were examined microscopically for *C. variabile* and *S. botryosum*.

Component freeze-blotter seed assays. To determine the degree to which *C. variabile* and *S. botryosum* may be present on or in the pericarp and/or embryo of spinach seed, component freeze-blotter seed assays were performed on five seed lots and compared with freeze-blotter assays of whole seed of each seed lot (Table 4). Whole seed assays were completed as described above. For the component seed assay of lot D1, 100 seed were rinsed in running deionized water for 60 min to soften the pericarps so that the pericarp and embryo of each seed could be separated manually using sterilized forceps. The components of each seed were then assayed separately using the freeze-blotter protocol. The procedure was repeated for each of seed lots B2, B5, and WSU1 to determine whether the location of these fungi in spinach seed might vary among cultivars or seed lots. To minimize contamination of the embryos with spores from the pericarps during manual separation of the seed components, the seed of each of these lots was soaked in 1.2% NaOCl for 60 s after the 60-min rinse in water. The seed were then triple-rinsed in sterile deionized water, and the pericarps and embryos were separated, dried, and plated individually. To determine whether the embryos might be infected internally versus contaminated on the surface, 100 seed of lot WSU2 were rinsed in water, the pericarp and embryo of each seed separated, and the components of each seed soaked separately in 1.2% NaOCl for 60 s in 5-ml microcentrifuge tubes (USA Scientific, Inc., Ocala, FL), triple-rinsed in sterile deionized water, dried, and plated.

Electron microscopy. Seed lot 03-409, harvested from a spinach seed crop fungicide trial at the WSU-NWREC in Mount Vernon, WA in September 2003, was determined by freeze-blotter assay to be infected with *S. botryosum* at 21.5% and *C. variabile* at 17.0% (10). In November 2003, an additional 50 seed of this lot were surface-sterilized and assayed using the freeze-blotter method. At 20 to 24 h after moving the seed from –20°C to 24°C, the seed were examined using a dissecting microscope for evidence of *S. botryosum* and *C. variabile*. Five seed with mycelium indicative of *S. botryosum* on the surface were each cut in half using an ethanol-disinfested surgical blade. Similarly, two seed with conidiophores indicative of *C. variabile* were cut in half so that approximately 50% of the conidiophores were present on each half of the seed. One-half of each seed was incubated as described for the freeze-blotter assay to monitor development of fungi. The other half was prepared for transmission electron microscopy (TEM) by fixing, washing, dehydrating, infiltrating, embedding, and curing the seed following the protocol of Bozzola and Russell (3). The pericarp and embryo of each half-seed were separated and fixed overnight in 2% glutaraldehyde and 2% paraformaldehyde in 50 mM pipes buffer (Electron Microscopy Sciences, Fort Washington, PA). The seed components were then rinsed once in 50 mM pipes buffer and twice in 25 mM phosphate buffer, followed by postfixation with 1% osmium tetroxide in 25 mM phosphate buffer for 2 h, and 0.1% tannic acid for 1 h. The seed components were then dehydrated sequentially for 10 min in each of 30, 50, 70, 80, and 95% acetone, followed

Table 4. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* or *C. macrocarpum* on whole seed, and on the pericarps and embryos of five spinach seed lots as determined by component seed assays

		Incidence (%)					
		S. botryosum			*C. variabile* and/or *C. macrocarpum*[a]		
Seed lot code[b]	Seed preparation for component seed assay[c]	Whole seed	Pericarp	Embryo	Whole seed	Pericarp	Embryo
D1	60-min rinse in water	49 ± 5[d]	54	29	0	0	0
B2	60-min rinse in water + 60-s soak of whole seed in 1.2% NaOCl	57 ± 8	45	36	0	0	0
B5	As for lot B2	95 ± 2	91	76	0	0	0
WSU1	As for lot B2	62	45	36	3	2	0
WSU2	60-min rinse in water + 60-s soak of separated pericarps and embryos in 1.2% NaOCl	26	13	5	20	19	1

[a] At the time seed assays were completed, isolates of *C. variabile* were not differentiated morphologically from *C. macrocarpum* (6,12).

[b] Seed lots are coded for proprietary purposes. D and B represent company codes (Table 2). WSU = seed harvested from Washington State University seed crop trials in Mount Vernon, WA. Numbers represent seed lots.

[c] Whole seed were subjected to a nonsterilized freeze-blotter seed assay as described in the text. For component seed assays, 100 seed of each lot were rinsed in water for 60 min to soften the pericarps, and the pericarps and embryos separated. Seed of lots B2, B5, and WSU1 were rinsed for 60 min, surface-sterilized in 1.2% NaOCl, triple-rinsed, dried, and separated into pericarps and embryos, which were subjected to a freeze-blotter assay. Pericarps and embryos of each seed of lot WSU2 were separated and then individually surface-sterilized, rinsed, and subjected to a freeze-blotter assay.

[d] Mean ± standard deviation of four replications of 100 seed for whole seed assays of lots D1, B2, and B5. One replication of 100 whole seed was assayed for lots WSU1 and WSU2.

by dehydration three times in 100% acetone for 10 min each time. After dehydration, the seed components were infiltrated overnight in each of 1:3, 1:2, 1:1, and 3:1 resin:acetone, followed by infiltration three times with 100% resin. Using 100% resin as embedding medium, the pericarp and embryo were then embedded separately and incubated overnight at 55°C for polymerization. Thin sections (70 to 100 nm) of each embryo and pericarp were prepared using an ultra microtome, placed on Formvar-coated grids (Ted Pella, Inc., Redding, CA), stained with Sato's lead stain procedure (35), and examined using a transmission electron microscope (JEOL USA, Inc., Peabody, MA). Images of mycelium in the pericarps were taken using a digital camera (Soft Imaging System Corp., Lakewood, CO). One of the half seeds was discarded because spores of an *Alternaria* sp. were observed on the corresponding half seed in the incubator.

An additional 50 seed of lot 03-409 were subjected to the freeze-blotter seed health assay. The seed were removed from the incubator 5 and 10 days after plating for examination at ×8 to ×100 magnification for development of *C. variabile* and *S. botryosum*, respectively. Three seeds with putative *C. variabile* and five seeds with *S. botryosum* or pseudothecia of *Pleospora herbarum*, the teleomorph of *S. botryosum* (1,37), developing on the pericarp were prepared for scanning electron microscopy (SEM) according to the protocol of Bozzola and Russell (2). The seed were fixed overnight at room temperature in 1% osmium tetroxide, placed on aluminum stubs, dehydrated overnight using a vacuum evaporator (Norton, Vacuum Equipment Division, Newton, MA), and coated with gold for 6 min using a sputter coater (Technics Hummer V, San Jose, CA). Conidiophores and conidia of *C. variabile* and *S. botryosum*, and pseudothecia of *P. herbarum,* were observed using a scanning electron microscope (S-570, Hitachi, Ltd., Tokyo, Japan).

RESULTS

Development of *S. botryosum* and *C. variabile* on spinach seed. Using the

Fig. 2. Symptoms of Cladosporium leaf spot (left) and Stemphylium leaf spot (right) approximately 3 weeks after inoculation of spinach plants with *Cladosporium variabile* and *Stemphylium botryosum*, respectively. Cladosporium leaf spots are small with discrete margins compared with the larger, diffuse lesions of Stemphylium leaf spot.

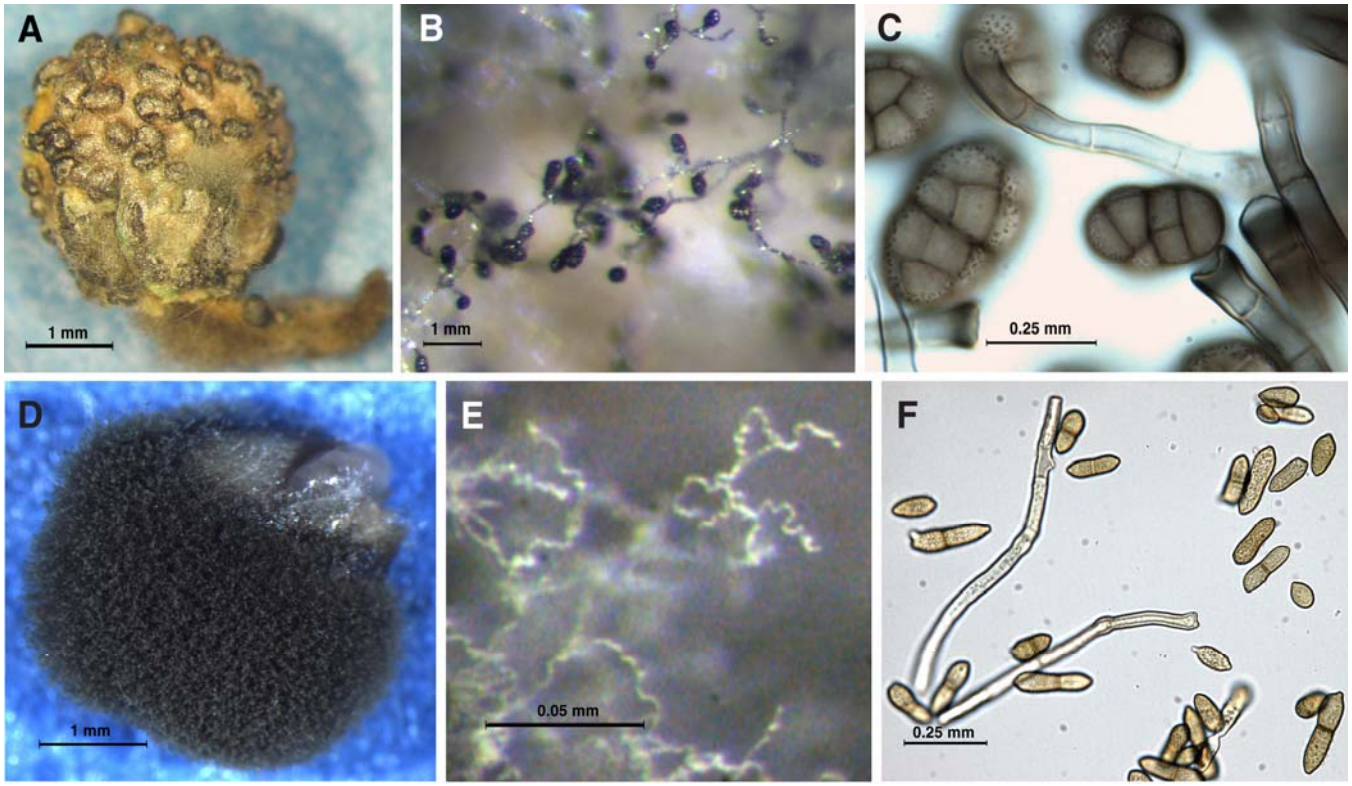

Fig. 1. Spinach seed infected with *Cladosporium variabile* and *Stemphylium botryosum*, and subjected to a freeze-blotter seed health assay. **A,** Erumpent pseudothecia of *Pleospora herbarum*, the teleomorph of *S. botryosum*, developing on the pericarp. **B,** Mycelium and conidia of *S. botryosum*. **C,** Conidiophores and conidia of *S. botryosum*. **D,** Dense sporulation of *C. variabile* observed 5 days after plating the seed. **E,** Torulose aerial hyphae produced apically on conidiophores of *C. variabile*, 7 days after plating the seed. **F,** Conidiophores and conidia of *C. variabile*.

freeze-blotter seed health assay, *S. botryosum* was observed on spinach seed in the form of erumpent black pseudothecia of the teleomorph, *P. herbarum* (Fig. 1A), and/or white mycelium on which conidiophores produced dark brown conidia characteristic of *S. botryosum* (Fig. 1B and C) (37). Asci and ascospores were not observed in the pseudothecia, even on seed incubated on the blotters for up to 2 months (data not shown). Pseudothecia developed on almost all seed infected with *S. botryosum*. On some seed, pseudothecia developed over most of the surface of the pericarp (Fig. 1A), whereas on other seed pseudothecia were observed on limited areas of the pericarp. Similarly, conidiophores and conidia were sometimes concentrated in one or a few areas of the seed surface, and sometimes covered most of the seed.

When dematiaceous conidia of *Cladosporium* measuring 15 to 25 µm × 7 to 10 µm were observed in chains of two to three on dark conidiophores that measured up to 350 µm (usually <150 µm), the fungus was initially identified as *C. variabile* (Fig. 1D and F). Isolates with longer chains of smaller, light-colored conidia were identified as other *Cladosporium* spp. (6,12). However, after completion of pathogenicity tests of a sample of the putative *C. variabile* isolates (see below), coupled with further microscopic examination of these isolates, some of the isolates proved to be *C. variabile* and others *C. macrocarpum*, two species that are not readily distinguished morphologically (6,12). According to Ellis (12), *C. variabile* can be differentiated from *C. macrocarpum* based on the presence of torulose aerial hyphae (Fig. 1E) produced by *C. variabile* but not by *C. macrocarpum*, and pathogenicity on spinach of the former species but not the latter species (6,12). Because these methods of differentiating the two species were not recognized at the time the seed assays were completed, results for putative isolates of *C. variabile* are presented as *C. variabile* or *C. macrocarpum* (Tables 1 to 4).

Prevalence of *S. botryosum* in spinach seed. *S. botryosum* was observed on all 11 seed lots assayed in the winter of 2002–2003, at incidences ranging from 0.3 to 86.0% (Table 1). Surface-sterilizing seed of lot US-3 for 10 min in 1.2% NaOCl reduced the incidence of *S. botryosum* from 54.8 to 23.3%. Increasing the duration of surface-sterilization beyond 10 min reduced the incidence of *S. botryosum* to <20%, but the fungus was not eradicated from seed by chlorine treatment, even after 40 min.

Each of the 66 spinach seed lots produced in 2003 and assayed in this study was infected with *S. botryosum*, at a mean incidence of 29.4% per lot and a range of 0.3 to 97.5% for individual lots (Table 2). Seed lots from Denmark and New Zealand had the highest mean incidence of this pathogen (58.4 and 58.5%, respectively), while the lowest mean incidence was detected in seed lots grown in the United States (6.1%). Seed lots from companies B and C had the highest mean incidence of infection (61.3 and 53.3%, respectively), followed by companies E (35.9%), D (12.6%), A (6.9%), and F (3.1%).

Duration of survival of *S. botryosum* in spinach seed. *S. botryosum* was detected in all 12 seed lots harvested in western Washington in 1993 to 2001, with a range of 0.8 to 54.8% for individual lots (Table 3). The pathogen was observed in two 11-year-old seed lots (1993 crops) at incidences of 7.0 and 7.5% (Table 3). For these 12 seed lots, a significant correlation was detected between the percentage of seedborne *S. botryosum* and the duration of storage measured as the year of harvest ($r = 0.70$, $P = 0.01$). For lot US-3 harvested in August 2001, *S. botryosum* was detected on 54.8, 2.5, and 0.3% of the seed when assayed 1 (fall 2001), 21 (summer 2003), and 28 months (fall 2004) after harvest, respectively.

Prevalence of *C. variabile* and/or *C. macrocarpum* in spinach seed. *C. variabile* and/or *C. macrocarpum* were observed on all 11 seed lots assayed in the winter of 2002–2003, at incidences ranging from 0.3 to 49.0% (Table 1). Surface-sterilizing the seed of lot US-3 for 10 min reduced the incidence from 49.0 to 0.3%. Increasing the duration of surface-sterilization to 20 min or longer completely eradicated *Cladosporium* from the seed.

In contrast to the results for *S. botryosum*, *C. variabile* and/or *C. macrocarpum* were detected in only 26 of the 66 seed lots produced in 2003 (39.4%), with a mean incidence of 0.4% and a range of 0.0 to 4.0% for individual lots (Table 2). Seed lots from the United States had the highest mean incidence (0.7%), followed by lots from Denmark (0.1%) and the Netherlands (0.1%). Of the 32 seed lots from the United States, 22 (68.8%) were infected with *C. variabile* and/or *C. macrocarpum*, compared with 1 of 6 lots (16.7%) and 3 of 27 lots (11.1%) from the Netherlands and Denmark, respectively. *C. variabile* and *C. macrocarpum* were not detected in the single seed lot from New Zealand. Seed lots from company F had the highest mean incidence of *C. variabile* and/or *C. macrocarpum* (2.9%), followed by companies A (0.5%) and C (0.1%). The two fungi were not detected in seed lots from companies B, D, and E, but were detected in 17 of 27 lots (63.0%) from company A and 6 of 24 lots (25.0%) from company C.

The correlation between incidence of *S. botryosum* and incidence of C. *variabile* and/or *C. macrocarpum* was not significant ($r = –0.04$) for all 11 seed lots produced in 2000 to 2002 (Table 1). However, lot US-3 was harvested from a research trial inoculated with *S. botryosum* and *C. variabile*, and excluding this lot resulted in a significant negative correlation ($r = –0.54$ at $P < 0.05$) between the incidences of *S. botryosum* and *C. variabile* or *C. macrocarpum*. Similarly, there was a significant negative correlation in the incidence of these fungi ($r = –0.27$ at $P < 0.05$) on the 2003 seed lots assayed (Table 2).

Duration of survival of *C. variabile* and/or *C. macrocarpum* in spinach seed. *C. variabile* and/or *C. macrocarpum* were detected in only 2 of the 12 seed lots produced in 1993 to 2001 in western Washington (Table 3). The fungi were observed at 0.5% incidence in an 8-year-old seed lot (1996 crop) and a 3-year-old lot (2001 crop). For seed lot US-3, the fungi were detected in 49.0, 2.3, and 0.0% of the seed when assayed 1 (fall 2001), 21 (summer 2003), and 28 months (fall 2004) after harvest, respectively.

Other fungi detected on spinach seed. Incidence of *Verticillium* in the seed lots listed in Tables 1 and 2 has been published (11). *Verticillium* was also present in 9 of the 12 seed lots produced in 1993 to 2001 in the United States, including two 11-year-old spinach seed lots harvested in 1993, at 7.0 and 19.0% incidence (data not shown). Most isolates resembled *V. dahliae* based on development of dark brown to black microsclerotia in the pericarps and the presence of hyaline conidiophores (18,39). There was no significant correlation between the percentage of seedborne *Verticillium* and the duration of storage (year of harvest) of the seed lot. *Colletotrichum* spp. were identified based on development of acervuli with black setae and single-celled, hyaline conidia (27). Isolates of this genus were observed on 3 of the 66 seed lots (4.5%) produced in 2003, including 2 of 27 seed lots (7.4%) from Denmark at a mean incidence of 0.04%, and 1 of 6 seed lots (16.7%) from the Netherlands at a mean incidence of 0.04%. All three lots were from the same company. *Colletotrichum* was not present in the seed lots from New Zealand or the United States. Species of *Acremonium*, *Alternaria*, *Bipolaris*, *Botrytis*, *Epicoccum*, *Fusarium*, *Gonatobotrys*, *Penicillium*, and *Ulocladium* completed the mycoflora observed on the seed lots (data not shown).

Pathogenicity of seed isolates of *S. botryosum*, *C. variabile*, and *C. macrocarpum*. All seed isolates of *S. botryosum* from the seed lots assayed in the preliminary survey (Table 1) proved pathogenic on spinach in the greenhouse, resulting in symptoms typical of Stemphylium leaf spot (Fig. 2) (7,22). *S. botryosum* was reisolated from the symptomatic leaves. Similarly, putative isolates of *C. variabile* from these seed lots were pathogenic on spinach, resulting in symptoms typical of Cladosporium leaf spot (Fig. 2) (7). *C. variabile* was reisolated from the symptomatic leaves. Plants inoculated with a small-spored *Cladosporium* sp. from seed

lot US-3 did not develop symptoms of Cladosporium leaf spot, and no fungi were reisolated from these plants. Noninoculated control plants did not develop symptoms, and no fungi were reisolated from the control plants.

Eleven of 24 isolates of *S. botryosum* (45.8%) obtained from seed lots produced in 2003 were pathogenic on spinach (Table 2): two from New Zealand, five from Denmark, and four from the Netherlands. None of the six isolates of *S. botryosum* from seed lots produced in the United States in 2003 was pathogenic on spinach. However, all 14 isolates of *S. botryosum* from seed lots produced in the United States from 1993 to 2001 were pathogenic on spinach (Table 3). Of the 15 putative isolates of *C. variabile* from the 2003 seed lots, five (33.3%) were pathogenic on spinach (Table 2). All five of these isolates were from seed lots grown in Denmark. Further microscopic examination of the 10 nonpathogenic isolates demonstrated the isolates to be *C. macrocarpum*, not *C. variabile*, based on the absence of torulose aerial hyphae (Fig. 1E) as well as a lack of pathogenicity on spinach (12). The coiled aerial hyphae were observed when *C. variabile* developed on symptomatic leaf tissue in moist chambers or on agar media, but did not develop when *C. variabile* grew directly on agar media. During subsequent freeze-blotter seed assays, it was noted that coiled aerial hyphae were only observed after at least 5 days of incubation of the seed. None of the small-spored *Cladosporium* spp. was pathogenic on spinach.

Component freeze-blotter seed assays. *S. botryosum* was detected in the pericarps and embryos of all five seed lots subjected to component seed assays (Table 4), with ranges of 13 to 91% and 5 to 76% of the pericarps and embryos infected, respectively. The incidence of *S. botryosum* in embryos was always lower than the incidence in pericarps, regardless of the method of preparation of the seed for component seed assays. The incidence of this fungus in embryos ranged from 38.5 to 83.5% of the incidence in the pericarps. *C. variable* and/or *C. macrocarpum* were present at a much lower incidence than *S. botryosum* in all five seed lots (Table 4). The *Cladosporium* spp. were observed in 2 and 19% of the pericarps of seed lots WSU1 and WSU2, respectively, but were not detected in the embryos of lot WSU1, and were observed on the embryo of only one seed of lot WSU2. Neither of these species was observed in the pericarps or embryos of lots D1, B2, and B5. The presence of *S. botryosum* and *C. variabile* or *C. macrocarpum* in embryos that had been surface-sterilized (lot WSU2) demonstrated internal infection of the embryos.

Electron microscopy. Intracellular hyphae were observed in the pericarps of all six seed from lot 03-409 that were examined by TEM (Fig. 3). In some cells, the intracellular hyphae were observed penetrating the pericarp cell walls. Intercellular hyphae were not observed by TEM. Fungal structures were not detected in any of the six embryos examined by TEM. Conidia and conidiophores of *S. botryosum* and *C. variabile* or *C. macrocarpum*, and immature pseudothecia of *P. herbarum,* were observed on the pericarps using SEM. Chains of up to three verrucose conidia of *C. variabile* or *C. macrocarpum*, single verrucose conidia of *S. botryosum*, and globose, immature, erumpent pseudothecia of *P. herbarum* were observed by SEM.

DISCUSSION

The prevalence of *S. botryosum* in spinach seed was demonstrated by detection of *S. botryosum* in each of 89 spinach seed lots assayed in this study, at incidences ranging from <1 to >95% for individual lots. These seed lots were produced in Denmark, the Netherlands, New Zealand, and the United States by six seed companies, representing the primary spinach seed production areas of the world at the time this survey was carried out. To our knowledge, *S. botryosum* has not previously been reported on spinach seed grown in Denmark, the Netherlands, or New Zealand. In contrast to *S. botryosum*, *C. variabile* and/or *C. macrocarpum* were detected in only 39 of the 89 spinach seed lots assayed (43.8%). *C. variabile* and/or *C. macrocarpum* were present in all 11 seed lots produced in 2000, 2001, or 2002, at a range of 0.3 to 49.0% for individual lots. However, these fungi were detected in only 26 of the 66 seed lots produced in 2003, at incidences of <1 to 3% for individual lots, and in only two of the 12 seed lots produced in 1993 to 2001, at 0.5% incidence for each lot.

Isolates of *S. botryosum* obtained from seed lots produced in 1993 to 2001 each proved pathogenic on spinach. However, only 11 of 24 isolates (<50%) of *S. botryosum* obtained from the 2003 seed lots proved pathogenic on spinach. The 12 isolates that were not pathogenic on spinach were morphologically similar to the pathogenic isolates, although conidia and conidiophores were not measured for each isolate. These isolates may not be pathogenic to the particular spinach inbred lines used in the pathogenicity tests, highlighting the need to investigate the genetic nature of this host–pathogen system. Another possibility is loss of pathogenicity of these isolates during storage on colonized filter disks at –80°C for 10 to 14 months before pathogenicity tests were completed, compared with 4 to 5 months of storage for the other isolates tested for pathogenicity. The authors have observed loss of pathogenicity of several spinach isolates of *S. botryosum* after about a year of storage at –20 or –80°C, but not for isolates of *C. variabile*.

Isolates of putative *C. variabile* from seed lots produced in 1993 to 2002 were pathogenic on spinach. In contrast, only 5 of the 15 isolates initially identified as *C. variabile* from the 2003 seed lots were pathogenic. Subsequent microscopic examination of the 10 nonpathogenic isolates revealed the absence of torulose aerial hyphae, indicating that these were isolates of *C. macrocarpum*, not *C. variabile* (6,12). Although Correll et al. (5), Hadzistevic (16), and Van Poeteren (40) reported that *C. macrocarpum* causes a leaf spot of spinach, Ellis (12) reported that *C. variabile* can be distinguished from *C. macrocarpum* by the presence of spirally coiled aerial hyphae, and pathogenicity to spinach of the former species only. We could not distinguish the two species based on morphology of conidia and conidiophores as observed in the freeze-blotter seed health assay until at least 5 days after plating the seed, when torulose aerial hyphae were produced by isolates of *C. variabile*. Based on morphological characteristics alone, these fungi could readily be misidentified using the freeze-blotter seed health assay. Research is needed to determine whether *C. variabile* and *C. macrocarpum* are genetically distinct. This information could aid in development of a more efficient and specific seed assay than the freeze-blotter seed

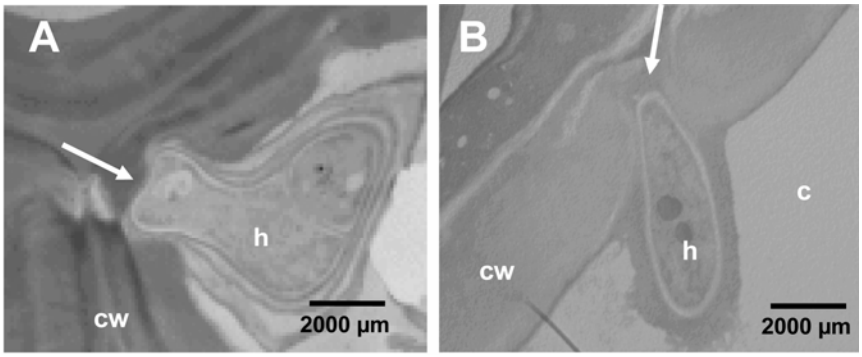

Fig. 3. Transmission electron micrographs of intracellular hyphae in the pericarp cells of spinach seed infected with *Stemphylium botryosum* **(A)** and *Cladosporium variabile* or *C. macrocarpum* **(B)**. (cw = pericarp cell wall, h = hyphal cell of the fungus, c = plant cytoplasm, and arrow = site of penetration through the pericarp cell wall.)

health assay, such as the PCR-based seed assay developed by Pryor and Gilbertson (32) for detection of *Alternaria radicina* on carrot seed.

Of the 66 seed lots assayed from the 2003 season, 59 (89%) were produced in western Washington or Denmark. Spinach seed crops in western Washington are typically planted in April or May and harvested in August or September. The average monthly temperatures in May, June, July, August, and September 2003 in western Washington were 0.2, 1.9, 2.0, 0.5, and 0.8°C higher, respectively, than the average monthly temperatures from 1992 to 2003; i.e., the 2003 season in western Washington was warmer than the previous 13 years (WSU Public Agricultural Weather System, Prosser, WA [published online]). Similarly, the Danish Meteorological Institute reported that 2003 was a very warm year in Denmark compared with the average temperatures from 1961 to 1990 (published online). This may account for the greater prevalence of *S. botryosum* than of *C. variabile* in the 2003 seed lots, as the optimal temperature range for growth of *S. botryosum* on agar media is 18 to 24°C (22) compared with 15 to 20°C for *C. variabile* (15). In contrast to results for the 2003 seed lots, individual seed lots produced in 2000, 2001, or 2002 were infected with *C. variabile* or *C. macrocarpum* at incidences up to 27.3%. Significant negative correlations detected in this study between the incidences of seedborne *S. botryosum* versus *C. variabile* or *C. macrocarpum* suggest that infection of the developing seed in spinach seed crops may be favored by different conditions for these fungi.

Conditions favorable for long-term storage of seed of most crops include 4 to 10°C and 50% relative humidity (29). Unfortunately, these conditions also favor survival of seedborne pathogens, with the duration of survival decreasing at higher temperatures and relative humidities in storage (29). Viable, pathogenic isolates of *S. botryosum* were isolated from spinach seed lots that had been stored by a seed company at 4.4°C and 60% relative humidity for 3, 8, 10, and 11 years. *C. variabile* and/or *C. macrocarpum* were detected in 3- and 8-year-old seed lots stored under the same conditions, although at much lower incidences than *S. botryosum*. These storage conditions also supported long-term survival of *Verticillium* in spinach seed, as demonstrated by detection of *Verticillium* in seed stored for 11 years. Fuentes-Davila (15) obtained pathogenic isolates of *C. variabile* from 6-year-old spinach seed lots. In this study, *S. botryosum* and *C. variabile* or *C. macrocarpum* were detected at incidences of 54.8 and 49.0%, respectively, in seed lot US-3 when assayed a month after harvest. By 28 months after harvest, the incidence of *S. botryosum* had dropped to 0.3%, and *C. variabile* or *C. macrocarpum* could no longer be detected. Lot US-3 was stored at a similar relative humidity but much higher mean temperature (18.8°C) than the 3- to 11-year-old commercial seed lots, because of the lack of optimum seed storage facilities at the WSU-NWREC. Although we do not know the original incidences of the fungi in these 12 seed lots, detection of *S. botryosum* at incidences of 7.0 and 7.5% in seed lots stored for 11 years demonstrates that storing spinach seed at optimum temperatures for maintaining seed viability also supports survival of *S. botryosum*, *C. variabile* or *C. macrocarpum*, and *V. dahliae*. Similarly, *Alternaria brassicae* (Berk.) Sacc. persisted for 10 months in rape and mustard seed stored at room temperature (11 to 25°C), but for only 6 months in seed stored at 30°C (36). The pathogen could not be detected on seed stored at 40°C for 4 months. Seedborne *Drechslera* spp. and *Fusarium* spp. were eliminated, and *Alternaria tenuis* Nees was reduced from 80 to 10% incidence when infected barley seed were stored for 24 weeks at 20°C and 14% relative humidity (26).

Component seed assays revealed that *S. botryosum* and *C. variabile* or *C. macrocarpum* can reside in the pericarp as well as the embryo of spinach seed, although the fungi were detected at higher incidences in the pericarps than the embryos. The fungi were detected in embryos that had been surface-sterilized, demonstrating that they can be internal and external seedborne fungi of spinach, as documented for several other seedborne pathogens (4,24,25,28). Using TEM in this study, intracellular hyphae were detected in the pericarps of spinach seed that appeared to be infected only by *S. botryosum* or *C. variabile* and/or *C. macrocarpum*. Hyphae were not observed in the embryos examined by TEM, but a very limited number of seed (six) were examined by TEM. *S. botryosum* was detected in a greater number of the embryos assayed than *C. variabile* or *C. macrocarpum*, suggesting that infection of spinach seed by *S. botryosum* may more frequently be internal than infection by *C. variabile* or *C. macrocarpum*. However, the incidence and location of these fungi in spinach seed may be influenced by maturity of the seed crop at the time of infection, and how favorable weather conditions are for infection of the developing seed.

The prevalence of *S. botryosum* in spinach seed lots, combined with routine interstate and international movement of spinach seed, might explain the recent first reports or observations of this pathogen in Arizona (23), California (22), Florida (34), Maryland and Delaware (13), Oregon (M. L. Putnam and L. J. du Toit, *unpublished data*), and Washington (7). Raid (33) reported that the nature of outbreaks of Stemphylium leaf spot in baby leaf spinach crops in Florida suggested that infected seed may be the source of inoculum. Anecdotal evidence of seed transmission of *C. variabile* in spinach was reported in Denmark in 1952 (17), but Fuentes-Davila (15) could not demonstrate seed transmission of *C. variabile*. Seed transmission of *S. botryosum* and *C. variabile* in spinach was recently demonstrated in greenhouse experiments (19,20). Additional research is warranted to identify thresholds for seedborne inoculum under different field production practices, and the potential efficacy of seed treatments (chlorine, hot water, biological, and fungicide) for eradication of the fungi from infected spinach seed or prevention of seed transmission. Furthermore, the significance of seedborne inoculum relative to other sources of inoculum (infested spinach residues or infected volunteer spinach [9]) remains to be determined. This information will help determine the role of infested or infected seed in dissemination of *C. variabile* and *S. botryosum* in the spinach seed industry, and the need for seed treatments to help manage these pathogens.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support for this project from the Robert MacDonald Vegetable Seed Memorial Fund, the Puget Sound Seed Growers' Association, the Washington State Commission for Pesticide Registration, and the Alfred Christianson Family Endowment; seed samples provided by seed companies; assistance from Frank Dugan at differentiating isolates of *C. variabile* from *C. macrocarpum*; and technical assistance from Louise Brissey, Kerri Brooks, Mike Derie, Barbara Holmes, and Nathan Lloyd. This project was supported by the Agricultural Research Center of the College of Agricultural, Human, and Natural Resource Sciences at Washington State University, and is listed as Plant Pathology New Series No. 0403.

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