MANAGEMENT OF SEEDBORNE *STEMPHYLIUM BOTRYOSUM* AND *CLADOSPORIUM VARIABILE* CAUSING LEAF SPOT OF SPINACH SEED CROPS IN WESTERN WASHINGTON

BY
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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of PABLO HERNANDEZ-PEREZ find it satisfactory and recommend that it be accepted.

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Chair

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MANAGEMENT OF SEEDBORNE *STEMPHYLIUM BOTRYOSUM* AND *CLADOSPORIUM VARIABILE* CAUSING LEAF SPOT OF SPINACH SEED CROPS IN WESTERN WASHINGTON

ABSTRACT

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Up to 50% of the spinach seed planted in the USA and up to 20% of the world supply of spinach seed is produced in the Pacific Northwest. Washington is the primary spinach seed producing state in the USA, where Cladosporium and Stemphylium leaf spots can cause significant losses in spinach seed crops.

Stemphylium botryosum was present in each of 66 spinach seed lots produced in 2003 in the USA, Denmark, the Netherlands, and New Zealand at a mean incidence of 29.38% per lot. *Cladosporium* resembling *C. variabile* were present in 26 of 66 lots at a mean incidence of 0.37% per lot. Pathogenicity tests with 24 isolates of *S. botryosum* and 15 isolates of *Cladosporium* resembling *C. variabile* demonstrated 11 and 5, respectively, were pathogenic on spinach; the 10 non-pathogenic isolates of *Cladosporium* proved to be *C. macrocarpum*. Pathogenic isolates of *S. botryosum* were also detected in 11-year-old seed lots. *Stemphylium botryosum* and *C. variabile* were more prevalent in the pericarps than the embryos of spinach seed.

Cladosporium variabile and *Verticillium* spp. (primarily *V. dahliae*) were largely eliminated from spinach seed soaked in 1.2% NaOCl for ≥10 min. Although the incidence of seedborne *S. botryosum* was reduced significantly, this pathogen was not eradicated from spinach seed by chlorine treatment. Seed germination was not affected by a 40-min soak in 1.2% NaOCl. *Cladosporium variabile* was eradicated from spinach seed soaked in water heated at 40°C for 10 min. *Stemphylium botryosum* was eradicated from a lightly infected seed lot by hot water treatment at ≥55°C for ≥10 min, but could not be eradicated from two heavily infected lots even when treated at 60°C for 40 min. Seed germination was adversely affected by hot water treatment at 50°C for 30 to 40 min, or 55 or 60°C for ≥10 min.

Cladosporium variabile and *S. botryosum* were transmitted from infected spinach seed planted in the greenhouse and maintained under misters at 14.4 to 21.4°C and 74.5 to 95.4% relative humidity. Lesions first appeared on cotyledon tips (direct seed transmission), followed by cotyledon blades and first true leaves (secondary spread). Seed transmission rates ranged from 0.4 to 18.1% and 3.7 to 10.3% of the incidence of planted seed infected with *C. variabile* and *S. botryosum*, respectively.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii
ABSTRACT iv
TABLE OF CONTENTS vi
LIST OF TABLES viii
LIST OF FIGURES ix
DEDICATION x
CHAPTER
1. Literature review
1.1. Spinach and spinach seed production 1
1.2. Fungal leaf spots of spinach 2
1.3. Cladosporium leaf spot (*Cladosporium variabile* (Cooke) de Vries) 2
1.3.1. History and geographic distribution 2
1.3.2. Importance 3
1.3.3. Causal agent 3
1.3.4. Host range 4
1.3.5. Symptoms 4
1.3.6. Epidemiology 5
1.3.7. Management 6
1.3.7.1. Chemical control 6
1.3.7.2. Cultural control 8
1.3.7.3. Resistant cultivars 8
1.4. Stemphylium leaf spot (*Stemphylium botryosum* Wallr.) 8
1.4.1. History and geographic distribution 8
1.4.2. Importance 9
1.4.3. Causal agent 10
1.4.4. Host range 11
1.4.5. Symptoms 11
1.4.6. Epidemiology 12
1.4.7. Management 13
1.4.7.1. Chemical control 13
1.4.7.2. Cultural control 14
1.4.7.3. Resistant cultivars 14
1.5. Cladosporium and Stemphylium leaf spot of spinach seed crops: Research needs 15
1.6. Literature cited 16

2. Prevalence of *Stemphylium botryosum* and *Cladosporium variabile* in commercial spinach seed lots
2.1. Introduction 21
2.2. Materials and methods 23
2.2.1. Spinach seed lots 23

2.2.2. Freeze-blotter seed assays 23
2.2.3. Pathogenicity of seed isolates of *S. botryosum* and *C. variabile* 25
2.2.4. Component freeze-blotter seed assays 27
2.2.5. Electron microscopy 28
2.3. Results 31
2.3.1. Prevalence of *Stemphylium botryosum* in spinach seed 31
2.3.2. Prevalence of *C. variabile* in spinach seed 31
2.3.3. Other fungi detected 32
2.3.4. Pathogenicity of seed isolates of *S. botryosum* and *C. variabile* 33
2.3.5. Component freeze-blotter seed assay 34
2.3.6. Electron microscopy 34
2.4. Discussion 35
2.5. Literature cited 42

3. Efficacy of hot water and chlorine seed treatments for eradication of *Stemphylium botryosum* and *Cladosporium variabile* from spinach seed
3.1. Introduction 53
3.2. Materials and methods 55
3.2.1. Chlorine seed treatments 55
3.2.2. Hot water seed treatments 57
3.2.3. Component freeze-blotter seed assay 59
3.3. Results 60
3.3.1. Chlorine seed treatments 60
3.3.2. Hot water seed treatments 62
3.3.3. Component freeze-blotter seed assay 66
3.4. Discussion 66
3.5. Literature cited 71

4. Seed transmission of *Stemphylium botryosum* and *Cladosporium variabile* in spinach
4.1. Introduction 83
4.2. Materials and methods 85
4.3. Results 90
4.4. Discussion 95
4.5. Literature cited 99

LIST OF TABLES

Chapter 2

Page

Table 1. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in 66 spinach seed lots produced in 2003 in Denmark, the Netherlands, New Zealand, and the USA by six seed companies. 46

Table 2. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in 12 spinach seed lots produced from 1993 to 2001 in western Washington, USA. 47

Table 3. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in spinach seed lot 01-101 produced in western Washington in 2001 and assayed in 2001, 2003, and 2004. 48

Table 4. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in the pericarps and embryos of five spinach seed lots. 49

Chapter 3

Table 1. Analyses of variance for germination and seed health assays of spinach seed lots treated with chlorine (1.2% NaOCl) for durations ranging from 0 to 40 (trial 1) or 60 min (trial 2). 73

Table 2. Efficacy of chlorine seed treatments for eradication of *Cladosporium variabile, Stemphylium botryosum, Verticillium* spp., and other fungi from spinach seed. 74

Table 3. Analyses of variance for germination and seed health assays of spinach seed treated with hot water. 75

Table 4. Efficacy of hot water treatments on spinach seed quality. 77

Table 5. Efficacy of hot water seed treatments for eradication of *Cladosporium variabile, Stemphylium botryosum,Verticillium* spp., and other fungi from spinach seed..... 80

Chapter 4

Table 1. Seed transmission and secondary spread of *Stemphylium botryosum* and *Cladosporium variabile* in spinach..... 102

Table 2. Temperature, relative humidity, and leaf wetness in greenhouse seed transmission trials for *Stemphylium botryosum* and *Cladosporium variabile* in spinach..... 103

LIST OF FIGURES

Chapter 2

Page

Fig. 1. Cross section of spinach fruit showing seed structure (from Peterson and Harris, 1997). 50

Fig. 2. Transmission electron micrographs of *Stemphylium botryosum* (A and C) and *Cladosporium variabile* (B and D) inside cells of the pericarps of spinach seed. cw = pericarp cell wall, h = hyphal cell of the fungus, c = plant cytoplasm, and arrow = site of penetration through the pericarp cell wall. 51

Fig. 3. Scanning electron micrographs of conidia and conidiophores of *Cladosporium variabile* (A and B), conidium and conidiophore of *Stemphylium botryosum* (C), and erumpent immature pseudothecium of *Pleospora herbarum* (D) on spinach seed..... 52

Chapter 4

Fig. 1. Lesions resulting from seed transmission of *Cladosporium variabile* and *Stemphylium botryosum* on spinach. A) Lesions caused by *S. botryosum* on cotyledon tips. B) Lesions caused by *S. botryosum* on cotyledon tips with the pericarp attached. C) Lesions caused by *C. variabile* on a cotyledon blade. D) Lesions caused by *C. variabile* on a true leaf. E) Lesions caused by *S. botryosum* on the first pair of true leaves. 104

DEDICATION

A mi increíblemente inteligente y dulce esposa Julie Kate y nuestros maravillosos hijos: Abel Guy y Grace Navidad. A Antonia, mi madre. A mis hermanos y sobrinos.

Chapter 1

Literature review

1.1. Spinach and spinach seed production.

Spinach (*Spinacia oleracea* L.), a member of the Chenopodiaceae (Sanders, 2003), is a source of various vitamins and minerals such as vitamin A, calcium, phosphorus, iron, and potassium, and is a significant source of protein (Mills, 2001). This plant was first cultivated in Iran around 400 AD. Around 1100 AD, spinach was introduced to Spain by the Arabs, and by 1400 AD spinach could be found throughout Europe. Spinach is believed to have been introduced to North America in 1828 (Anonymous, 2003). Consumption of spinach was promoted commercially in the USA beginning in the 1920s (Mills, 2001).

Spinach is now an important leafy vegetable consumed in many countries of the world (Lucier, 1993). In the USA, the principal states producing spinach are Arizona, Arkansas, California, Colorado, Maryland, New Jersey, Oklahoma, Texas, and Virginia (Correll et al., 1994; Ryder, 1976), where spinach is grown for the fresh and processed (frozen and canned) markets (Lucier, 1993), with a combined cultivated area of 16,000 ha and an annual value of $184 million (Lucier, 2000).

Spinach is considered a small-seeded vegetable compared to large-seeded vegetables such as cucurbits and legumes. Up to 50% of the spinach seed planted in the USA and up to 20% of the world supply of spinach seed is produced in the Pacific Northwest, where long summer daylength and moderate temperatures favor production of spinach seed crops (Foss & Jones, 2000; Thomas et al., 1997). In Washington, the primary spinach-seed-producing state in the USA, small-seeded vegetable seed production takes place on approximately 15,000 acres with 6,000 acres in western Washington (Thomas et al., 1997). About 2000 to 4000 acres of spinach

seed crops are grown annually in Washington, making spinach the most economically important small-seeded vegetable seed crop grown in western Washington. The market value of spinach seed sold to commercial growers is $1,000 to $1,200 per acre, with seed growers' production costs averaging $900 per acre (Foss and Jones, 2000; Thomas et al, 1997).

1.2. Fungal leaf spots of spinach.

Spinach can be infected by several fungi that cause leaf spot diseases (Correll et al., 1994): *Alternaria spinaciae* Allescher and Noack, *Ascochyta spinaciae* Bondartzeva-Monteverde, *Cercospora bertrandii* Chupp, *Phyllosticta chenopodii* Saccardo, *Ramularia spinaciae* Nypels, *Entyloma ellisii* Haltsed (Sherf and MacNab, 1986), *Cladosporium macrocarpum* Preuss, *Cladosporium variabile* (Cooke) de Vries, *Colletrotichum dematium* (Pers.) Grove f. sp. *spinaciae* (Ellis & Halst.), and *Stemphylium botryosum* Wallr. (Correll et al., 1994; Inglis et al., 1997; Koike et al., 2001; Sherf and MacNab, 1986). *Stemphylium botryosum* and *C. variabile* have been identified as the principal pathogens causing leaf spot of spinach seed crops in western Washington (du Toit and Derie, 2001).

1.3. Cladosporium leaf spot (*Cladosporium variabile* (Cooke) de Vries).

1.3.1. History and geographic distribution. *Cladosporium variabile*, causing a leaf spot disease on spinach also known as "rust", was described as *Heterosporium variabile* by Cooke in 1877 in Great Britain (Reed and Cooley, 1911). Before then, Cooke had described the fungus as *Helminthosporium variabile* (Reed and Cooley, 1911). In the USA, the fungus was first reported in 1906 in Connecticut by Clinton (1906). In 1911, the fungus was reported on spinach in Virginia by Reed and Cooley (1911). David (1995) reported the presence of *C. variabile* in the

USA, Austria, Belgium, Canada, Great Britain, France, Hungary, India, Italy, Japan, Spain, Turkey, China, Denmark, Germany, Romania, and the Ukraine. Subsequent to the first reports of Cladosporium leaf spot of spinach in the USA, the disease has been reported in California, Idaho, Massachusetts, Montana, New Jersey, New York, Oklahoma, Texas, and Washington (Fuentes-Davila, 1988).

1.3.2. Importance. Cladosporium leaf spot of spinach is of significance when spinach is grown for its leaves, because the disease can cause spotting of spinach leaves and, under severe disease pressure, infected leaves may dry and dehisce (Mathur and Sehgal, 1965), reducing the market value of the crop (Fuentes-Davila, 1988). *Cladosporium variabile* has caused major outbreaks of leaf spot in spinach seed crops in western Washington, which affected seed production significantly (Inglis et al., 1997). Japan, a primary importer of spinach seed from the USA, has established that this pathogen is seedborne and seed transmitted, which affected some export markets for spinach seed lots from the Pacific Northwest (Fuentes Davila, 1988). However, no phytosanitary restrictions have been issued for the import countries of spinach seed produced in the USA regarding *C. variabile* (Phillip Brown, *personal communication*).

1.3.3. Causal agent. de Vries (1952) established that *C. variabile* develops woolly-felty, grayish, white or pale red colonies after 11 days of growth at 18°C on glucose agar. Unbranched conidiophores develop from hyphae immersed in the media and measure up to 350 μ in length (usually <150 μm) and 3 to 5 μm in diameter (up to 6 or 8 μm in natural substratum). Conidiophores are thick-walled and regularly septate, producing short chains of up to five conidia (usually three). Conidia are densely verrucose, olivaceous brown, ovate, citriform to cylindrical in shape (de Vries, 1952), and may be of three types: 1) very dark conidia that are thick-walled, multi-celled, and 10 to 16 μm in length; 2) paler, narrower conidia, 5 to 8 μm wide,

and multi-celled; and 3) smooth, oval, one-celled conidia (Jacques, 1941). Ellis (1971) reported that conidia are 0- to 3-septate, 5 to 30 μm in length and 3 to 13 μm in diameter (usually 15 to 25 μm in length and 7 to 10 μm in diameter), and that *C. variabile* develops tortuous and spirally coiled aerial hyphae. The author also notes that these coiled hyphae, along with pathogenicity to spinach, are primary reasons *C. variabile* is recognized as distinct from *C. macrocarpum*.

1.3.4. Host range. In addition to *S. oleracea*, *C. variabile* has been reported to be pathogenic on beet (*Beta vulgaris* L.), goosefoot weed (*Chenopodium amaranticolor* Coste & Reyn.), cabbage (*Brassica oleracea* var. *capitata* L.), shinleaf (*Pyrola grandiflora* R.), star-thistle (*Centaurea diffusa* Lam), *Sambucus* spp., *Galtonia* spp., and *Iris* spp. (Jaques, 1941; Frank DiCosmo et al., 1982).

1.3.5. Symptoms. When spinach is infected by *C. variabile*, the leaves develop circular spots, often on lower and older leaves first, that are initially white to yellow and later turn tan in color, rarely exceeding 6 mm in diameter (Anonymous, 2001; Fuentes-Davila and Gabrielson, 1987; Inglis et al., 1997). Each spot usually develops a narrow brown margin (du Toit and Derie, 2001). The disease may progress from older to younger leaves if conditions remain conducive. Individual spots may coalesce, forming irregular spots (Mathur et al., 1969; Inglis et al., 1997). Dark green sporulation consisting of conidia and conidiophores occurs in the lesions as they mature, a feature that can distinguish this disease from other leaf spots of spinach such as anthracnose and Stemphylium leaf spot (Anonymous, 2001). Older leaves may die prematurely and, when disease is severe, all leaves of the plant may perish (Reed and Cooley, 1911; Fuentes-Davila and Gabrielson, 1987; Inglis et al., 1997).

1.3.6. Epidemiology. Infection of spinach by *C. variabile* is favored by temperatures between 15 and 20°C and relative humidity >80%. However, the pathogen is able to infect

spinach at temperatures ranging from 10 to 30°C (Fuentes-Davila, 1988). Fuentes Davila (1988) reported that conditions with both favorable temperature and relative humidity for Cladosporium leaf spot do not always occur concurrently in Skagit County, WA. Therefore, this area does not always experience epiphytotics of Cladosporium leaf spot in spinach seed crops. Nonetheless, severe outbreaks of the disease have been reported, and infection can occur on seed developing in the crop (Inglis et al, 1997).

Mathur and Sehgal (1969) reported that *C. variabile* penetrates the spinach leaf through the cuticle. They did not observe appressoria during the infection process. Fuentes-Davila (1988) suggested that different physiological forms of the fungus may exist with the different forms possibly infecting the host by different methods. He observed penetration of *C. variabile* through stomata and detected structures resembling appressoria on the leaf surface. Once infection is established, *C. variabile* produces vesicles that develop hyphae that grow inter- and intra-cellularly in the plant (Mathur et al., 1969; Fuentes-Davila, 1988). Penetration via stomata was observed to occur 48 h after inoculation of the fungus onto spinach plants, and two-celled conidia infected the leaves more frequently than one- or three-celled conidia (Fuentes-Davila, 1988). Fuentes-Davila (1988) observed symptoms of infection by *C. variabile* in spinach three days after inoculation followed by incubation in dew chambers, and in seven to 10 days after inoculation and incubation in growth chambers.

Sporulation occurs within leaf lesions. Spores of *C. variabile* are disseminated by wind, splashing water, and farm equipment. Severity of Cladosporium leaf spot may increase slightly in the presence of spinach pollen (du Toit and Derie, 2002, Inglis et al., 1997). *Cladosporium variabile* survives the winters in western Washington on infected volunteer spinach (du Toit and Derie, 2003b; Fuentes Davila, 1988), and may survive on weed hosts, plant residues, and

discarded seed (Inglis et al., 1997). The fungus can survive in dry, infected spinach leaves for at least 18 months without losing virulence (Fuentes-Davila, 1988).

Cladosporium variabile is seedborne in spinach. However, although seed transmission of *C. variabile* in spinach has been documented by citation, it has not been demonstrated conclusively (du Toit and Derie, 2003a and 2003b; Fuentes-Davila, 1988; Hansen et al., 1952; Inglis et al., 1997).

1.3.7. Management.

1.3.7.1. Chemical control. Prior to 2002, growers in Washington relied primarily on two registered fungicides to help manage Cladosporium leaf spot in spinach seed crops: chlorothalonil (Bravo Weather Stik, Syngenta Crop Protection, Inc., Greensboro, NC) at 1.42 liters/A on a 7- to 14- day spray interval, and mancozeb (Dithane DF Rainshield NT, Dow Agrosciences LLC, Indianapolis, IN) at 907.20 g/A [WSU Pesticide Information Center On-Line (PICOL), TriCities, WA (http://picol.cahe.wsu.edu/labels/Labels.php?SrchType=C)]. These preventative fungicides were typically applied early in the season before symptoms were observed in the crops (Inglis et al., 1997). Currently, azoxystrobin (e.g., Amistar, Syngenta Crop Protection, Inc.), is also registered in Washington to manage leaf spot in spinach (PICOL, 2004). These fungicides provide effective control of Cladosporium and Stemphylium leaf spots in spinach seed crops if applied appropriately (du Toit and Derie, 2003a; du Toit et al., 2004, 2005a, 2005b, 2005c). Other formulations of mancozeb and chlorothalonil are also registered in Washington to manage leaf spot in spinach seed crops (PICOL, 2004).

In 1970, bordeaux mixture was shown to inhibit significantly germination of *C. variabile* spores and provide good protection of spinach leaves against infection by the fungus (Mathur and Sehgal, 1970). Fuentes-Davila (1988) reported that leaf spot caused by *C. variabile* was

completely controlled on inoculated plants in the greenhouse with applications of benomyl (Benlate 50WP, DuPont Chemical Co., Wilmington, DE) and chlorothalonil alone or in combination. In field trials, Fuentes-Davila (1988) found that best control of the disease was achieved with foliar applications of benomyl + iprodione (Rovral 4F, Bayer Crop Science, Pittsburgh, PA), chlorothalonil, benomyl + chlorothalonil, and benomyl + flusilazol (FMC Corporation, Philadelphia, PA). Fungicide trials performed in the greenhouse and in the field over several years by du Toit and Derie (2003a), and du Toit et al. (2004, 2005a and b) demonstrated that fungicides in the strobilurin family [i.e., azoxystrobin (Quadris 2.08 FL, Syngenta Crop Protection, Inc.), pyraclostrobin (Cabrio EG, BASF Corp., Research Triangle Park, NC), pyraclostrobin + boscalid (Pristine, BASF Corp.), and kresoxim-methyl (Sovran, BASF Corp.)], as well as iprodione (Rovral 4F) and thiophanate-methyl (Topsin M 70WP, Cerexagri, Inc., King of Prussia, PA) were highly efficacious against Cladosporium leaf spot, significantly reducing leaf spot severity in the crops and incidence of *C. variabile* on the harvested seed.

Spinach seed treatment with captan (2.5 g a.i./kg seed), benomyl, and thiram (each at 5.0 g a.i./kg seed), and baytan, bitertanol, and flusilazol (each at 0.6 g a.i./kg seed) completely prevented detection of *C. variabile* on the seed (Fuentes-Davila, 1988). Treating infected spinach seed with chlorine (1.2% NaOCl) for up to 20 minutes eliminated *C. variabile* from the seed (du Toit and Derie, 2003b). Benomyl has been cancelled by the Enviromental Protection Agency (http://www.epa.gov/fedrgstr/EPAFR-CONTENTS/2002/January/Day-15/contents.htm). The evaluation of fungicides less environmentally harmful to treat spinach seed would be valuable. The report of chlorine spinach seed treatment by du Toit and Derie (2003b) is the only report of chlorine spinach seed treatment available to the public. Further research on the efficacy of seed

treatments (e.g., hot water and fungicides) for management of *C. variabile* in spinach seed is needed.

1.3.7.2. Cultural control. To manage Cladosporium leaf spot regionally in the area of spinach seed production, volunteer spinach that develops from seed shattered onto the ground at harvest of seed crops must be controlled by plowing and disking before cultivation the following spring, in order to remove potential overwintering sources of inoculum (Inglis et al., 1997). Removal of small, undersized, and damaged spinach seed during sizing and cleaning of the seed after harvest, may also remove some infected seed (Inglis et al., 1997).

1.3.7.3. Resistant cultivars. Differences in the degree of resistance of commercial spinach cultivars to *C. variabile* remain to be determined definitively (du Toit and Derie, 2002). The semi-savoy open pollinated cultivar 'Winter Bloomsdale' was more resistant to the pathogen than two other semi-savoy open pollinated cultivars 'Fall Green' and 'Ozarka II', in studies performed in growth chambers (Inglis et al., 1997).

1.4. Stemphylium leaf spot (*Stemphylium botryosum* Wallr.).

1.4.1. History and geographic distribution. *Stemphylium botryosum* infects a broad range of plants world-wide (Booth and Pirozynski, 1967). *Pleospora herbarum* (Fr.) Rab., the teleomorph of *S. botryosum*, is distributed world-wide, most commonly in temperate and sub-tropical areas (Booth and Pirozynski, 1967). Isolates of this fungus causing leaf spot of spinach were first detected in the USA in California in 1997 (Koike et al., 2001). The fungus has since been found as a pathogen of spinach in Arizona (L.J. du Toit and S.T. Koike, *unpublished data*), Maryland and Delaware (Everts and Armentrout, 2001), Oregon (M.L. Putnam and L.J. du Toit,

unpublished data), Florida (Raid and Kucharek, 2003), and in spinach seed crops in Washington (du Toit and Derie, 2001).

Koike et al. (2001) demonstrated that four isolates of *S. botryosum* from spinach were not pathogenic to 18 plant species inoculated, including other members of the Chenopodiaceae, except in the case of one isolate that developed small leaf spots (<2 mm) on fava bean (*Vicia faba* L. cv. Bell). Therefore, the authors suggested that isolates of *S. botryosum* pathogenic to spinach belong to a *forma specialis* of the pathogen, and the name *Stemphylium botryosum* f. sp. *spinacia* was proposed. The distribution of the teleomorph and the anamorph may not reflect the distribution of isolates of *S. botryosum* pathogenic specifically to spinach. Understanding the host specificity of *S. botryosum* isolates pathogenic to spinach will assist in management of the disease, e.g., by crop rotation (Koike et al., 2001).

Although Correll et al. (1994) mentioned that *Stemphylium* spp. can damage spinach seed crops, similarities in symptomatology of Cladosporium leaf spot and Stemphylium leaf spot may have resulted in the two diseases being misidentified at times. Alternatively, co-infections by *C. variabile* and *S. botryosum* may have been attributed to *C. variabile* alone because of the greater propensity for sporulation of *C. variabile* compared to *S. botryosum*. A third possibility is that Stemphylium leaf spot is a relatively new pathogen of spinach in the USA (du Toit and Derie, 2001; Koike et al., 2001).

1.4.2. Importance. Before being reported as a pathogen of spinach, *S. botryosum* was considered a weak pathogen or saprobe on spinach (Raid and Kucharek, 2003). However, du Toit et al., (2005a and b) demonstrated that if the pathogen is not controlled in a spinach seed crop, significant losses may occur. Low incidences of the disease may cause significant losses in

fresh market and processing crops as a result of reduction in quality of the spinach or the necessity for additional hand sorting (Everts and Armentrout, 2001; Koike et al., 2001).

Stemphylium botryosum was detected in commercial spinach seed grown in the USA as well as commercial seed lots from the European Union (EU), suggesting that *S. botryosum* may be prevalent in the global spinach seed industry. This possibility could explain the recent reports of the pathogen in spinach crops in other states (du Toit and Derie, 2001). The fungus was recently found on spinach seed grown in Washington (du Toit and Derie, 2003a). Monitoring seed lots for infection may help minimize further spread of the fungus by identifying infected seed lots to be treated (e.g., with hot water, chlorine, or fungicide).

1.4.3. Causal agent. *Stemphylium botryosum* developing from the holotype produces conidia 33 to 35 μm in length and 24 to 26 μm wide. A majority of the conidia are almost as broad as they are long (length/width ratio is 1.0 to 1.5) (Simmons, 1985). Conidia are oblong, olive to brown, ovoid to subdoliiform, and may be constricted at 1 to 3 transverse septa and at 1 to 3 longitudinal septa. Conidia each have a single basal pore. Conidiophores are erect, flexuous, 1- to 7-septate, 20 to 72 μm in length and 4 to 6 μm wide, brown, and with a swollen apical cell 7 to 11 μm in diameter (Booth and Pirozynski, 1967). The mature ascospores of *Pleospora herbarum* are 32 to 35 μm long and 13 to 15 μm wide, medium brown in color, and obovoid in shape. Ascospores have seven transverse septa (three to five major, others secondary), and one longitudinal septum in the initial four transverse divisions of the ascospore. Mature ascospores are constricted at each of the three initial transverse septa. Mature asci measure approximately 16 x 25 μm, are bitunicate, tubular, with parallel walls and a rounded apex, and gradually narrows at the base (Simmons, 1985). Pseudothecia are globose to flattened, 100 to 500 μm in diameter, and immersed to erumpent in the host tissue (Booth and Pirozynski, 1967).

1.4.4. Host range. Isolates of *S. botryosum* have been reported as pathogens of a broad range of plants, including alfalfa (*Medicago sativa* L.), bean (*Phasaeolus vulgaris* L.), carrot (*Daucus carota* L. subsp. *sativus* (Hoffm.)), endive (*Cichorium endivia* L.), fava bean (*Vicia faba* L.), lettuce (*Lactuca sativa* L.), landino clover (*Trifolium repens* L.), lupine (*Lupinus* spp.), onion (*Allium cepa* L.), parsley (*Petroselinum crispum* (Mill.) Nym. Ex A. W. Hill), pea (*Pisum sativum* L.), radish (*Raphanus sativus* L.), yellow sweet clover (*Melilotus officinalis* (L.) Lam), tall fescue (*Festuca arundinacea* Schreb.), tomato (*Lycopersicon esculentum* Miller), gladiolus (*Gladiolus italicus* Mill.), and sanfoin (*Onobrychis* spp.) (Booth and Pirozynski, 1967; Koike et al., 2001).

1.4.5. Symptoms. du Toit and Derie (2001) reported that symptoms of Stemphylium leaf spot developed within 80 h of inoculation of spinach plants in the greenhouse. Spots on the leaves were initially small (1 to 2 mm in diameter) and sunken, circular, gray-green in color, and changed to a white color 24 to 48 h later (du Toit and Derie, 2001; Raid and Kucharek, 2003). The spots then became dry and bleached, light tan in color, papery, and typically showed no apparent sign of fungal infestation except in very humid conditions, and were more abundant on older leaves (du Toit and Derie, 2001; Everts and Armentrout, 2001; Koike et al., 2001; Raid and Kucharek, 2003). Older leaves of spinach plants appear to be more susceptible than younger leaves (L.J. du Toit, *personal communication*). The lack of sporulation on the lesions (unless under wet or very humid conditions) helps distinguish this disease from other leaf spots of spinach, such as Cladosporium leaf spot, downy mildew (*Peronospora farinosa* (Fr.:Fr.) Fr. f. sp. *spinaciae* Byford), and anthracnose (*Colletotrichum dematium* (Pers.) Grove f. sp. *spinaciae* (Ellis & Halst.) Arx), in which fungal growth typically can be observed in the spots (Koike et al., 2001). Stemphylium leaf spots enlarge and coalesce rapidly, are irregular in shape, and do not

develop the narrow brown margin that is distinctive of Cladosporium leaf spot (du Toit and Derie, 2001). Stemphylium leaf spot can resemble herbicide and/or fertilizer damage (Koike et al., 2001).

1.4.6. Epidemiology. Infection of spinach by *S. botryosum* can occur over a wide range of temperatures. However, moderate to warm temperatures (18 to 24°C) favor development of Stemphylium leaf spot (Koike et al., 2001). Prolonged periods of leaf wetness are also favorable for the disease (Koike et al., 2001; Raid and Kucharek, 2003). Koike et al. (2001) observed that Stemphylium leaf spot was enhanced during rainy periods and in fields irrigated with overhead sprinklers.

du Toit and Derie (2003a, 2003b) found pseudothecia of *P. herbarum* on spinach stem debris that was left on the soil surface through the winter in fields in which spinach seed crops had been grown the previous season. Isolates of *S. botryosum* generated from ascospores discharged from pseudothecia onto agar in petri plates inverted over this debris were pathogenic on spinach in the greenhouse. du Toit and Derie (2003b) also reported that *S. botryosum* can be seedborne in spinach. Therefore, infected seed may be a source of inoculum of the pathogen. The presence of pollen on spinach plants enhances aggressiveness of the pathogen significantly (du Toit and Derie, 2002). Dissemination of the pathogen occurs through spores spread by wind, rain splash, irrigation, and farm equipment (Booth and Pirozynski, 1967; Koike et al., 2001; Raid and Kucharek, 2003).

1.4.7. Management.

1.4.7.1. Chemical control. Similar to Cladosporium leaf spot, chlorothalonil (Bravo Weather Stick) and mancozeb (Dithane DF Rainshield NT) were the fungicides growers in Washington relied on prior to 2002 to help manage Stemphylium leaf spot in spinach seed crops

(PICOL, 2001). Azoxystrobin is now also registered in Washington to manage leaf spot in spinach (PICOL, 2004). du Toit and Derie (2003a) and du Toit et al. (2004, 2005a and b) documented that fungicides in the strobilurin family as well as iprodione were highly efficacious against Stemphylium leaf spot. Azoxystrobin (Quadris 2.08 FL) is registered in Florida to manage Stemphylium leaf spot of spinach (Raid and Kucharek, 2003). Foliar applications of azoxystrobin, acibenzolar-S-methyl (Actigard 50 WG, Syngenta Crop Protection, Inc.), or azoxystrobin + acibenzolar-S-methyl, significantly reduced severity of Stemphylium leaf spot in Maryland (Everts and Armentrout, 2002a and 2002b).

Stemphylium botryosum is more aggressive on spinach in the presence of pollen (du Toit and Derie, 2002). Therefore, fungicide applications should be initiated just prior to pollen shed in spinach seed crops (du Toit and Derie, 2002) and repeated at appropriate intervals if conditions are conducive for this disease (e.g., wet). To avoid development of resistance to fungicides by *S. botryosum*, fungicides with different modes of action should be alternated or mixed to manage this disease for the long term (du Toit and Derie, 2003a). Treatment of infected seed with chlorine (1.2% NaOCl) for up to 40 minutes reduced the incidence of *S. botryosum* from 54.8% for the non-treated seed to18.3% (du Toit and Derie, 2003b).

1.4.7.2. Cultural control. Because free moisture on plants provides an environment favorable for development of *S. botryosum*, agricultural practices that increase the duration of leaf wetness (e.g., overhead irrigation) should be avoided (Raid and Kucharek, 2003). *Stemphylium botryosum* is not a soilborne pathogen. Furthermore, pseudothecia of *P. herbarum* only have been detected on spinach stem debris remaining on the soil surface after harvest of seed crops (du Toit and Derie, 2003b). Therefore, spinach debris should be incorporated into the soil following harvest of spinach seed crops in the fall, to allow microbes to degrade the debris

and resident inoculum, reducing the amount of overwintering inoculum surviving to the next season and minimizing ascospore dispersal that spring (du Toit and Derie, 2003a).

1.4.7.3. Resistant cultivars. Currently, the resistant response of spinach cultivars to Stemphylium leaf spot only has been examined preliminarily. The savoy spinach cultivar 'Vienna' showed slightly less development of the disease compared to other varieties tested in California (Koike et al., 2001).

1.5. Cladosporium and Stemphylium leaf spot of spinach seed crops: Research needs.

In order to manage Cladosporium and Stemphylium leaf spots more efficiently in spinach crops, additional research is needed to determine the seedborne nature (internal and/or external) of these two fungi, the efficacy of seed treatments for eradicating inoculum from stock and harvested seed lots, and the epidemiology of *S. botryosum* and *C. variabile*, including the potential for seed transmission. Knowledge of these aspects of both fungi, combined with determining potential yield losses associated with the pathogens, will facilitate development of an effective integrated disease management program for the spinach seed industry. In addition, the prevalence of these pathogens in the commercial spinach seed industry must be assessed to determine the usefulness of seed treatments and to develop plans to avoid spreading these fungi via infected seed lots. In consideration of these research needs, the objectives of this thesis project are to:

1. assess the prevalence of *S. botryosum* and *C. variabile* in commercial spinach seed lots,
2. determine the location of *C. variabile* and *S. botryosum* within spinach seed,

3. clarify whether *C. variabile* and *S. botryosum* can be transmitted from seed to seedlings, and
4. evaluate the efficacy of seed treatments for control of seedborne *C. variabile* and *S. botryosum.*

1.6. Literature cited.

1. Anonymous. 2001. Spinach Cladosporium leaf spot. University of California Statewide Integrated Pest Management Program. http://www.ipm.ucdavis.edu/PMG/r732100311.html.
2. Anonymous. 2003. Spinach. Atlantic Provinces Vegetable Crops Guide to Pest Management, 2002-2003. Agriculture and Forestry Farm Extension Services, Prince Edward Island, Canada. http://www.gov.pe.ca/af/agweb/library/vegetable/spinach02_new.php3.
3. Booth, C., and Pirozynski, K.A. 1967. *Pleospora herbarum*. Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria No. 150. Surrey, England.
4. Clinton, G.P. 1906. Notes on fungous diseases. Page 275, In: Report of the Connecticut Agricultural Experiment Station, 1905, Part V. Report of the Station Botanist. Connecticut.
5. Correll, J.C., Morelock, T.E., Black, M.C., Koike, S.T., Brandenberger, L.P., and Dainello, F.J. 1994. Economically important diseases of spinach. Plant Dis. 78:653-660.
6. David, J.C. 1995. *Cladosporium variabile*. International Mycological Institute Descriptions of Pathogenic Fungi and Bacteria No. 1229. Surrey, England.

7. de Vries, G.A. 1952. Contribution to the Knowledge of the Genus *Cladosporium* Link ex Fr. Ph.D. Thesis, University of Utrecht. Utrecht, the Netherlands. 121 pp.
8. du Toit, L.J., and Derie, M.L. 2001. *Stemphylium botryosum* pathogenic on spinach seed crops in Washington. Plant Dis. 85:920.
9. du Toit, L.J., and Derie, M.L. 2002. Leaf spot of spinach seed crops in Washington State. Phytopathology 92:S21.
10. du Toit, L.J., and Derie, M.L. 2003a. Leaf spot of spinach seed crops: Research results from 2002. Pages 61-64, In: Proceedings of the Annual Meeting of the Western Washington Horticulture Association, 9-11 January 2003, SeaTac, WA.
11. du Toit, L.J., and Derie, M.L. 2003b. Inoculum sources of *Stemphylium botryosum* and *Cladosporium variabile* in spinach seed crops. Phytopathology 93:S22.
12. du Toit, L.J., Derie, M.L., and Hernández-Pérez, P. 2004. Evaluation of fungicides for control of leaf spot in spinach seed crops, 2003. F&N Tests 59:V115.
13. du Toit, L.J., Derie, M.L., and Hernández-Pérez, P. 2005a. Evaluation of fungicides for control of leaf spot in spinach seed crops, 2004. F&N Tests 60:V044.
14. du Toit, L.J., Derie, M.L., and Hernández-Pérez, P. 2005b. Evaluation of yield loss caused by leaf spot fungi in spinach seed crops, 2004. F&N Tests 60:V047.
15. du Toit, L.J., Derie, M.L., and Hernández-Pérez, P. 2005c. Leaf spot of spinach: 3rd year report. Pages 39-48, In: Proceedings of the Annual Meeting of the Western Washington Horticulture Association, 11-13 January 2005, SeaTac, WA.
16. Ellis, M.B. 1971. *Cladosporium*. Page 315, In: Dematiaceous Hyphomycetes. Kew Commonwealth Mycological Institute. Surrey, England.

17. Everts, K.L., and Armentrout, D.K. 2001. Report of leaf spot of spinach caused by *Stemphylium botryosum* in Maryland and Delaware. Plant Dis. 85:1209.
18. Everts, K.L., and Armentrout, D.K. 2002a. Evaluation of fungicides for control of Stemphylium leaf spot and anthracnose of spinach. F&N Tests 57:V088.
19. Everts, K.L., and Armentrout, D.K. 2002b. Evaluation of fungicides for control of Stemphylium leaf spot of spinach. F&N Tests 57:V089.
20. Foss, C.R., and Jones, L.J. 2000. Crop Profile for Spinach Seed in Washington. http://cipm.ncsu.edu/cropprofiles/docs/waspinachseed.html.
21. Frank DiCosmo, G.H., Towers, N., and Lam, J. 1982. Photo-induced fungicidal activity elicited by naturally occurring thiophene derivatives. Pest. Sci. 13:589-594.
22. Fuentes-Davila, G. 1988. Studies on a Leaf Spot Disease of Spinach Caused by *Cladosporium variable* (Cooke) de Vries. Ph.D. Thesis, Washington State University. Pullman, WA. 49 pp.
23. Fuentes-Davila, G., and Gabrielson, R.L. 1987. Leaf spot of spinach caused by *Cladosporium variabile*. Pages 177-180, In: Proceedings of the 1987 Annual Meeting of the Western Washington Horticulture Association, 6-7 January, SeaTac, WA.
24. Hansen, H.R., Weber, A., and Kristensen, H.R. 1952. Plant diseases in Denmark in 1950. Annual survey compiled by the State Phytopathological Experiment Station. Tidsskr. Planteavl. 56:1-59. (Translated abstract from Rev. Appl. Mycol. 32:234).
25. Inglis, D.A., Derie, M.L., and Gabrielson, R.L. 1997. Cladosporium Leaf Spot on Spinach Seed Crops and Control Measures. Washington State University Extension Bulletin No. 865. Pullman, WA.

26. Jacques, G.W. 1941. Studies on the genus *Heterosporium*. Contr. Inst. Bot. Montreal. 39:46.

27. Koike, S.T., Henderson, D.M., and Butler, E.E. 2001. Leaf spot disease of spinach in California caused by *Stemphylium botryosum*. Plant Dis. 85:126-130.

28. Lucier, G. 1993. Vegetables and Specialties Situation and Outlook Year-Book. Commodity Economic Division, Economic Research Service, U.S. Department of Agriculture, Washington, D.C.

29. Lucier, G. 2000. Vegetables and Specialties Situation: An Outlook Report. Commodity Economic Division, Economic Research Service, U.S. Department of Agriculture, Washington, D.C.

30. Mathur, R.L., Mathur, B.L., and Sehgal, S.P. 1969. Studies on a leaf spot disease of spinach caused by *Cladosporium variabile* (Cooke) de Vries in Rajasthan. Ind. Phytopath. 18:215-216.

31. Mathur, R.L., and Sehgal, S.P. 1970. Effect of fungicides on germination and size of germ tubes of conidia of *Cladosporium variabile* (Cooke) de Vries. Labdev J. Sci. Tech. 8-A:214-216.

32. Mathur, R.L., and Sehgal, S.P. 1965. Relative efficacy of fungicides for the control of *Cladosporium variabile* causing leaf spot disease of spinach (*Spinacia oleracea*). Ind. Phytopath. 12:161-163.

33. Mills, H.A. 2001. Vegetable Crops. Spinach. University of Georgia. Athens, GA. http://www.uga.edu/vegetable/spinach.html.

34. Pscheidt, J.W., and Ocamb, C.M. (Editors). 2004. Spinach - Leaf spot. Page 409. Spinach - Stemphylium leaf spot. Page 410. In: Pacific Northwest Plant Disease Management

Handbook. Oregon State University, Washington State University, and University of Idaho. Corvallis, OR.

35. Raid, R.N, and Kucharek, T. 2003. 2003 Florida Plant Disease Management Guide: Spinach. http://edis.ifas.ufl.edu/BODY_PG054.
36. Reed, H.S, and Cooley, J.S. 1911. *Heterosporium variabile* Cke: Its relation to *Spinacia oleracea* and environmental factors. Pages 78-99, In: Annual Report of the Virginia Polytechnic Institute Agricultural Experiment Station, Lynchburg, VA.
37. Ryder, E.J. 1976. Leafy Salad Vegetables. AVI Publishing, Westport, CT.
38. Sanders, D.C. 2003. Spinach. North Carolina State University Horticulture Information Leaflets. http://www.ces.ncsu.edu/depts/hort/hil/hil-17.html.
39. Sherf, A.F., and MacNab, A.A. 1986. Spinach. Pages 544-554, In: Vegetable Diseases and Their Control, 2nd Edition. John Wiley & Sons, New York, NY.
40. Simmons, E.G. 1985. Perfect states of *Stemphylium*. II. Sydowia 38:284-293.
41. Thomas, J., Schreiber, A., Pelter, G., and Havens, D. 1997. Washington's Small–Seeded Vegetable Seed Industry. Washington State University Extension Bulletin No. 1829. Pullman, WA.

Chapter 2

Prevalence of *Stemphylium botryosum* and *Cladosporium variabile* in commercial spinach seed lots

2.1. INTRODUCTION

Cladosporium variabile (Cooke) de Vries and *Stemphylium botryosum* Wallr have been identified as important foliar pathogens of spinach seed crops in western Washington (du Toit and Derie, 2001; du Toit and Derie, 2002; Fuentes-Davila, 1988). Outbreaks of Cladosporium and Stemphylium leaf spots in western Washington can have significant negative impacts on spinach seed production (du Toit and Derie, 2002; Inglis et al., 1997). These diseases may also reduce the quality of processing spinach crops and may necessitate additional hand sorting for fresh market spinach crops (Everts and Armentrout, 2001; Fuentes-Davila, 1988). Fuentes-Davila (1988) reported *C. variabile* to be seedborne in spinach. du Toit and Derie (2003) detected *S. botryosum* and *C. variabile* in spinach seed lots produced in the EU and in Washington State, the primary region of spinach seed production in the USA.

What it is commonly called a spinach "seed" actually is a fruit with a seed enclosed by the pericarp (thick, corky structure surrounding the seed) (Fig. 1). The true seed consists of a testa, embryo, and non-living perisperm (maternal storage tissue) (Peterson and Harris, 1997; Singh and Mathur, 2004b). For this thesis, the spinach fruit (consisting of the pericarp, testa, embryo, and perisperm) is referred to as a seed. The prevalence of *S. botryosum* and *C. variabile* in commercial spinach seed lots is not known. Information on prevalence of these pathogens will help determine the need for spinach seed treatments and assist with development of strategies to avoid spreading these fungi via infected seed lots. The location of these pathogens within

spinach seed also remains to be determined. Such information may help determine the potential efficacy of seed treatments and the potential for seed transmission of these pathogens in spinach. Therefore, the objectives of this study were to assess:

1. the incidence of *S. botryosum* and *C. variabile* in commercial spinach seed lots, and
2. the location of *C. variabile* and *S. botryosum* within spinach seed.

2.2. MATERIALS AND METHODS

2.2.1. Spinach seed lots. In July 2003, a letter was sent to six companies that produce or distribute spinach seed, requesting samples of commercial seed lots to assay for *C. variabile* and *S. botryosum*. A total of 77 stock or harvested seed lots were collected from six companies (Alf Christianson Seed Company, Mount Vernon, WA; Daehnfeldt, Odense, Denmark; Enza Zaden, Enkhuizen, Holland; Pop Vriend, Andijk, Holland; Schafer Ag Services LLC, Mount Vernon, WA; and Seminis Vegetable Seeds, Inc., Enkhuizen, Holland). The seed lots were produced in Denmark, the Netherlands, New Zealand or the USA (western Washington or western Oregon) with some companies producing seed in multiple countries (Tables 1 and 2). Sixty-six of the seed lots were harvested in 2003 (Table 1), and eleven from 1993 to 2001 (Table 2). The seed lots harvested from 1993 to 2001 were stored in a seed vault at 4.4^{o}C and 60% relative humidity (data provided by the seed company). Seed company names have been coded for proprietary reasons (Table 1). Seed lot 01-101, harvested in western WA in 2001, was also used in this study. Datalogger records (Model 425, Spectrum Technologies, Inc., Plainfield, IL) from May to September of 2002 indicate this seed lot was stored at 18.8 ± 2.9^{o}C and 61.5 ± 5.0% relative humidity.

2.2.2. Freeze-blotter seed assays. A sample of each seed lot was subjected to a freeze-blotter seed health assay during the winter or spring of 2003-04, following the method described by du Toit et al. (2005). Each seed assay consisted of four replications of 100 seeds. A replication was established with five 10-cm-diameter plastic Petri dishes, each containing 20 seeds. The seed for each replication was placed into a mesh tea strainer (Model 101, Venalicia Tea, Neuss, Germany). Working in a laminar flow hood, the tea strainer was placed in 150 ml of 1.2% NaOCl and shaken manually for 60 s. 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 5 ml of sterile deionized water in a Petri dish. The Petri dishes were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI). The seed were incubated in the dark at 24ºC for 24 h to imbibe, 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 13 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 using a dissecting microscope (8 to 100x magnification) approximately 4, 7, and 14 to 15 days after plating, for development of *C. variabile*, *S. botryosum*, and other fungi. The mean and standard deviation of seedborne infection by each fungus were calculated for each seed lot; and the mean, standard deviation, and range of seedborne infection were calculated for seed lots from each company and country. Spinach seed lot 01-101, harvested from a fungicide trial in western Washington in 2001 and assayed for *C. variabile* and *S. botryosum* in 2001 using the protocol described above, was assayed again in 2003 and 2004 to determine the duration of survival of *C. variabile* and *S. botryosum* in the seed lot.

2.2.3. Pathogenicity of seed isolates of *S. botryosum* and *C. variabile*. 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. Non-clarified 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] was used for isolating *S. botryosum,* and Difco potato dextrose agar (PDA) (Becton, Dickinson and Co., Sparks, MD) was used for *C. variabile* and other *Cladosporium* spp. Approximately 1 week after isolation, spores were removed from the culture of each isolate using a sterilized inoculating loop, and streaked onto a Petri dish containing PDA or V8 agar. The plates were kept at room temperature (approximately 12 h/12 h day/night cycle). Two days later, a single spore germinating on the medium was transferred to a second Petri dish of 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 and the fungi were allowed to colonize the disks. For *C. variabile* cultures, 1.5 mm of deionized sterile water was added to each plate 7 days after the single spore was transferred, and the Petri plates were shaken to disperse conidia over the medium and the filter disks. After the fungi had colonized the filter disks, the disks were removed from the plates and dried overnight in a laminar flow hood in sterilized coin envelopes (5.7 cm x 8.9 cm) (Westvaco Envelope Division, Springfield, MA). The dried colonized disks were stored with desiccant at –20^{o}C, following the method described by Peever et al. (1999).

Two-week-old cultures of each of five isolates of *S. botryosum* from seed lots from each of companies A, B, C, and D, and four isolates from seed lots from company E were tested for pathogenicity on spinach plants in the greenhouse (Table 1). These isolates comprised nine from

Denmark, seven from the Netherlands, six from the USA, and two from New Zealand. Similarly, eight isolates of *C. variabile* from seed lots from companies A and seven from company C were selected randomly and tested for pathogenicity on spinach plants in the greenhouse (Table 1). These isolates of *C. variabile* represented nine from the USA and six from Denmark. In addition, five isolates of *Cladosporium* spp. other than *C. variabile* from seed lots from each of companies B, D, and E (seven isolates from Denmark, six from the Netherlands, and two from New Zealand were tested for pathogenicity. Two-week-old cultures of isolates of *S. botryosum* from seed lots produced in 1993 (three isolates), 1994 (five isolates), 1996 (three isolates), and 2001 (three isolates) were also tested for pathogenicity on spinach plants in the greenhouse (Table 2). The latter isolates were stored at -20^{o}C for 4 to 5 months before pathogenicity tests were carried out, compared to 10 to 14 months of storage for the isolates from the 2003 seed lots.

A randomized complete block design with three replications per isolate (one plant/replication) was used for each set of pathogenicity tests. Six- to 10-week-old plants of the proprietary, smooth leaf, medium-long standing, female spinach inbred lines A or B (obtained from a local seed company as a source of thrips-tolerant spinach lines) were inoculated with a conidial suspension (20 ml/three plants at approximately 10^5 conidia/ml) of the appropriate isolate using an atomizer (Badger Air-Brush Co., Franklin Park, IL). Control plants were atomized with sterile deionized water. A known pathogenic isolate of *S. botryosum*, referred to hereafter as 'Cheetah' because it was originally 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*, referred to hereafter as '00-304' because it was originally obtained from an infected spinach seed crop grown in western Washington in 2000, served as the control isolate for *C. variabile*. Plants were rated visually one,

two, and three weeks after inoculation, for percentage of leaves covered with leaf spot symptoms. Isolations were made approximately 21 days after inoculation from plants that developed symptoms of leaf spot, by cutting sections (2 mm x 2 mm) from the margins of leaf symptoms, surface-sterilizing the sections in 0.6% NaOCl for 1.5 min, triple-rinsing the sections in sterile deionized water, drying them on sterile paper towel in a laminar flow hood, and placing the dry sections onto V8 agar (for isolations of *S. botryosum*) or PDA (for isolations of *C. variabile*). Isolations were also made from leaves of the control plants.

Plates were kept at room temperature and exposed to an approximately 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 (Petri dish lined with damp paper towel). The plates were examined microscopically 3 days later for the development of conidia of *S. botryosum* or *C. variabile*. The pathogenicity on spinach of other fungi observed on the seed was not tested.

2.2.4. Component freeze-blotter seed assays. To determine if *C. variabile* and *S. botryosum* may be present in the pericarp and/or embryo of spinach seed (Fig. 1), component freeze-blotter seed assays were performed. The pericarp and embryo of each of 100 seed of spinach seed lots V, W, X, Y, and Z (Table 4) were separated manually using forceps flame-sterilized between each seed. The pericarp and embryo of each seed were then assayed separately using the freeze-blotter protocol described above. The seed of lot V were rinsed in running deionized water for 60 min to soften the pericarps prior to separation of the pericarps and embryos. The seed of lots W, X, and Y were also rinsed in running deionized water for 60 min, and then soaked in 1.2% NaOCl for 60 s before the pericarps and embryos were separated, to minimize the possibility of contaminating the embryo with spores on the surface of the pericarp

when separating the two components. The seed of lot Z were rinsed in running deionized water for 60 min before the pericarp and embryo of each seed were separated, and then the two components of each seed were soaked separately in 1.2% NaOCl for 60 s in 5 ml microcentrifuge tubes (USA Scientific, Inc., Ocala, FL). The pericarps and embryos were individually triple-rinsed in sterile deionized water, dried and plated as described in this section. This procedure was used to determine whether the embryos and pericarps might each be infected internally or infested with the pathogens.

2.2.5. Electron microscopy. Fifty seed of lot 03-409 (from a spinach seed crop fungicide trial carried out at Washington State University - Northwestern Washington Research and Extension Center in Mount Vernon, WA in 2003), which had been determined to be infected with *S. botryosum* at 21.5% and *C. variabile* at 17.0% (du Toit et al., 2004), were subjected to the freeze-blotter seed health assay described in section 2.2.2. Twenty-four h after moving the seed from –20ºC to an incubator set at 24ºC, the seed were examined microscopically for evidence of *S. botryosum* and *C. variabile*. Five seed on which mycelium indicative of *S. botryosum* was developing on the surface of the pericarps, were each cut in half using a surgical blade. The blade was disinfected with 70% ethyl alcohol between each seed. Similarly, two seeds on which conidiophores indicative of *C. variabile* were developing 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 then taken to the Washington State University - Electron Microscopy Center (WSU-EMC) laboratory and the other half of each seed was kept in the Petri plate in the incubator to allow the fungi to develop further. The two halves of each seed were labeled for identification.

In the WSU-EMC laboratory, the seed were prepared for transmission electron microscopy (TEM) by fixing, washing, dehydrating, infiltrating, embedding, and curing the seed following

the protocol described by Bozzola and Russell (1999b). 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) at room temperature. The seed components were then rinsed once in 50 mM pipes buffer and twice in 25 mM phosphate buffer, followed by post-fixation with 1% osmium tetroxide in 25 mM phospate buffer for two 2 h at room temperature, and 0.1% tannic acid for 1 h at room temperature. After fixation, the seed components were dehydrated sequentially for 10 min in each of 30, 50, 70, 80, and 95% acetone, followed 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 (Electron Microscopy Sciences, Fort Washington, PA), followed by infiltration with 100% resin three times. After infiltration, the pericarp and embryo of each half seed were embedded separately in labeled flat molds (Ted Pella, Inc., Redding, CA) using 100% resin as embedding medium, and incubated overnight at 55^{o}C for polymerization. Thin sections (70 to 100 nm) of embryos and pericarps were prepared using an ultra microtome, picked up on formvar coated grids (Ted Pella, Inc., Redding, CA), and stained with Sato's lead stain procedure (Sato, 1968) before they were examined using a transmission electron microscope (JEOL USA, Inc., Peabody, MA).

Images of mycelium of *C. variabile* and *S. botryosum* in the pericarps were taken using a digital camera (Soft Imaging System Corp., Lakewood, CO). One of the half seeds prepared for electron microscopy was discarded because *Alternaria* spp. were observed on the corresponding half seed maintained in the incubator.

An additional 50 seed of lot 03-409 were subjected to the freeze-blotter seed assay.

Five and 10 days after plating, the seed were removed from the incubator and examined using a dissecting microscope (8 to 100x magnification) for development of *C. variabile* and *S. botryosum*, respectively. Three seeds with *C. variabile* and five seeds with *S. botryosum* (or pseudothecia of *P. herbarum*) developing on the pericarp were prepared for scanning electron microscopy (SEM) according to the protocol of Bozzola and Russell (1999a). The seed were fixed overnight at room temperature in 1% osmium tetroxide. The fixed seed were placed on stubs of aluminum (Ted Pella, Inc., Redding, CA) and dehydrated overnight using a vacuum evaporator (Norton, Vacuum Equipment Division, Newton, MA). After dehydration, the seed were gold coated with a sputter coater (Technics Hummer V, San Jose, CA) for 6 min. 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). Images were captured using a digital camera (Quartz Imaging Corporation, Vancouver, BC, Canada).

2.3. RESULTS

2.3.1. Prevalence of *S. botryosum* in spinach seed. *Stemphylium botryosum* was identified based on conidiophore and conidial characteristics (Booth and Pirozynski, 1967), and by the development of immature pseudothecia of the teleomorph, *Pleospora herbarum* (Simmons, 1985), on the pericarps of the seed. All 77 spinach seed lots assayed were infected with *Stemphylium botryosum* (Tables 1 and 2). The 66 seed lots produced in 2003 had a mean incidence of infection of 29.38% with a range from 0.25 to 97.50% for individual lots (Table 1). Seed lots from Denmark and New Zealand had the highest mean incidence of this pathogen (58.36% and 58.50%, respectively) while the lowest mean incidence was detected in seed lots

grown in the USA (6.10%). Spinach seed lots from companies B and C had the highest mean incidence of infection (61.30% and 53.32%, respectively), followed by companies E (35.92%), D (12.63%), A (6.85%), and F (3.08%).

Stemphylium botryosum was detected in all twelve seed lots harvested between 1993 and 2001, with a range from 0.75 to 54.75% for individual lots (Table 2). The pathogen was observed in two 11-year-old seed lots (1993 crops) at incidences of 7.00 and 7.50% (Table 2). For these twelve seed lots, a significant correlation was detected between the percentage seedborne *S. botryosum* and the year of harvest ($r = 0.70$, $P = 0.01$). For seed lot 01-101 harvested in 2001, *S. botryosum* was detected at a mean incidence of 60.00, 2.50, and 0.25% in fall 2001, summer 2003, and fall 2004, respectively (Table 3).

2.3.2. Prevalence of *C. variabile* in spinach seed. When short chains of relatively large conidia (15 – 25 x 7 – 10 µm) of *Cladosporium* developed on long conidiophores (up to 350 µm, usually less than 150 µm), the fungus was identified as *C. variabile*. Isolates with smaller conidia developing on shorter conidiophores belonged to other *Cladosporium* spp. (David, 1995; Ellis, 1971). Isolates identified as *C. variabile* displayed slower growth on PDA compared to the other *Cladosporium* spp. *Cladosporium variabile* was detected in 26 (39.39%) of the 66 seed lots produced in 2003, with a mean incidence of 0.37% and a range from 0.00 to 4.00% for individual lots (Table 1). Seed lots from the USA had the highest mean incidence of this fungus (0.68%), followed by lots from Denmark (0.08%), and the Netherlands (0.04%). Of the 32 seed lots from the USA, 22 (68.75%) were infected with this pathogen, compared to 1 of 6 lots (16.67%) and 3 of 27 lots (11.11%) produced in the Netherlands and Denmark, respectively. *Cladosporium variabile* was not detected in the single seed lot from New Zealand. Seed lots from company F had the highest mean incidence of *C. variabile* (2.92%), followed by company

A (0.45%) and company C (0.14%). The fungus was not detected in the seed lots from companies B, D, and E, but was detected in 17 of 27 (62.96%) of the lots from company A, and 6 of 24 (25.00%) of the lots from company C.

Cladosporium variabile was detected in two of 12 seed lots produced between 1993 and 2001 (Table 2). The fungus was observed at 0.50% incidence in an 8-year-old seed lot (1996 crop) and a 3-year-old lot (2001 crop) produced in western Washington (Table 2). For seed lot 01-101, *C. variabile* was detected in a mean of 17.00, 2.25, and 0.00% of the seed when assayed in fall 2001, summer 2003, and fall 2004, respectively (Table 3).

2.3.3. Other fungi detected. Besides *S. botryosum* and *C. variabile*, *Verticillium* spp. were also observed on the seed lots. *Verticillium* spp. were identified based on the development of hyaline verticillate conidiophores with 3 to 4 phialides at most nodes. Most *Verticillium* spp. resembled *V. dahliae* based on the development of dark brown to black microsclerotia in the pericarps of the seed and the presence of hyaline conidiophores (Hawksworth and Talboys, 1970; Smith, 1965). Of the 66 seed lots produced in 2003, 61 (92.42%) were infected with *Verticillium*, at a range in incidence from 0.00 to 84.75% for individual lots. All seed lots from companies D and F were infected with *Verticillium*, while of the lots from companies A, C, B, and E, 96.30 (26 lots), 91.67 (22 lots), 80.00 (4 lots), and 66.67% (2 lots), respectively, were infected with *Verticillium*. Seed lots from companies D, F, A, B, E, and C had a mean incidence of seedborne *Verticillium* of 34.25, 30.75, 26.22, 18.05, 9.25, and 5.89%, respectively. The mean incidence of seedborne *Verticillium* was 26.33% for lots from the Netherlands, 25.05% for lots from the USA, and 8.78% for lots from Denmark. Even though seed lots from Denmark had the lowest mean incidence of seedborne *Verticillium*, one lot from this country had the highest incidence of *Verticillium* in the study (84.75%). All of the seed lots produced in the Netherlands in 2003 were

infected with *Verticillium*, while 93.75 and 92.59% of the lots harvested in the USA and Denmark, respectively, were infected. *Verticillium* was not detected in the seed lot from New Zealand. *Verticillium* was present in 9 of the 12 spinach seed lots produced between 1993 and 2001 in western Washington. *Verticillium* was observed in two 11-year-old spinach seed lots (harvested in 1993) at 7.00 and 19.00% incidence. There was no significant correlation between the percentage seedborne *Verticillium* and the year of harvest of the seed.

Colletotrichum spp. were identified based on the development of acervuli with black setae and single-celled, hyaline conidia (Mathur and Kongsdal, 2003). *Colletotrichum* spp. were only observed in 2 of 27 seed lots (7.41%) produced in Denmark at a mean incidence of 0.04%, and in 1 of 6 seed lots (16.67%) from the Netherlands at a mean incidence of 0.04%. All three infected seed lots were from company C. *Colletotricum* spp. were not present in the seed lot from New Zealand nor in lots from the USA. Species of *Acremonium*, *Alternaria*, *Bipolaris, Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Gonatobotrys*, *Penicillium*, and *Ulocladium* completed the mycoflora observed on the seed lots assayed (data not provided).

2.3.4. Pathogenicity of seed isolates of *S. botryosum* and *C. variabile*. Twenty four isolates of *S. botryosum* from seed lots produced by companies A to E in 2003 were inoculated onto spinach plants in the greenhouse. Eleven (45.83%) were pathogenic (two from New Zealand, five from Denmark, and four from the Netherlands) (Table 1). None of the six isolates of *S. botryosum* from seed lots produced in the USA in 2003 was pathogenic on spinach. However, all 14 of the isolates of *S. botryosum* from seed lots produced in the USA from 1993 to 2001 were pathogenic on spinach. Of 15 isolates of *C. variabile* tested for pathogenicity on spinach, five (33.33%) were pathogenic. All five of these isolates were from seed lots grown in

Denmark (Table 1). None of the isolates of other *Cladosporium* spp. from seed lots produced by companies B, D, and E was pathogenic to spinach plants in the greenhouse.

2.3.5. Component freeze-blotter seed assays. *Stemphylium botryosum* was detected in the pericarps and embryos of all five seed lots subjected to component seed assays (Table 4), with a range from 13.00 to 91.00% and 5.00 to 76.00% mean incidence in the pericarps and embryos, respectively. The incidence of embryos infected with *S. botryosum* ranged from 38.46 to 83.52% of that detected in the pericarps. Similarly, the incidence of embryos infected with *C. variable* was much lower than that of the pericarps. *Cladosporium variable* was present in 2.00% of the pericarps of seed lot Y and 19.00% of the pericarps of lot Z, compared to none of the embryos of lot Y, and 1.00% of the embryos of lot Z. Both *C. variabile* and *S. botryosum* were detected in embryos that had been surface-sterilized in 1.2% NaOCl for 60 s (lot Z), demonstrating internal infection of the embryos.

2.3.6. Electron microscopy. Intracellular hyphae of both *S. botryosum* and *C. variabile* were observed in the pericarps of seed lot 03-409 (Fig. 2), but neither fungus was detected in the embryos by TEM. Intracellular hyphae of both fungi were observed penetrating through pericarp cell walls (Fig. 2A and 2B). Intercellular hyphae were not observed.

Conidia and conidiophores of *C. variabile* and *S. botryosum*, and immature pseudothecia of *S. botryosum* were observed on the pericarps of seed lot 03-409 using SEM (Fig. 3). Chains of up to three conidia of *C. variabile* (Fig. 3A and 3B); single verrucose conidia of *S. botryosum* (Fig. 3C); and globose, immature, erumpent pseudothecia of *P. herbarum* (Fig. 3D) were observed on the pericarps of the seed.

2.4. DISCUSSION

The prevalence of *S. botryosum* in commercial spinach seed lots is reflected in the fact that *S. botryosum* was detected in each of the 66 seed lots assayed that had been produced in Denmark, the Netherlands, New Zealand, and the USA in 2003 by six seed companies, with a wide range in incidence for individual lots (<1 to >95%). However, only 11 of 24 isolates (<50%) of *Stemphylium* (two isolates from New Zealand, five from Denmark, and four from the Netherlands) proved pathogenic on spinach plants in the greenhouse. These isolates produced symptoms typical of Stemphylium leaf spot (du Toit and Derie, 2001; Koike et al., 2001), proving to be *S. botryosum*. The 12 isolates of *Stemphylium* that were not pathogenic on spinach were morphologically similar to the pathogenic isolates, although spores and conidiophores were not measured. These non-pathogenic isolates may belong to a different species than *S. botryosum*. Alternatively, these isolates may not be pathogenic to the spinach inbred lines used in the pathogenicity test. Another possibility is the loss of pathogenicity of these isolates because they were stored for 10 to 14 months before pathogenicity tests were completed. Other pathogenic spinach isolates of *S. botryosum* stored at –20 or –80°C have been demonstrated to lose pathogenicity within a year of storage (L.J. du Toit, *personal communication*). To our knowledge, *S. botryosum* was not previously reported on spinach seed grown in Denmark, the Netherlands, or New Zealand.

Cladosporium variabile was detected at a very low incidence in the seed lots produced in 2003 and from 1993 to 2001, compared to the incidence of *S. botryosum* observed in these lots. The isolates of *Cladosporium* forming short chains of relatively large conidia on long conidiophores were identified as *C. variabile*. Of the 15 isolates of *C. variabile* tested for pathogenicity on spinach in the greenhouse, only five (all from seed lots produced in Denmark)

were pathogenic. Cultures of the 10 isolates not pathogenic on spinach were determined to be *C. macrocarpum*, not *C. variabile*, based on a more detailed examination of conidia and conidiophores, the lack of aerial hyphae developing from the tips of mature conidiophores, and the lack of pathogenicity on spinach (David, 1995; Ellis, 1971). Ellis (1971) reported that *C. variabile* and *C. macrocarpum* are similar morphologically, but that the presence of spirally coiled aerial hyphae and pathogenicity to spinach plants distinguish *C. variabile* from *C. macrocarpum*. The coiled aerial hyphae of *C. variabile* were observed when the pathogen developed on necrotic leaf spots on spinach leaf tissue in moist chambers and on sections of symptomatic leaf tissue placed on agar media. However, during the freeze-blotter seed assays, coiled aerial hyphae were only observed after ≥ 5 days of incubation. Aerial hyphae were not evident when *C. variabile* was growing on agar media. The aerial hyphae of *C. variabile* appear to develop only when the fungus is growing on spinach tissues. *Cladosporium variabile* and *C. macrocarpum* isolates together were detected at a mean incidence of 0.37% in 26 of the 66 seed lots assayed from the 2003 season.

Correll et al. (1994), Hadzistevic (1955), and Van Poeteren (1932) reported that *C. macrocarpum* causes leaf spot of spinach. However, Ellis (1971) indicated that *C. macrocarpum* is not pathogenic on spinach. Therefore, it is possible that isolates of *C. variabile* have been misidentified as *C. macrocarpum*. *Cladosporium variabile* and *C. macrocarpum* could not be distinguished based on development of conidia and conidiophores in the freeze-blotter seed health assay until at least 5 days of incubation of the seed, when coiled aerial hyphae developed for isolates of *C. variabile*. Based on morphological characteristics alone these fungi could readily be misidentified, demonstrating the need for further research to determine if *C. variabile*

and *C. macrocarpum* are genetically distinct and, if so, the necessity of a more effective assay to detect *C. variabile* in spinach seed (e.g., a molecular assay).

The seed companies that participated in this survey produce a majority of the world supply of spinach seed. Therefore, this survey probably is an accurate reflection of the prevalence of these two leaf spot pathogens in commercial spinach seed lots produced in 2003. The prevalence of seedborne *S. botryosum* within commercial spinach seed lots and the commercial movement of spinach seed might explain the recent first reports or observations of this pathogen in Arizona (L.J. du Toit and S.T. Koike, *unpublished data*), California (Koike et al., 2001), Maryland and Delaware (Everts and Armentrout, 2001), Oregon (M.L. Putnam and L.J. du Toit, *unpublished data*), Florida (Raid and Kucharek, 2003), and Washington (du Toit and Derie, 2001). The results also demonstrate the need for monitoring seed lots to decrease dissemination of *S. botryosum*.

The 2003 season in which 66 seed lots assayed in this study were produced, was characterized by unusually warm and dry weather for the region of spinach seed production in the USA [WSU Public Agricultural Weather System, Prosser, WA (http://frost.prosser.wsu.edu/paws/paidusers)], and by very warm (but not dry) conditions in Denmark [Danish Meteorological Institute (http://glwww.dmi.dk/f+u/publikationer/tr/2004/tr04-02/html/chapter02.htm)]. Of the 66 seed lots from 2003 assayed in this study, 59 (89%) were produced in these two regions. Spinach seed crops in western Washington are typically planted in April or May and harvested in August or September. Differences between the average monthly temperatures in 2003 vs. average monthly temperatures from 1992 to 2003 for April, May, June, July, August, and September in western Washington were: 0.0, 0.2, 1.9, 2.0, 0.5, and 0.8^{o}C, respectively, i.e., the 2003 season in western Washington was warmer than the previous

13 years. Similarly, the Danish Meteorological Institute reported that 2003 was a warm year in Denmark compared to the average temperatures from 1961 to 1990. This may account for the greater prevalence of *S. botryosum* compared to *C. variabile* in the 2003 survey as the optimal temperatures for growth of *S. botryosum* are 18 to 24°C (on agar media) (Koike et al., 2001) compared with 15 to 20°C for *C. variabile* (Fuentes-Davila, 1988). du Toit and Derie (2003) reported that individual seed lots produced in the EU in 2002 were infected with *C. variabile* at incidences up to 27.3%, which is higher than the incidences of *C. variabile* observed on seed lots produced in the EU in 2003. They also detected up to 86.0% incidence of *S. botryosum* in these seed lots.

Conditions favorable for storage of seed of most crops include 4 to 10°C and 50% relative humidity (not exceeding 70% relative humidity) (Neergaard, 1977). Survival of fungi in seed is also affected by seed storage conditions (Neergaard, 1977). Viable, pathogenic isolates of *S. botryosum* were isolated from spinach seed lots that had been stored at 4.4°C and 60% relative humidity for 3, 8, 10, and 11 years. Fungi resembling *C. variabile* were detected in 3- and 8-year-old seed lots stored under the same conditions, although the pathogenicity of these isolates on spinach was not verified and *C. variabile* was present at much lower incidences than *S. botryosum*. Fuentes-Davila (1988) isolated *C. variabile* from 6-year-old spinach seed lots and demonstrated pathogenicity of the isolates on spinach plants. In the fall of 2001, du Toit and Derie (2003) detected *C. variabile* and *S. botryosum* at incidences of 17.0 and 60.0%, respectively, in spinach seed lot 01-101. By the fall of 2004, the incidence of *S. botryosum* had dropped to 0.25% and *C. variabile* could not be detected. Seed lot 01-101 was stored at a similar relative humidity but a higher mean temperature (18.8°C) than the 11-year-old lots from which *S. botryosum* was isolated. Although we do not know the original incidences of *S. botryosum* in

these 11-year-old lots, incidences of 7.0 and 7.5% *S. botryosum* detected 11 years after harvest suggest that the temperature at which seed lots are stored may influence the duration of survival of the fungus in spinach seed. *Alternaria brassicae* (Berk.) Sacc. persisted for 10 and 6 months in rape seed and mustard seed stored at room temperature (11 to 25^{o}C) and 30^{o}C, respectively, but the pathogen was eliminated when the seed was stored at 40^{o}C for 4 months (Shrestha, 2003). *Drechslera* spp. and *Fusarium* spp. were eliminated from infected barley kernels, and *Alternaria tenuis* Nees was reduced from 80 to 10% incidence when seed were stored for 24 weeks at 20^{o}C and 14% relative humidity (Lutey and Christensen, 1963).

The component seed assays revealed that *S. botryosum* and *C. variabile* can reside in the pericarp as well as the embryo of spinach seed. Both pathogens were detected in the pericarps and embryos even when the two components were surface-sterilized separately in 1.2% NaOCl. This demonstrates that *S. botryosum* and *C. variabile* can be internal and external seedborne pathogens of spinach. However, both fungi were detected at higher incidences in the pericarps than the embryos. Intracellular hyphae of both pathogens were detected in the pericarps but not in the embryos by TEM, although not enough pericarps and embryos were examined by TEM in this study. Additional work is warranted to determine the location and nature of infection of *S. botryosum* and *C. variabile* in spinach seed. Detection of a higher incidence of *S. botryosum* than *C. variabile* in the embryos of spinach seed suggests that infection of spinach seed by *S. botryosum* may typically be more internal than that of *C. variabile*. However, the location of *C. variabile* and *S. botryosum* in spinach seed may also be influenced by maturity of the seed crop at the time of infection, as well as how favorable conditions are for *C. variabile* vs. *S. botryosum* at the time of infection of the developing seed. Leach and Borthwick (1934) reported the presence of hyphae of *Peronospora effusa* (Grev.) Tul. in the nucellus of spinach seed.

Colletotrichum dematium f. sp. *spinaciae* has been detected in the pericarp and seed coat of beet (*Beta vulgaris*), another member of the Chenopodiaceae (Chikuo and Sugimoto, 1989). Leach (1931) observed hyphae and oospores of *Peronospora farinosa* (Fr.:Fr.) Fr. in the seed coat of sugar beet, and Melhus (1931) detected hyphae of *Albugo bliti* (Biv.-Bern.) Kuntze in the seed coat of *Amaranthus* spp., and *Cystopus bliti* (Biv.-Bern.) Lév in the seed coat of *Amaranthus retroflexus* L.

The fact that *S. botryosum* and *C. variabile* can be internal and external seedborne pathogens of spinach raises questions about the potential for these pathogens to be transmitted to seedlings during germination, as documented for spinach downy mildew (Inaba et al., 1983). Seed transmission of *S. botryosum* in spinach has not been reported, but anecdotal evidence of seed transmission of *C. variabile* in spinach has been reported in Denmark (Hansen et al., 1952). Research is needed to assess the potential for seed transmission of these two leaf spot fungi. The presence of *S. botryosum* and *C. variabile* in the pericarps vs. embryos of spinach seed may also affect the potential efficacy of seed treatments (e.g., chlorine and/or hot water soaks, and fungicide seed treatments) for eradication of the fungi from the seed or for preventing seed transmission of these fungi.

The presence of *Verticillium* in a majority of the seed lots produced in Denmark, the USA, and the Netherlands, representing lots produced by all the seed companies that participated in this study, indicates that *Verticillium* too was prevalent in commercial spinach seed lots in 2003. Furthermore, *Verticillium* was shown to survive for at least 11 years in spinach seed. The *Verticillium* results of this survey have been published along with *Verticillium* pathogenicity and seed transmission trials completed by du Toit and Derie (du Toit et al., 2005).

2.5. LITERATURE CITED

1. Booth, C., and Pirozynski, K.A. 1967. *Pleospora herbarum*. Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria No. 150, Surrey, England.
2. Bozzola, J.J., and Russell, L.D. 1999a. Specimen preparation for scanning electron microscopy. Pages 49-71, In: Electron Microscopy. Principles and Techniques for Biologists, 2nd Edition. Jones and Bartlett Publishers, Sudbury, MA.
3. Bozzola, J.J., and Russell, L.D. 1999b. Specimen preparation for transmission electron microscopy. Pages 19-47, In: Electron Microscopy. Principles and Techniques for Biologists, 2nd Edition. Jones and Bartlett Publishers, Sudbury, MA.
4. Chikuo, Y., and Sugimoto, T. 1989. Histopathology of sugar beet flowers and seed balls infected with *Colletotrichum dematium* f. sp. *spinaciae*. Ann. Phytopathol. Soc. Japan 55:404-409.
5. Correll, J.C., Morelock, T.E., Black, M.C., Koike, S.T., Brandenberger, L.P., and Dainello, F.J. 1994. Economically important diseases of spinach. Plant Dis. 78:653-660.
6. David, J.C. 1995. *Cladosporium variabile*. International Mycological Institute Descriptions of Pathogenic Fungi and Bacteria No. 1229. Surrey, England.
7. du Toit, L.J., and Derie, M. L. 2001. *Stemphylium botryosum* pathogenic on spinach seed crops in Washington. Plant Dis. 85:920.
8. du Toit, L.J., and Derie, M.L. 2002. Leaf spot of spinach seed crops in Washington State. Phytopathology 92:S21.
9. du Toit, L.J., and Derie, M.L. 2003. Inoculum sources of *Stemphylium botryosum* and C*ladosporium variabile* in spinach seed crops. Phytopathology 93:S22.

10. du Toit, L.J., Derie, M.L., and Hernandez-Perez, P. 2004. Evaluation of fungicides for control of leaf spot in spinach seed crops, 2003. F&N Tests 59:V115.
11. du Toit, L.J., Derie, M.L., and Hernández-Pérez, P. 2005. Verticillium wilt in spinach seed production. Plant Dis. 89:4-11.
12. Ellis, M.B. 1971. *Cladosporium*. Page 315, In: Dematiaceous Hyphomycetes. Kew Commonwealth Mycological Institute. Surrey, England.
13. Everts, K.L., and Armentrout, D.K. 2001. Report of leaf spot of spinach caused by *Stemphylium botryosum* in Maryland and Delaware. Plant Dis. 85:1209.
14. Fuentes-Davila, G. 1988. Studies on a Leaf Spot Disease of Spinach Caused by *Cladosporium variable* (Cooke) de Vries. Ph.D. thesis, Washington State University, Pullman, WA. 49 pp.
15. Hadzistevic, D. 1955. The incidence of plant pests and diseases in the PR of Serbia in 1953. Plant Prot., Beograd. (Translated abstract from Rev. Appl. Mycol. 35:91).
16. Hansen, H.R., Weber, A., and Kristensen, H.R. 1952. Plant diseases in Denmark in 1950. Annual survey compiled by the State Phytopathological Experiment Station. Tidsskr. Planteavl. 56:1-59. (Translated abstract from Rev. Appl. Mycol. 32:234).
17. Hawksworth, D.L., and Talboys, P.W. 1970. *Verticillium dahliae*. Commonwealth Mycological Institute Description of Pathogenic Fungi and Bacteria No. 256. Surrey, England.
18. Inaba, T., Takahashi, K., and Morinaka, T. 1983. Seed transmission of spinach downy mildew. Plant Dis. 67:1139-1141.

19. Inglis, D.A., Derie, M.L., and Gabrielson, R.L. 1997. Cladosporium leaf spot on spinach seed crops and control measures. Washington State University Extension Bulletin No. 865. Pullman, WA.
20. Koike, S.T., Henderson, D.M., and Butler, E.E. 2001. Leaf spot disease of spinach in California caused by *Stemphylium botryosum*. Plant Dis. 85:126-130.
21. Leach, L.D. 1931. Downy mildew of beet caused by *Peronospora schachtii* Fuckel. Hilgardia 6:203-251.
22. Leach, L.D., and Borthwick, H.A. 1934. Distribution of downy mildew mycelium in spinach fruits. Phytopatholoy 24:1021-1025.
23. Lutey, R.W., and Christensen, C.M. 1963. Influence of moisture content, temperature and length of storage upon survival of fungi in barley kernels. Phytopathology 53:713-717.
24. Mathur, S.B., and Kongsdal, O. 2003. *Colletotrichum dematium*. Pages 182-183, In: Common Laboratory Seed Health Testing Methods for Detecting Fungi. International Seed Testing Association, Bassersdorf, Switzerland.
25. Melhus, I.E. 1931. The presence of mycelium and oospores of certain downy mildews in the seeds of their hosts. Iowa St. Coll. J. Sci. 5:185:188.
26. Neergaard, P. 1977. Management of seed storage. Pages 574-594, In: Seed Pathology. Volume I. John Wiley & Sons, New York, NY.
27. Peever, T.L., Canihos, Y., Olsen, L., Ibañez, A., Liu, Y.C., and Timmer, L.W. 1999. Population genetic structure and host specificity of *Alternaria* spp. causing brown spot of minneola tangelo and rough lemon in Florida. Phytopathology 89:851-860.

28. Peterson, P.S., and Harris, E. 1997. Notes on seed physiology, germination, and quality assessment. Page 67, In: Workshop of California Seed Analysts and Researchers, 26-28 March, Fresno, CA.

29. Raid, R.N, and Kucharek, T. 2003. 2003 Florida Plant Disease Management Guide: Spinach. http://edis.ifas.ufl.edu/BODY_PG054.

30. Sato, T. 1968. A modified method for lead staining of thin sections. J. Electron Microscopy 17:158-159.

31. Shrestha, S.K. 2003. Survival of *Alternaria brassicae* in seeds and a crop debris of rapeseed and mustard in Nepal. Seed Sci. & Technol. 31:103-109.

32. Simmons, E.G. 1985. Perfect states of *Stemphylium*. II. Sydowia 38:284-293.

33. Singh, D., and Mathur, S.B. 2004a. Location of fungal hyphae in seeds. Pages 101-168, In: Histopathology of Seed-Borne Infections. CRC Press, Boca Raton, FL.

34. Singh, D., and Mathur, S.B. 2004b. Structure of seeds. Page 55, In: Histopathology of Seed-Borne Infections. CRC Press, Boca Raton, FL.

35. Smith, H.C. 1965. The morphology of *Verticillium albo-atrum, V. dahliae,* and *V. tricorpus*. N. Z. J. Agric. Res. 8:450-478.

36. Van Poeteren, N. 1932. Report of the activities of the phytopathological service in the year 1931. Versl. en Meded. Plantenziektenkundigen Dienst Te Wageningen 66. (Translated abstract from Rev. Appl. Mycol. 11:766-767).

Table 1. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in 66 spinach seed lots produced in 2003 in Denmark, the Netherlands, New Zealand, and the USA by six seed companies

Company or country[a]	No. of seed lots infected/no. assayed (%)[b]	Incidence (%)[c]					No. of pathogenic isolates /no. tested[d]
		Mean	Standard deviation	Median	Min.	Max.	
		S. botryosum					
Company							
A	27/27 (100.00)	6.85	2.16	4.75	0.50	19.00	0/5
B	5/5 (100.00)	61.30	4.99	63.00	8.00	97.50	4/5
C	24/24 (100.00)	53.32	3.08	49.63	0.25	96.00	0/5
D	4/4 (100.00)	12.63	2.44	14.88	1.00	19.75	4/5
E	3/3 (100.00)	35.92	4.56	31.75	17.50	58.50	3/4
F	3/3 (100.00)	3.08	1.76	2.00	1.00	6.25	-
Country							
Denmark	27/27 (100.00)	58.36	3.54	63.00	8.00	97.50	5/9
The Netherlands	6/6 (100.00)	18.21	3.03	18.00	1.00	31.75	4/7
New Zealand	1/1 (100.00)	58.50	7.51	58.50	8.50	58.50	2/2
USA	32/32 (100.00)	6.10	2.02	4.13	0.25	19.00	0/6
Total	66/66 (100.00)	29.38	2.82	16.63	0.25	97.50	11/24
		C. variabile[e]					
Company							
A	17/27 (62.96)	0.45	0.49	0.25	0.00	2.50	0/8
B	0/5 (0.00)	0.00	0.00	0.00	0.00	0.00	-
C	6/24 (25.00)	0.14	0.13	0.00	0.00	1.00	5/7
D	0/4 (0.00)	0.00	0.00	0.00	0.00	0.00	-
E	0/3 (0.00)	0.00	0.00	0.00	0.00	0.00	-
F	3/3 (100.00)	2.92	1.82	3.25	1.50	4.00	-
Country							
Denmark	3/27 (11.11)	0.08	0.06	0.00	0.00	1.00	5/6
The Netherlands	1/6 (16.67)	0.04	0.08	0.00	0.00	0.25	-
New Zealand	0/1 (0.00)	0.00	0.00	0.00	0.00	0.00	-
USA	22/32 (68.75)	0.68	0.61	0.25	0.00	4.00	0/9
Total	26/66 (39.39)	0.37	0.33	0.00	0.00	4.00	5/15

[a] Seed company identities are coded to ensure producer anonymity.

[b] All 66 seed lots were harvested from commercial seed crops. None of the lots was treated with fungicides.

[c] Four sub-samples of 100 seed were surface-sterilized in 1.2% NaOCl for 60 s, triple-rinsed in sterile-deionized water, dried on sterile paper towel, and subjected to a freeze-blotter assay as described in the text. Min. and Max. = minimum and maximum incidence, respectively.

[d] Plants were inoculated with 20 ml of a conidial suspension (approximately 10^5 conidia/ml). A randomized complete block design with three replications per isolate (one plant/replication) was used.

[e] When short chains of large conidia (15 – 25 x 7 - 10µ) were observed on long conidiophores (about 150µ long) of *Cladosporium* isolates, *C. variabile* was recorded as the species.

Table 2. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in 12 spinach seed lots produced from 1993 to 2001 in western Washington, USA

Year[a]	Stock or harvested seed[b]	Incidence (%)[c]				
		S. botryosum			*C. variabile*[d]	
		Mean	Standard deviation	No. of pathogenic isolates/no. tested[e]	Mean	Standard deviation
1993	Stock	7.00	2.58	2/2	0.00	0.00
1993	Stock	7.50	3.11	1/1	0.00	0.00
1994	Unknown	8.50	2.38	2/2	0.00	0.00
1994	Stock	0.75	0.50	2/2	0.00	0.00
1994	Stock	0.75	0.50	1/1	0.00	0.00
1996	Stock	27.75	2.22	1/1	0.50	1.00
1996	Stock	17.25	5.38	2/2	0.00	0.00
1997	Stock	54.75	8.30	-	0.00	0.00
1998	Stock	36.50	5.20	-	0.00	0.00
1998	Unknown	20.25	4.11	-	0.00	0.00
2001	Stock	54.00	4.97	1/1	0.50	0.58
2001	Stock	19.50	1.91	2/2	0.00	0.00

[a] Year the seed was grown and harvested.

[b] Stock seed is seed of male or female inbred lines used to grow a hybrid seed crop. Harvested seed is the seed harvested from the female inbred line and subsequently sold to growers for fresh market or processing spinach production.

[c] Four sub-samples of 100 seed were surface-sterilized in 1.2% NaOCl for 60 s, triple-rinsed in sterile-deionized water, then dried, plated, and subjected to a freeze-blotter assay as described in the text.

[d] When short chains of large conidia (15 – 25 x 7 - 10µ) were observed on long conidiophores (usually 150µ long) of *Cladosporium* isolates, *C. variabile* was recorded as the species.

[e] A randomized complete block design with three replications per isolate (one plant/replication) was used to test pathogenicity of a sample of isolates on spinach. Three plants/isolate were inoculated with 20 ml of a conidial suspension (approximately 10^5 conidia/ml).

Table 3. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in spinach seed lot 01-101 produced in western Washington in 2001 and assayed in 2001, 2003, and 2004

	Incidence (%)[a]			
	S. botryosum		***C. variabile*[b]**	
Date of seed assay	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**
October 2001	60.00	-	17.00	-
June 2003	2.50	0.58	2.25	1.50
January 2004	0.25	0.50	0.00	0.00

[a] Four sub-samples of 100 seed were surface-sterilized in 1.2% NaOCl for 60 s, triple-rinsed in sterile-deionized water, then dried, plated, and subjected to a freeze-blotter assay as described in the text.

[b] When short chains of large conidia (15 – 25 x 7 - 10µ) were observed on long conidiophores (usually 150µ long) of *Cladosporium* isolates, *C. variabile* was assumed to be the species.

Table 4. Incidence of *Stemphylium botryosum* and *Cladosporium variabile* in the pericarps and embryos of five spinach seed lots

Seed lot[a]	Seed treatment [b]	Incidence (%)			
		S. botryosum		*C. variabile*	
		Pericarp	Embryo	Pericarp	Embryo
V	60 min rinse in deionized water	54	29	-	-
W	60 min rinse in deionized water + soak of whole seed in 1.2% NaOCl for 60 s	45	36	-	-
X	As for lot W	91	76	0	0
Y	As for lot W	45	36	2	0
Z	60 min rinse in deionized water + soak of separated pericarps and embryos in 1.2% NaOCl for 60 s	13	5	19	1

[a] Seed lots are coded for producer anonymity.

[b] All seed were rinsed in running deionized water for 60 min to soften the pericarps for manual separation from the embryos. The pericarps and embryos were separated manually for 100 seed/lot. Seed of lots W, X and Y were surface-sterilized in 1.2% NaOCl, then triple-rinsed in sterile-deionized water, dried, and separated into pericarps and embryos which were then plated and subjected to a freeze-blotter assay as described in the text. Seed lot V was not surface-sterilized in 1.2% NaOCl. Pericarps and embryos of each seed of lot Z were first separated and then surface-sterilized separately in 1.2% NaOCl.

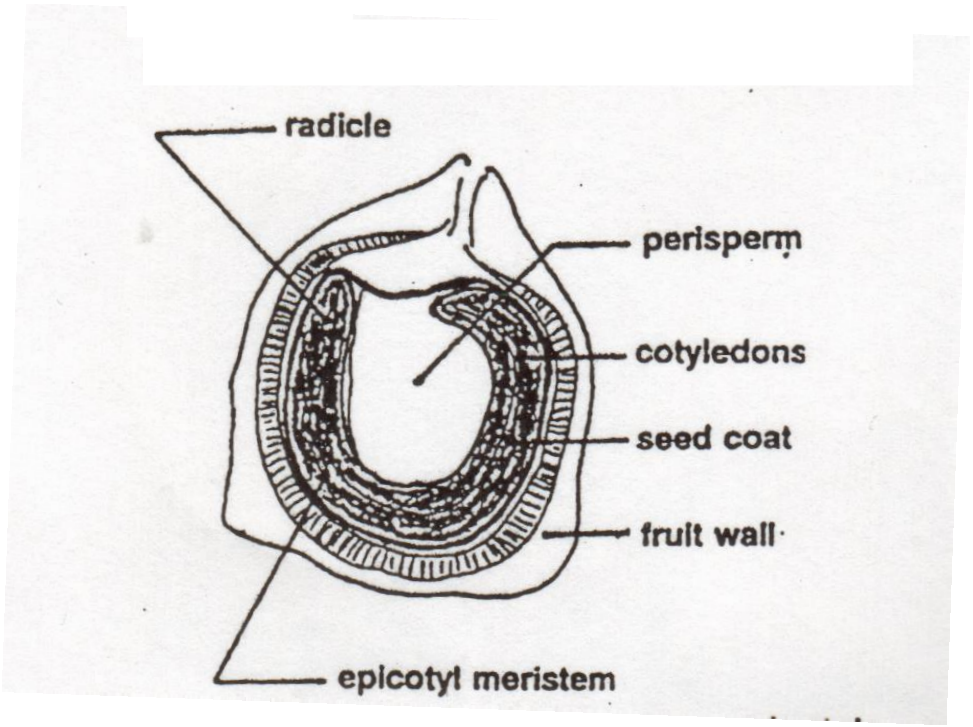

Fig. 1. Cross section of spinach fruit showing seed structure (from Peterson and Harris, 1997).

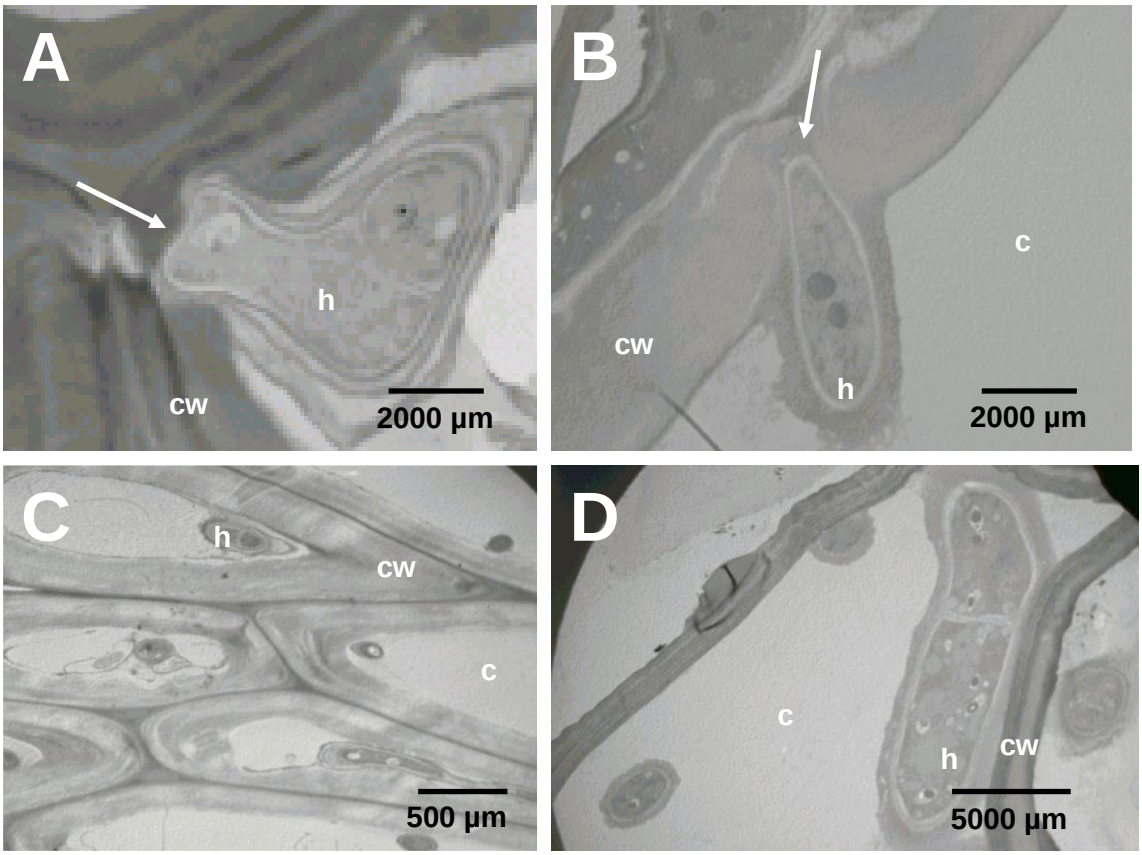

Fig. 2. Transmission electron micrographs of *Stemphylium botryosum* (A and C) and *Cladosporium variabile* (B and D) inside cells of the pericarps of spinach seed. cw = pericarp cell wall, h = hyphal cell of the fungus, c = plant cytoplasm, and arrow = site of penetration through the pericarp cell wall.

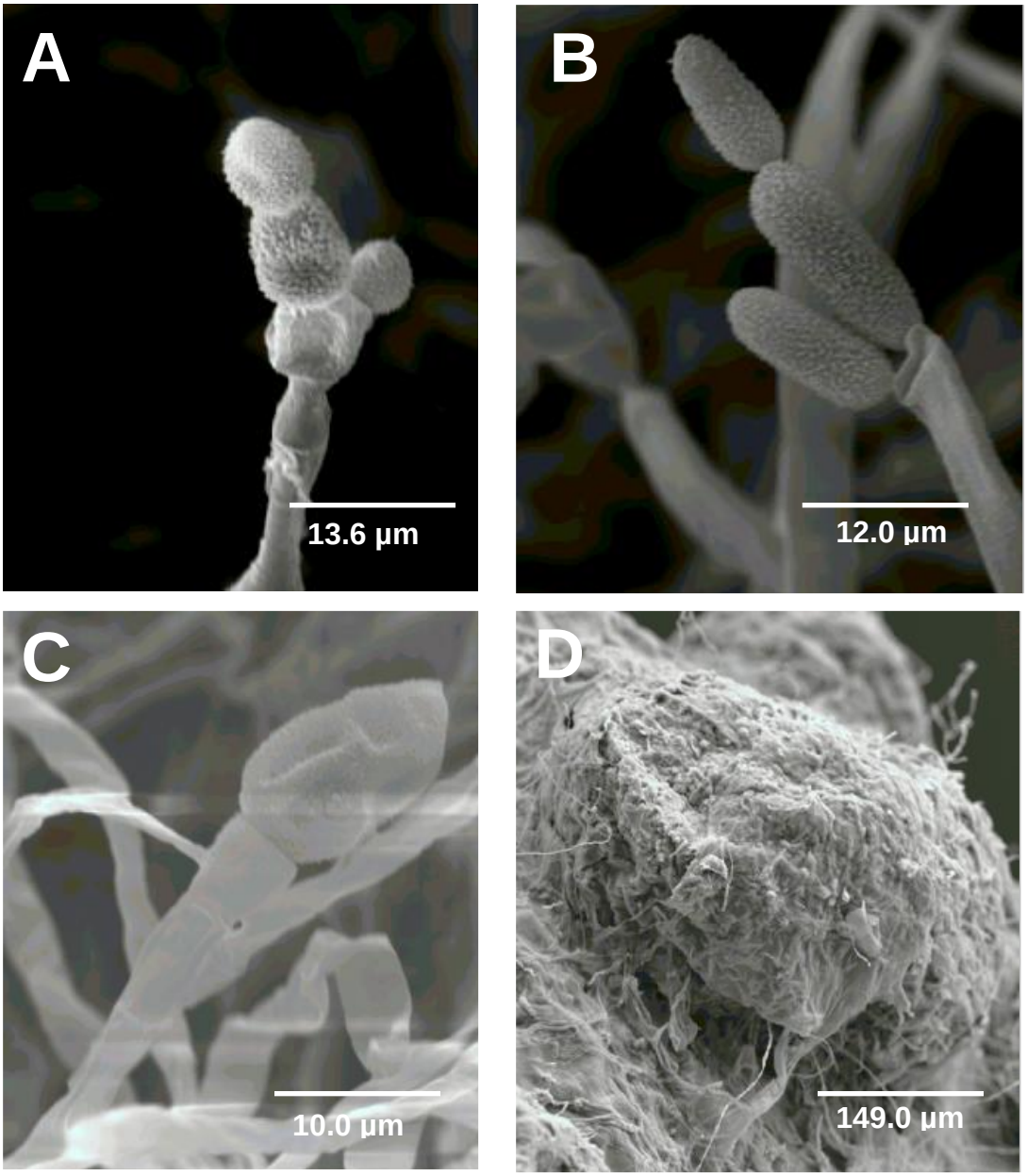

Fig. 3. Scanning electron micrographs of conidia and conidiophores of *Cladosporium variabile* (A and B), conidium and conidiophore of *Stemphylium botryosum* (C), and erumpent immature pseudothecium of *Pleospora herbarum* (D) on spinach seed.

Chapter 3

Efficacy of hot water and chlorine seed treatments for eradication of *Stemphylium botryosum* and *Cladosporium variabile* from spinach seed.

3.1. INTRODUCTION

Biological, chemical, and physical seed treatments are used to prevent transmission of internal or external seedborne pathogens from seed to the seedlings (Agarwal and Sinclair, 1997; Neergaard, 1977). Biological seed treatments involve the use of one or more organisms antagonistic to the pathogen. Chemical seed treatments involve the use of chemicals applied to the seed to control pathogens in the seed or in the soil. Fungicide (contact or systemic) seed treatment is the most common type of chemical seed treatment used. Other types of chemical seed treatments include disinfectants such as chlorine (Agarwal and Sinclair, 1997). Deep-seated seedborne pathogens are not readily affected by contact fungicides, so systemic fungicides are needed for internally infected seed. Biological and chemical seed treatments may also protect seed and seedlings from infection by soilborne pathogens, and subsequent dispersion of the pathogens within the developing crops (Agarwal and Sinclair, 1997; Neergaard, 1977; Maude, 1996).

Physical seed treatment is the use of hot water, saturated water vapor, dry heat, oil, microwaves, radiation, etc., for the treatment of seed under physical conditions that inhibit or kill the pathogens without damaging the seed (Agarwal and Sinclair, 1997; Maude, 1996). Hot water treatment is recommended for seed with surface or deep-seated infections. The higher the moisture content of the seed, the lower the temperature necessary to kill the seed using hot water treatment (Agarwal and Sinclair, 1997). The technique was developed by J. L. Jensen in 1888 in

Denmark as a method to control cereal smut (Neergaard, 1977). Small quantities of seed are recommended for hot water treatment because seed temperatures must be raised quickly and then dropped quickly after treatment. Nonetheless, even at temperatures that do not injure the embryo, the seed can be damaged as a result of imbibing water if the seed is not dried rapidly and adequately after treatment (Agarwal and Sinclair, 1997). Nega et al. (2003) stated that effective methods of seed treatment certified for organic farming are needed to control seedborne pathogens, and that hot water seed treatment is a potential method.

Hot water treatment has been reported to significantly reduce the incidence of *Alternaria dauci*, *A. radicina*, and *A. alternata* in carrot seed, *A. brassicicola* and *Phoma lingam* in cabbage seed, *P. valerianella* in lamb's lettuce seed, *A. radicina* in parsley seed and *A. porri* and *Stemphylium vesicarium* in onion seed (Aveling et al., 1993; Hermansen et al., 2000; Nega et al., 2003; Strandberg, 1988).

Chlorine seed treatment is a chemical method of treatment in which seeds are soaked in some form of chlorine (e.g., NaOCl) (Agarwal and Sinclair, 1997). In a preliminary study, du Toit and Derie (2003) detected 23.3, 16.8, 19.0, and 18.3% incidence of *S. botryosum* in spinach seed soaked in 1.2% NaOCl for 10, 20, 30, and 40 min, respectively, compared to 54.8% for non-treated seed. In contrast, *C. variabile* was detected at an incidence of 0.3% in spinach seed treated for 10 min or longer in 1.2% NaOCl, and was not detected in seed treated for 20, 30 or 40 min, compared to 49.0% for the non-treated seed. Treatment of seed with hot chlorinated water has been investigated for some seedborne pathogens. *Alternaria radicina* was eradicated from carrot seed in a 1.0% NaOCl solution heated at 50^{o}C for 20 min, without affecting germination significantly (Pryor et al., 1994). However, significant reduction in germination was observed when the seed was exposed to this treatment for 30 min.

To our knowledge, there are no published reports of the efficacy of hot water treatment of spinach seed. Although some seed companies currently utilize hot water and/or chlorine treatments for spinach seed, the details of these treatment protocols are not available to the public for proprietary reasons (L.J. du Toit, *personal communication*). Hence, there is a need for publically available research results on the efficacy of hot water and chlorine treatments for control of seedborne pathogens of spinach. The objective of this study was to determine the efficacy of hot water and chlorine seed treatments on eradication of *S. botryosum* and *C. variabile* from spinach seed.

3.2. MATERIALS AND METHODS

3.2.1. Chlorine seed treatments. To determine the efficacy of chlorine seed treatments on eradication of *S. botryosum* and *C. variabile* from spinach seed, four replications of five durations of seed treatment (0, 10, 20, 30, and 40 min) in 1.2% NaOCl were evaluated in the fall of 2004 using a randomized complete block design. The seed lot used was harvested from a spinach seed crop fungicide trial carried out at the Washington State University - NWREC in Mount Vernon in 2003. It was determined by freeze-blotter assay in November 2003 to be infected with *C. variabile* and *S. botryosum* at 17.0 and 21.5%, respectively (du Toit et al., 2004). For each replication of each treatment, a mesh tea strainer containing 220 seed of spinach seed lot 03-409 was soaked in a 250 ml glass beaker containing 100 ml of 1.2% NaOCl.The mouth of the beaker was immediately covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI), and the seed were immersed for the appropriate duration on a gyrotery shaker at 220 rpm. After the appropriate length of time, the seed were triple-rinsed while still in the tea strainer using sterile deionized water. For the control treatment, 220 seed were triple-rinsed in

sterile deionized water without hot water treatment. The seed were then dried on sterile paper towel in a laminar flow hood and placed in a sterile disposable Petri dish. The dish was sealed with Parafilm and stored at 24ºC in the dark.

For each replication of each treatment, 100 seed were then subjected to a freeze-blotter seed health assay following the method described in Chapter 2 and by du Toit et al. (2005b). *Stemphylium botryosum, C. variabile*, *Verticillium* spp., *Colletotricum* spp., and other fungi were identified as described in Chapter 2. For each replication of each treatment, 100 seed were subjected to a germination seed assay based on the Association of Official Seed Analysts (AOSA) protocol described by Yaklich (1985). Fifty seed were placed onto each of two double layers of Anchor seed germination blotters (38 # regular weight, Anchor Paper Co., St. Paul, MN) that had been moistened with deionized water and placed over a single sheet of wax paper. The seed on each blotter were covered with a germination blotter that had been moistened with deionized water. The blotters were rolled, secured with a rubber band and stored in an upright position. The blotter rolls were inserted into a plastic bag (ten rolls per plastic bag). Each bag was closed with a rubber band and placed in a plastic bucket in a seed germinator (Stults Scientific Engineering Corp., Springfield, IL) set at 15ºC with no light. Counts of germinated, non-germinated, abnormal, and rotten seed were carried out 4, 7, and 14 days later.

The experiment was repeated five weeks later. For the second trial, 50 and 60 min durations of treatment in 1.2% NaOCl were added for a total of four replications of six durations of seed treatment.

3.2.2. Hot water seed treatments. To determine the effect of hot water treatment on eradication of *S. botryosum* and *C. variabile* from spinach seed, a split plot design arranged in randomized complete blocks with four replications was established. The water temperature (40,

45, 50, 55, and 60°C) was the whole plot factor, and duration of treatment (10, 20, 30, and 40 min) at each temperature was the sub-plot factor. Four mesh tea strainers, each containing 220 spinach seed of lot 03-409, were immersed in deionized water heated to the appropriate temperature in a programmable circulating water bath (Fisher Scientific International, Inc., Hampton, NH). One tea strainer was removed from the water bath after each duration of treatment and immediately rinsed in running deionized water for 5 min at room temperature. The procedure was repeated for each temperature. The seed were dried and stored for two to five days as described for the chlorine seed treatments. The water was removed from the circulating bath between whole plot treatments (temperature), and the bath was disinfected with 70% ethyl alcohol and triple-rinsed with deionized water. For the control treatment for each replication, 220 seed were rinsed for 5 min in deionized water, dried and stored.

Freeze-blotter seed health assays and germination assays were performed as described for the chlorine seed treatments. Extra heavy weight (76 #) Anchor seed germination blotters (Anchor Paper Co.) were used instead of regular weight (38 #) blotters, so a single moistened blotter was used for each of the lower and top layers. *Stemphylium botryosum, C. variabile, Verticillium* spp., and other fungi were identified as described in Chapter 2.

The hot water seed treatment experiment was repeated twice. Seed lots 04-305 and 04-407, both harvested from a spinach seed crop fungicide trial carried out at the Washington State University - NWREC in Mount Vernon in 2004 (du Toit et al., 2005a), were used in trials 2 and 3, respectively, because the incidence of *S. botryosum* in seed lot 03-409 had dropped from 16.75% in the second chlorine trial to 4.75% in the first hot water trial, four months later.

Analyses of variance, means comparisons by Fisher's protected least significance difference (LSD), correlation coefficients, and regression analyses were calculated for the seed health and

germination assays using PROC GLM of SAS Version 8.2 (SAS Institute, Cary, NC). To generate homogeneous variances and normal residuals for parametric analyses of results of the chlorine trials, the dependent variable was calculated as a percentage of the control treatment: $(X_i - X_o) / X_o * 100$, where X_i = dependent variable at i min in 1.2% NaOCl, and X_o = dependent variable for the control treatment (0 min in 1.2% NaOCl) of the same replication. For the chlorine trials, the incidences (%) of *Fusarium* spp. and non-germinated seed detected in trial 2 were subjected to square root transformation to generate homogeneous variances. Friedman's non-parametric rank test was used instead of Fisher's protected LSD to compare means for the incidences of *C. variabile* and *Fusarium* spp. detected in trial 1, and the incidence of *C. variabile* and seed germination after 7 days in trial 2, because transformations did not generate homogeneous variances and/or normal residuals. For the hot water trials, each dependent variable was analyzed using Friedman's non-parametric rank test because of non-normal data, except for the following variables: abnormal germination, rotten seed, and incidence of other *Cladosporium* spp. detected in trial 1; and total germination in trial 2. These variables were each calculated as a percentage of the control treatment for each replication as described above, and the following transformations were used to generate homogeneous variances and/or normal residuals: square root transformation for rotten seed in trial 1 and total germination in trial 2, and arcsin transformation for the incidence of *Cladosporium* spp. other than *C. variabile* detected in trial 1. The original means are presented for each of the dependent variables (Tables 2, 4, and 5), but means separations were calculated as explained above.

3.2.3. Component freeze-blotter seed assay. To determine the incidence of *C. variabile* and *S. botryosum* in the pericarps and embryos of lot 04-407 used in the third hot water trial, a component freeze-blotter seed assay was performed in February 2005 for 100 seed of this lot as

described in Chapter 2. The seed were first soaked in deionized water for 60 min to soften the pericarps for manual separation from the embryos. The separated pericarps and embryos were then surface-disinfected individually in 1.2% NaOCl for 60 s, triple-rinsed in sterile deionized water, dried, plated, and examined microscopically as described in Chapter 2.

3.3. RESULTS

3.3.1. Chlorine seed treatments. Chlorine seed treatments did not have a significant effect on seed quality in trial 1, whether measured as percentage germinated, non-germinated, abnormal, or rotten seed (Tables 1 and 2). Similar results were observed in the second chlorine trial, except for the percentage non-germinated seed ($P = 0.0324$ for duration effect in the ANOVA) (Table 1). However, the variation among replications for this variable was far greater than that among treatments (Table 1), and percentage non-germinated seed for the control treatment (16.00%) was not significantly different from that of the 60 min chlorine treatment (12.75%) (Table 2). In contrast, chlorine treatments significantly reduced the incidence of *C. variabile* detected in the seed in both trials (Table 1), regardless of the duration of chlorine treatment (Table 2). The incidence of *C. variabile* was reduced from 34.50% for non-treated seed to ≤ 1.00% for seed treated for 10 to 40 min in 1.2% NaOCl in trial 1, and from 28.25% for the non-treated seed in trial 2 to ≤ 0.50% (Table 2). There was no significant difference in incidence of *C. variabile* on seed treated with 1.2% NaOCl for 10, 20, 30, 40, 50, or 60 min (Table 2). The relationship between the percentage *C. variabile* detected in the treated seed (Y) and the duration of treatment in 1.2% NaOCl (X) was described by the following linear regressions: $Y = 34.50 – 6.73X + 0.45X^2 - 0.10X^3$ ($R^2 = 0.97$, CV = 38.58%) for trial 1, and $Y = 27.86 – 4.50X + 0.23X^2$ ($R^2 = 0.81$, CV = 121.58%) for trial 2.

Although the mean incidence of *S. botryosum* was reduced from 16.75% for the non-treated seed to 9.50% for seed treated with chlorine for 10 min, and <7.00% for seed treated for 20 to 40 min, in trial 1, there was no significant difference in incidence of *S. botryosum* on seed treated with chlorine for 10, 20, 30, or 40 min in this trial (Tables 1 and 2). In trial 2, carried out 5 weeks later than trial 1, the mean incidence of *S. botryosum* had dropped to 7.75% for the non-treated seed. There was no significant difference in incidence of *S. botryosum* detected on seed treated with chlorine for durations of 10, 20, 30, 40, 50, or 60 min (Table 2). The relationship between the percentage *S. botryosum* observed on the treated seed (Y) and the duration of treatment in 1.2% NaOCl (X) was shown by the following linear regressions: $Y = 16.56 – 0.82X + 0.10X^2$ ($R^2 = 0.75$, CV = 30.78%) for trial 1, and $Y = 8.24 – 0.08X$ ($R^2 = 0.32$, CV = 43.41%) for trial 2.

The incidence of *Verticillium* spp. [primarily *V. dahliae*, a known pathogen of spinach (du Toit, et al., 2005b)] observed on the seed was reduced from 33.75% for the non-treated seed to <4.00% for seed treated with chlorine in trial 1, and from 47.00 to <3.00% in trial 2 (Table 2). However, as for *C. variabile*, there was no significant difference in incidence of *Verticillium* spp. among the durations of chlorine seed treatment in either trial (Table 1). The relationship between the percentage *Verticillium* spp. observed on the treated seed (Y) and the duration of treatment in 1.2% NaOCl (X) was described by the following linear regressions: $Y = 33.47 – 4.62X + 0.20X^2$ ($R^2 = 0.95$, CV = 39.09%) for trial 1, and $Y = 46.53 – 7.10X + 0.36\ X^2 + 0.01\ X^3$ ($R^2 = 0.88$, CV = 76.90%) for trial 2.

The mean incidence of *Cladosporium* spp. other than *C. variabile* observed on the spinach seed was reduced significantly in both trials as a result of chlorine treatment, from 76.50% for non-treated seed to <11.00% in trial 1 and from 44.75 to <7.00% in trial 2 (Table 2). Significant

differences among the durations of chlorine treatment were only detected in trial 1 (Table 1), in which the incidence of these other *Cladosporium* spp. decreased incrementally from 10.25 to 5.25% with increasing duration of chlorine treatment from 10 to 40 min (Table 2). Similar results were observed for the mean incidence of *Fusarium* spp. detected in both trials (Tables 1 and 2), although a low incidence of *Fusarium* spp. was present in the non-treated seed (mean of 3.00 and 1.25% in trials 1 and 2, respectively). No significant differences among durations of chlorine treatment were detected for the mean incidence of *Alternaria* spp. in either trial (Table 1) However, the mean incidence of *Alternaria* spp. was significantly lower for all durations of chlorine seed treatment compared to the control treatment (58.75 and 57.25% for trials 1 and 2, respectively) (Table 2).

A significant correlation was detected in the first chlorine trial between the percentage rotten seed in the germination assay and the percentage of each of the fungi enumerated in the seed health assay: r = 0.51 at $P = 0.0214$ for *C. variabile*, r = 0.48 at $P = 0.0311$ for *S. botryosum*, r = 0.52 at $P = 0.0200$ for *Verticillium* spp., r = 0.54 at $P = 0.0100$ for other *Cladosporium* spp., r = 0.69 at $P = 0.0007$ for *Fusarium* spp., and r = 0.53 at $P = 0.0100$ for *Alternaria* spp. Significant correlations were also detected in trial 2 between the percentage non-germinated seed and percentage other *Cladosporium* spp. (r = 0.41 at $P = 0.0283$) and *Alternaria* spp. (r = 0.50 at $P = 0.0063$), and between the percentage abnormal seed and percentage *S. botryosum* (r = -0.44 at $P = 0.0187$).

3.3.2. Hot water seed treatments. Of the four variables analyzed using parametric analyses for the hot water trials (Table 3), the main (whole plot) factor of water temperature was significant for three variables: percentage abnormal germination and other *Cladosporium* spp. in trial 1, and percentage total germination in trial 2. The sub-plot factor of duration of treatment at

each temperature was significant for the percentage other *Cladosporium* spp. detected in trial 1 and total germination in trial 2. However, a significant ($0.01 < P \leq 0.05$) or highly significant ($P \leq 0.01$) interaction was detected between the hot water temperatures evaluated and the durations of seed treatment, for each of the seed quality variables measured in all these trials, except percentage rotten seed in trial 1 ($P = 0.7500$) (Table 3).

In trial 1, a significant delay in seed germination at 4 days was observed for seed heated to 50ºC for 40 min, 55ºC for 20 min or longer, or 60ºC for any duration compared to non-treated seed (Table 4). Total seed germination was not significantly different from that of the non-treated seed (mean of 80.25%) when the seed was treated at 40, 45, or 50ºC for 10, 20, 30, or 40 min, or at 55ºC for 10 or 20 min. However, a significant reduction in total germination was detected for seed heated at 55ºC for 30 or 40 min, and at 60ºC for any duration of treatment. None of the seed germinated that was soaked for 30 or 40 min in water heated to 60ºC (Table 4). Similar total germination results were observed in trials 2 and 3 (Table 4), although germination at 4 days was reduced significantly at 50ºC for only 20 min in these two trials (compared to 40 min in trial 1), and at all durations of treatment at 55 and 60ºC. Seed lots 04-305 and 04-407 used in trials 2 and 3, respectively, had lower germination for non-treated seed (48.00 and 40.75%, respectively) compared to lot 03-409 used in trial 1 (80.25%). The percentages of rotten seed were not significantly different from that of the non-treated seed (mean of 0.75%) for all the hot water treatments in trial 1. The percentage abnormal seed was reduced significantly from 3.50% for non-treated seed to 0.00% for seed treated at 60 ºC for 30 to 40 min, in trial 1. Similar results for percentage non-germinated, rotten, and abnormal seed were detected in trials 2 and 3, except that percentage non-germinated seed was also significantly affected at 50ºC treatment for

30 or 40 min, and the percentage rotten seed was reduced significantly at 60ºC for 40 min in trial 2 and for 20, 30, or 40 min in trial 3 (Table 4).

For all the variables recorded in the freeze-blotter seed health assays, a highly significant interaction was detected between water temperature and the duration of treatment at each temperature (Table 3). In all three trials, *C. variabile* was eradicated from the seed by all hot water treatments, except at 50ºC for 40 min in trial 2, when *C. variabile* was detected at an incidence of 0.25% (Table 5). The mean incidence of *S. botryosum* detected in the control treatment was only 4.75% in trial 1. Consistent and significant reductions in the mean incidence of this pathogen were detected on seed heated to 50ºC or higher for any duration in trial 1, and the pathogen was eradicated from seed heated to 55ºC for at least 20 min, or 60ºC for at least 10 min (Table 5). In trials 2 and 3, *S. botryosum* was observed on 65.75 and 66.75% of the non-treated seed, respectively. In both trials, significant reductions in incidence of *S. botryosum* were detected on seed heated to 40ºC for 40 min, or at higher temperatures for any duration (Table 5). However, *S. botryosum* was not eradicated from the spinach seed in either trial, even for seed heated to 60ºC for 40 min (at which temperature and duration the incidence of *S. botryosum* was reduced to 10.75 and 12.00% in trials 2 and 3, respectively).

The relationship between the incidence of *C. variabile* on the treated seed (Y), the temperature of the water (X), and the duration of treatment at that temperature (Z), was best described by the following linear regressions: $Y = 26.34 – 3.98Z + 0.17Z^2$ ($R^2 = 0.76$, CV = 76.90%) for trial 1, $Y = 1.16 – 0.17Z + 0.01Z^2$ ($R^2 = 0.57$, CV = 319.12%) for trial 2, and $Y = 1.40 – 0.21Z + 0.01Z^2$ ($R^2 = 0.59$, CV = 370.67%) for trial 3. Similarly, the linear regressions for *S. botryosum* were: $Y = 6.72 – 0.11X - 0.02Z$ ($R^2 = 0.47$, CV = 108.73%) for trial 1, Y = 106.15

– 2.10X + 4.32Z - 0.18Z^2 (R^2 = 0.86, CV = 23.77%) for trial 2, and Y = 100.76 – 1.86X + 4.32Z - 0.20Z^2 (R^2 = 0.85, CV = 19.56%) for trial 3.

The mean incidence of *Verticillium* spp. detected in the non-treated seed was 39.75, 23.00, and 38.75% in trials 1, 2, and 3, respectively (Table 5). Significant reductions in incidence of this fungus on the seed were observed for seed treated at 45ºC or higher in all three trials, and at 40ºC for at least 40 min in trial 2 or 20 min in trial 3. *Verticillium* spp. were eradicated from spinach seed treated at 50ºC or higher in trial 1; 50ºC for 40 min, 55ºC for at least 30 min, or 60ºC for any duration in trial 2; and 55ºC for at least 20 min or 60ºC for any duration in trial 3 (Table 5). The relationship between the percentage *Verticillium* spp. detected on the treated seed (Y), temperature of the water (X), and duration of hot water treatment (Z), was shown in the following linear regressions: Y = 3329.97 – 190.25X + 3.60X^2 – 0.02X^3 (R^2 = 0.64, CV = 144.50%) for trial 1, Y = 34.71 – 0.61X + 0.26Z – 0.02Z^2 (R^2 = 0.65, CV = 87.64%) for trial 2, and Y = 71.68 – 2.06X + 0.01X^2 – 0.43Z – 0.01Z^2 + 0.01XZ (R^2 = 0.90, CV = 61.82%) for trial 3.

The mean incidence of *Cladosporium* spp. other than *C. variabile* observed on the seed was reduced significantly from that of the control seed by hot water treatment at 40ºC for only 10 min, similar to the effects of hot water treatment on *C. variabile*. A low incidence (0.25 to 2.00%) of these other *Cladosporium* spp. could still be detected on seed treated at 60ºC in each trial (Table 5). A significant reduction in the mean incidence of *Fusarium* spp. compared to the non-treated seed was observed on seed treated at 45ºC and higher in trial 1, and at 40ºC for at least 20 min in trial 2 or 30 min in trial 3. However, the mean incidence of *Fusarium* spp. on non-treated seed was low in trial 1 (2.50%) compared to trials 2 and 3 (52.75 and 60.75%, respectively) (Table 5). The mean incidence of *Alternaria* spp. was reduced significantly from

that of the non-treated seed on seed treated at 40ºC for at least 20 min in trials 1 and 3, and at least 30 min in trial 2 (Table 5). *Alternaria* spp.were mostly eradicated from seed treated in water heated to 55 or 60ºC (Table 5).

In several hot water trials, significant (negative) correlations were detected between seed germination and the mean incidence of *S. botryosum* (r = 0.86 at *P* = 0.0300 for trial 2, and r = 0.94 at *P* = 0.0042 for trial 3), *Fusarium* spp. (r = 0.83 at *P* = 0.0422 in trial 3), and *Alternaria* spp. (r = 0.83 at *P* = 0.0403 in trial 3). Significant correlations were also detected between the percentage rotten seed and each of the fungi observed on the seed in trial 3 (r = 0.88 at *P* = 0.0206 for *C. variabile*, r = 0.94 at *P* = 0.0047 for *S. botryosum*, r = 0.95 at *P* = 0.0034 for *Verticillium* spp., r = 0.91 at *P* = 0.0126 for other *Cladosporium* spp., r = 0.94 at *P* = 0.0046 for *Fusarium* spp., and r = 0.94 at *P* = 0.0053 for *Alternaria* spp.).

3.3.3. Component freeze-blotter seed assay. *Cladosporium variabile* was not observed in the pericarps or embryos of the component seed assay for lot 04-407 completed in February 2005, three months after the third hot water trial was completed. *Stemphylium botryosum* was detected in 53.92% of the pericarps and 39.21% of the embryos.

3.4. DISCUSSION

Chlorine seed treatment for durations of 10 to 60 min significantly affected the incidences of *C. variabile* and *S. botryosum* detected on spinach seed. The mean incidence of *C. variabile* detected on spinach seed was significantly reduced for infected seed soaked in 1.2% NaOCl for as little as 10 min. In contrast, the reduction in incidence of *S. botryosum* by chlorine seed treatment was far less than that of *C. variabile*. At a low level of seedborne *S. botryosum* (7.75% in trial 2), there was no significant difference in the mean incidence of the fungus detected on

seed treated with chlorine for durations of 10 to 60 min. However, with a higher incidence of *S. botryosum* on the seed (16.75% in trial 2), the incidence was reduced by 43 to 67% of that detected on the non-treated seed, with the greatest reduction in seedborne infection observed for the longest duration of chlorine treatment (60 min). Seed quality was not adversely affected by chlorine treatment for any duration (10 to 60 min). Similar results were obtained by du Toit and Derie (2003) using 10 to 40 min of seed treatment in 1.2% NaOCl, where *C. variabile* was mostly eradicated from seed following 20 min or longer of chlorine treatment. In that study, the incidence of *S. botryosum* was also reduced significantly using 10 to 40 min of chlorine treatment, with the greatest reduction (66.60% of that detected on the non-treated seed) at 40 min of chlorine treatment.

Cladosporium variabile was eradicated from spinach seed treated with hot water at 40^{o}C for only 10 min, with no significant effect on seed germination at that temperature and duration of treatment. In contrast, *S. botryosum* was not eradicated from spinach seed by hot water treatment for the seed lots heavily infected with this fungus (65.75% for lot 04-305 and 66.75% for lot 04-407). *Stemphylium botryosum* was eradicated from a spinach seed lot with a low incidence of this fungus (4.75% for lot 03-409) when the seed was placed in water heated to 55^{o}C for at least 20 min or 60^{o}C for at least 10 min. This suggests that *S. botryosum* may be eradicated from spinach seed using hot water treatment if the seed lots have low levels of infection. Nonetheless, the results of hot water trials 2 and 3 suggest that *S. botryosum* is relatively heat tolerant, as the fungus was observed developing on spinach seed that had been heated to 60^{o}C for 40 min.

The more effective reduction in incidence of *C. variabile* compared to *S. botryosum* on spinach seed using chlorine or hot water treatments, supports the results of the component seed assays in Chapter 2 and in this chapter. The component seed assays described in Chapter 2

demonstrated that both *C. variabile* and *S. botryosum* can be internal and external pathogens of spinach seed, but that infection of spinach by *S. botryosum* appeared to be more deep-seated than that of *C. variabile*. The component seed assay results of this chapter corroborate those of Chapter 2 with respect to the prevalence of *S. botryosum* in the pericarps and embryos of the seed, although in all component assays the incidence of pericarps infected was greater than the incidence of embryos infected. Therefore, *C. variabile* may be more vulnerable than *S. botryosum* in spinach seed to the fungicidal effects of chlorine solutions and hot water.

Verticillium spp. and *Cladosporium* spp. other than *C. variabile* observed on spinach seed in this study were similarly sensitive to chlorine seed treatments as *C. variabile*. The mean incidences of these fungi were reduced significantly after chlorine treatment. Hot water seed treatment at 40°C was not as effective as chlorine seed treatment for eradicating *Verticillium* spp. However, hot water treatments at 45, 50, 55, or 60°C were effective against these fungi, with efficacy increasing as the duration of treatment increased from 10 to 40 min at each temperature. *Cladosporium variabile* was slightly more sensitive to hot water treatment than the other *Cladosporium* spp. observed on the spinach seed.

The mean incidence of *Fusarium* spp. detected on spinach seed was significantly reduced by chlorine treatments, but *Alternaria* spp. exhibited less of a response to chlorine treatment. In contrast, the *Fusarium* spp. and *Alternaria* spp. detected were mostly eradicated by hot water treatment at 55°C or 60°C. Significant reductions in incidence of these two genera were also detected at hot water temperatures of 45 and 50°C, with increasing efficacy of the treatments at higher temperatures and for longer durations.

Several studies have evaluated the efficacy of hot water treatments on different *Alternaria* spp. in small-seeded vegetables. The temperatures and durations of hot water treatment at which

significant reductions in the mean incidence of these fungi were achieved are similar to what was detected in this study on spinach seed. Nega et al. (2003) found that hot water treatment at 50ºC for 20 to 30 min, or at 53ºC for 10 to 30 min for carrot, cabbage, celery, parsley, and lamb's lettuce seed controlled *A. dauci, A. radicina, A. alternata,* and *A. brassicicola*. Hermansen et al. (2000) showed that heating carrot seed to 54ºC in water for 20 min eradicated *A. dauci*. Aveling et al. (1993) treated onion seed at 50ºC for 20 min to control *A. porri* and *Stemphylium vesicarium*. Strandberg (1988) treated carrot seed at 50ºC for 12 min to control *A. dauci* effectively. Pryor et al. (1994) used 50ºC hot water treatment of carrot seed for 20 min to control *A. radicina*.

Seed germination was not affected on spinach seed treated with 1.2% NaOCl for as long as 60 min. The results suggest that if a spinach seed lot infected with *C. variabile, S. botryosum*, and *Verticillium* spp. is treated in 1.2% NaOCl for ≥ 20 min, *C. variabile* and *Verticillium* spp. may largely be eradicated from the seed, and the mean incidence of *S. botryosum* will be reduced significantly with no adverse effect on germination. In contrast, a significant reduction in germination was detected for spinach seed lot 03-409 treated in water heated to 55ºC for 30 min or longer. *Stemphylium botryosum* was largely eradicated when the seed was heated to 50ºC for at least 20 min or to 55ºC or 60ºC for as little as 10 min. These results suggest that seed lots with a low incidence of *S. botryosum* can perhaps be treated effectively using hot water at 50ºC for 20 min with a significant reduction in incidence of this fungus, at well as eradicating *Verticillium* spp. from the seed and reducing the incidence of other *Cladosporium* spp., *Fusarium* spp., and *Alternaria* spp. without affecting seed germination. Similarly, a significant effect on germination was detected for spinach seed lot 04-305 treated with hot water at 55ºC for 20 min. This finding suggests that this lot could be treated at 55ºC for 10 min to achieve a significant reduction in

incidence of *S. botryosum* and highly significant reductions in the incidences of *Verticillium* spp., *Cladosporium* spp., *Fusarium* spp., and *Alternaria* spp. without a significant adverse effect on germination. Overall, results of the hot water seed treatment trials suggest that a spinach seed lot infested with *C. variabile*, *S. botryosum*, and *Verticillium* spp. may be treated with hot water at 50ºC for 20 min to eradicate *C. variabile* and reduce the incidence of *S. botryosum*, *Verticillium* spp., *Fusarium* spp., *Alternaria* spp., and other *Cladosporium* spp. with no adverse effect on seed germination. The results of this study suggest that chlorine and hot water seed treatments can be used effectively to reduce infection of spinach seed by *C. variabile* and *S. botryosum*. Both methods of seed treatment were more effective at controlling *C. variabile* than *S. botryosum* in spinach seed, but the temperature and duration of hot water treatements must be controlled precisely to avoid injuring the seed.

Hot water seed treatments could be utilized in conventional and organic production. Agarwal and Sinclair (1997) mentioned that such physical methods of seed treatment are usually used for control of seedborne pathogens when chemical seed treatments are not available. Hence there is a need to evaluate conventional, biological, and organic fungicide seed treatments (particularly systemic fungicides) as a method of treating infected spinach seed. This is particularly relevant given the predominant internal nature of infection of some seedborne pathogens of spinach such as *S. botryosum*. The efficacy of hot chlorine solutions on seedborne pathogens of spinach, particularly recalcitrant internal infections of fungi like *S. botryosum*, also remains to be investigated.

3.5. LITERATURE CITED

1. Agarwal, V.K., and Sinclair, J.B. 1997. Control of seedborne pathogens. D. Mechanical methods. Pages 453-459, In: Principles of Seed Pathology, 2nd Edition. CRC Press, Inc., Boca Raton, FL.
2. Aveling, T.A.S., Snyman, H.G., and Maude, S.P. 1993. Evaluation of seed treatments for reducing *Alternaria porri* and *Stemphylium vesicarium* on onion seed. Plant Dis. 77:1009.
3. du Toit, L.J., and Derie, M.L. 2003. Inoculum sources of *Stemphylium botryosum* and *Cladosporium variabile* in spinach seed crops. Phytopathology 93:S22.
4. du Toit, L.J., Derie, M.L., and Hernandez-Perez, P. 2004. Evaluation of fungicides for control of leaf spot in spinach seed crops, 2003. F&N Tests 59:V115.
5. du Toit, L.J., Derie, M.L., and Hernandez-Perez, P. 2005a. Evaluation of fungicides for control of leaf spot in spinach seed crops, 2004. F&N Tests 60:V044.
6. du Toit, L.J., Derie, M.L., and Hernández-Pérez, P. 2005b. Verticillium wilt in spinach seed production. Plant Dis. 89:4-11.
7. Hermansen, A., Brodal, G., and Balvoll, G. 2000. Hot water treatment of carrot seeds: Effects on seed-borne fungi, germination, emergence and yield. Seed Sci. Technol. 27:599-613.
8. Maude, R.B. 1996. Disease control: Eradication and reduction of inoculum by seed treatment. Pages 114-116, In: Seedborne Diseases & Their Control. Principles & Practice. CAB International, Oxon, UK.
9. Neergaard, P. 1977. Seed treatment, procedures and equipment. Pages 595-613, In: Seed Pathology. Volume I. John Wiley & Sons, New York, NY.

10. Nega, E., Ulrich, R., Werner, S., and Jahn, M. 2003. Hot water treatment of vegetable seed: An alternative seed treatment method to control seed-borne pathogens in organic farming. J. Plant Dis. Prot. 10:220-234.
11. Pryor, B.M., Davis, R.M., and Gilbertson, R.L. 1994. Detection and eradication of *Alternaria radicina* on carrot seed. Plant Dis. 78:452-456.
12. Strandberg, J.O. 1988. Detection of *Alternaria dauci* on carrot seed. Plant Dis. 72:531-534.
13. Yaklich, R.W. 1985. Germination tests. Pages 30-36, In: Rules for Testing Seeds. Association of Official Seed Analysts. J. Seed Technol. 8:2.

Table 1. Analyses of variance for germination and seed health assays of spinach seed lots treated with chlorine (1.2% NaOCl) for durations ranging from 0 to 40 (trial 1) or 60 min (trial 2)[a]

	Trial 1				Trial 2			
			Mean square probability >F				Mean square probability > F	
Dependent variable[b]	**CV (%)**	**R^2**	**Replication**	**Duration**	**CV (%)**	**R^2**	**Replication**	**Duration**
Germination assay (%)[c]								
Germination at 4 days	-67.56	0.359	0.7512	0.3416	-102.13	0.610	0.0040	0.7450
Germination at 7 days	-81.72	0.385	0.5167	0.4114	57.58	0.140	1.0000	0.8100
Total germination	-84.30	0.722	0.0081	0.8270	-89.03	0.446	0.3300	0.2020
Non-germinated seed	152.92	0.638	0.0245	0.9050	26.57	0.735	0.0076	0.0324
Abnormal germination	-196.19	0.538	0.1613	0.3248	-148.52	0.748	0.0002	0.3260
Rotten seed	-2293.19	0.690	0.0327	0.1702	-227.54	0.611	0.0058	0.4653
Seed health assay (% of seed infected)[d]								
Cladosporium variabile	32.63	0.671	1.0000	0.0063	2.04	0.632	1.0000	0.0030
Stemphylium botryosum	13.13	0.940	<0.0001	0.0148	243.03	0.638	0.0120	0.1072
Verticillium spp.	4.07	0.635	0.0805	0.1700	2.96	0.295	0.6640	0.4960
Other *Cladosporium* spp.	3.09	0.694	0.1233	0.0390	4.50	0.696	0.0010	0.2910
Fusarium spp.	19.84	0.843	1.0000	<0.0001	36.44	0.454	0.0700	0.5890
Alternaria spp.	15.42	0.579	0.4666	0.0760	14.52	0.467	0.1270	0.3180

[a] Each trial consisted of a randomized complete block design with four replications of each duration of treatment in 1.2% NaOCl (0, 10, 20, 30, and 40 min for trial 1; and 0, 10, 20, 30, 40, 50, and 60 min for trial 2). CV = coefficient of variation. R^2 = coefficient of determination.

[b] To generate homogeneous variances and normal residuals, the dependent variable was calculated as a % of the control treatment: $(X_i - X_o) / X_o * 100$, where X_i = measurement at i min in 1.2% NaOCl, and X_o = measurement for the control treatment (0 min) of that replication. Results for % *Fusarium* spp. and non-germinated seed in trial 2 were square-root-transformed to generate homogeneous variances. Friedman's non-parametric analyses were used for % *C. variabile* and *Fusarium* spp. in trial 1, and % *C. variabile* and germination at 7 days in trial 2, because transformations did not generate homogeneous variances.

[c] For each replication of each treatment, 100 seed were subjected to a germination assay (Yaklich, 1985).

[d] A freeze-blotter seed health assay was used as described in the text.

Table 2. Efficacy of chlorine seed treatments for eradication of *Cladosporium variabile*, *Stemphylium botryosum*, *Verticillium* spp., and other fungi from spinach seed[a]

Trial and duration in NaOCl (min)[b]	Germination assay (% of seed)[c]						Seed health assay (% of seed infected)[d]					
	Germinated											
	4 days	7 days	Total	Non-germinated	Abnormal	Rotten	*C. variabile*	*S. botryosum*	*Verticillium* spp.	Other *Cladosporium* spp.	*Fusarium* spp.	*Alternaria* spp.
Trial 1												
0	22.50[e]	54.00	75.25	14.75	3.50	6.00	34.50 a	16.75	33.75	76.50	3.00 a	58.75
10	26.25 a	65.25 a	80.25 a	11.00 a	3.75 a	4.50 a	1.00 b	9.50 a	3.25 a	10.25 a	0.25 bc	31.25 a
20	28.25 a	71.00 a	81.00 a	12.50 a	3.75 a	2.25 a	0.00 b	5.50 b	1.00 a	9.00 a	0.50 b	24.75 a
30	24.50 a	60.50 a	79.25 a	11.25 a	7.25 a	2.00 a	0.50 b	5.50 b	2.25 a	7.25 ab	0.00 c	21.50 a
40	26.75 a	70.75 a	82.75 a	12.00 a	4.25 a	1.00 a	0.50 b	6.25 b	1.50 a	5.25 b	0.00 c	22.25 a
Trial 2												
0	22.25	60.50 a	86.50	16.00 a	4.75	3.00	28.25 a	7.75	47.00	44.75	1.25	57.25
10	25.00 a	64.00 a	83.75 a	11.25 bc	3.75 ab	1.25 a	0.00 b	6.75 a	2.75 a	5.75 a	0.75 a	24.75 a
20	25.25 a	64.25 a	79.25 a	12.00 ab	6.00 a	2.75 a	0.25 b	8.50 a	1.25 a	6.25 a	0.25 a	17.50 a
30	28.75 a	66.25 a	86.50 a	7.50 c	5.00 ab	1.00 a	0.25 b	4.75 a	2.00 a	6.25 a	0.00 a	24.25 a
40	23.75 a	63.50 a	82.25 a	10.00 c	4.50 ab	3.25 a	0.00 b	5.75 a	2.25 a	3.75 a	0.25 a	18.75 a
50	26.25 a	62.75 a	83.25 a	12.25 ab	2.50 b	2.00 a	0.25 b	4.00 a	1.50 a	5.50 a	0.25 a	18.00 a
60	26.50 a	62.75 a	79.25 a	12.75 ab	4.75 ab	3.25 a	0.50 b	2.75 a	0.75 a	5.00 a	0.25 a	20.00 a

[a] Original means are presented for all variables, but means separations were based on means calculated as a percentage of the control treatment for each replication in order to meet requirements for parametric statistical analyses; except for the incidence of *C. variabile* and *Fusarium* spp. detected in trial 1, for which Friedman's non-parametric rank test was used because the data could not be normalized.

[b] Seed were treated for the appropriate duration in 1.2% NaOCl, triple-rinsed in sterile deionized water, and dried on sterile paper towel as described in the text.

[c] Seed were subjected to a blotter germination assay (Yaklich, 1985).

[d] Seed were subjected to a freeze-blotter seed health assay as described in the text.

[e] Means followed by different letters within a column are significantly different based on Fisher's protected least significance different ($P \leq 0.05$) or Friedman's non-parametric rank test.

Table 3. Analyses of variance for germination and seed health assays of spinach seed treated with hot water[a]

Trial and dependent variable[b]	R^2	CV (%)	Mean square probability > F				
			Tempe-rature	Replica-tion	Replication-by-temp.	Duration	Temp.-by-duration
Trial 1							
Germination assay (%)[c]							
Total germination	0.77	30.54	-	1.0000	-	-	<0.0001
Germination at 4 days	0.80	27.56	-	1.0000	-	-	<0.0001
Germination at 7 days	0.79	29.83	-	1.0000	-	-	<0.0001
Non-germinated seed	0.99	13.87	-	0.1562	-	-	<0.0001
Abnormal germination[c]	0.82	311.60	0.0492	<0.0001	0.1314	0.4530	<0.0001
Rotten seed[d]	0.80	13.46	0.1761	<0.0001	0.1724	0.4321	0.7500
Seed health assay (% of seed infected)[d]							
Stemphylium botryosum	0.57	37.57	-	1.0000	-	-	<0.0001
Cladosporium variabile	0.82	246.14	-	0.3992	-	-	<0.0001
Verticillium spp.	0.94	13.38	-	1.0000	-	-	<0.0001
Other *Cladosporium* spp.	0.69	5.89	0.0143	<0.0001	0.1001	0.0026	0.0009
Fusarium spp.	0.70	28.21	-	1.0000	-	-	<0.0001
Alternaria spp.	0.88	21.80	-	1.0000	-	-	<0.0001
Trial 2							
Germination assay (%)							
Total germination	0.89	14.81	<0.0001	<0.0001	0.1831	<0.0001	0.0200
Germination at 4 days	0.85	25.10	-	1.0000	-	-	<0.0001
Germination at 7 days	0.81	28.47	-	1.0000	-	-	<0.0001
Non-germinated seed	0.77	31.12	-	1.0000	-	-	<0.0001
Abnormal germination	0.71	35.02	-	1.0000	-	-	<0.0001
Rotten seed	0.37	5.53	-	1.0000	-	-	<0.0001
Seed health assay (% of seed infected)							
S. botryosum	0.91	37.57	-	1.0000	-	-	<0.0001
C. variabile	0.61	14.60	-	1.0000	-		<0.0001
Verticillium spp.	0.91	18.43	-	1.0000		-	<0.0001
Other *Cladosporium* spp.	0.63	36.41	-	1.0000	-	-	<0.0001
Fusarium spp.	0.96	13.45	-	1.0000	-	-	<0.0001
Alternaria spp.	0.96	12.32	-	1.0000	-	-	<0.0001
Trial 3							
Germination assay (%)							
Total germination	0.77	31.25	-	1.0000	-	-	<0.0001
Germination at 4 days	0.83	26.47	-	1.0000	-	-	<0.0001
Germination at 7 days	0.77	31.42	-	1.0000	-	-	<0.0001
Non-germinated seed	0.71	35.28	-	1.0000	-	-	<0.0001
Abnormal germination	0.56	42.92	-	1.0000	-	-	<0.0001
Rotten seed	0.24	56.42	-	1.0000	-	-	<0.0001
Seed health assay (% of seed infected)							
S. botryosum	0.90	20.63	-	1.0000	-	-	<0.0001
C. variabile	0.75	10.41	-	1.0000			<0.0001
Verticillium spp.	0.90	19.79	-	1.0000	-	-	<0.0001
Other *Cladosporium* spp.	0.66	32.89	-	1.0000	-	-	<0.0001
Fusarium spp.	0.97	11.76	-	1.0000	-	-	<0.0001
Alternaria spp.	0.95	14.05	-	1.0000	-	-	<0.0001

[a] Each trial consisted of a split plot randomized complete block design with four replications. Temp. = hot water temperature (whole plot factor). Duration = duration of treatment at each temperature = sub-plot factor. For each replication of each treatment, 100 seed were treated at 40, 45, 50, 55, or 60ºC for 10, 20, 30, or 40 min in deionized water, rinsed for 5 min, dried, and plated onto blotters as described in the text. Seed lots 03-409, 04-305, and 04-407 were used in trials 1, 2, and 3, respectively. CV = coefficient of variation. R^2 = coefficient of determination.

[b] Except for % abnormal germination, rotten seed, and other *Cladosporium* spp. in trial 1, and % total germination in trial 2, the dependent variables were analyzed using Friedman's non-parametric rank test because of non-normal data. Variables subjected to parametric analyses were calculated as a percent of the control treatment of each replication. Square root transformations were used for % rotten seed in trial 1, and % total germination in trial 2, and arcsin transformation was used for % other *Cladosporium* spp. detected in trial 1, to generate homogeneous variances and/or normal residuals.

[c] Seeds were subjected to a blotter germination assay (Yaklich, 1985).

[d] Freeze-blotter seed health assays were carried out as described in the text.

Table 4. Efficacy of hot water treatments on spinach seed quality[a]

Trial and hot water temp. (ºC)	Duration (min)	Germination assay (% of seed)[b]					
		Germinated					
		4 days	7 days	Total	Non-germinated	Abnormal	Rotten
Trial 1							
Control		8.00 abc[c]	58.00 abc	80.25 cde	15.50 ef	3.50	0.75 a
40	10	10.00 a	60.50 a	85.75 a	9.00 g	4.00 cdef	1.00 a
	20	9.75 a	59.75 a	81.75 bcd	12.00 efg	4.25 cdef	1.75 a
	30	8.75 abc	56.50 abc	80.75 cde	12.75 efg	5.25 abcde	1.25 a
	40	7.00 bcd	52.00 bcd	78.50 de	15.00 ef	5.25 abcde	1.25 a
45	10	13.00 ab	54.25 ab	86.00 ab	10.50 fg	2.75 efgh	0.75 a
	20	8.75 ab	57.50 ab	82.25 abcd	11.25 fg	5.00 abcde	1.25 a
	30	6.00 a	61.75 a	82.75 bcd	12.25 efg	3.75 def	1.25 a
	40	7.25 ab	60.50 ab	83.00 abcd	12.00 efg	3.50 def	1.25 a
50	10	8.75 a	59.00 a	84.50 abc	11.25 fg	3.75 def	0.50 a
	20	10.75 a	57.75 a	83.00 abcd	12.75 efg	3.50 def	0.75 a
	30	3.50 a	65.00 a	83.25 abcd	12.75 efg	3.00 efgh	0.50 a
	40	3.00 cde	56.00 cde	76.50 efg	16.75 e	5.25 abcde	1.50 a
55	10	6.75 a	63.50 a	84.75 abc	13.00 efg	1.75 fgh	0.50 a
	20	1.25 def	54.25 bcd	79.50 def	14.50 ef	4.75 bcdef	1.25 a
	30	0.00 efg	31.50 efg	63.00 gh	28.50 d	7.50 ab	1.00 a
	40	0.00 fg	14.25 fg	47.25 gh	41.75 c	8.00 a	0.00 a
60	10	0.00 defg	34.50 defg	67.50 fgh	25.25 d	7.00 abc	0.25 a
	20	0.00 g	0.75 g	10.50 h	83.00 b	6.50 abcd	1.00 a
	30	0.00 g	0.00 g	0.00 h	99.50 a	0.00 gh	0.50 a
	40	0.00 g	0.00 g	0.00 h	99.50 a	0.00 h	0.50 a
Trial 2							
Control		43.00 a	47.75 a	48.00	31.25 hi	8.75 bcdef	12.00 abcd
40	10	40.75 ab	45.75 ab	46.50 a	31.75 hi	7.00 fgh	14.75 ab
	20	45.50 a	51.25a	52.50 a	25.75 i	7.75 defg	14.00 abc
	30	37.50 abcd	44.50 bc	45.25 a	33.50 fghi	3.50 hi	17.50 a
	40	42.75 ab	48.75 ab	49.00 ab	32.25 hi	5.75 ghi	13.00 abc
45	10	38.75 abc	43.50 bc	44.00 bc	36.25 efgh	8.25 defg	11.50 bcde
	20	39.25 abc	45.25 bc	45.75 c	29.50 hi	9.25 bcdef	15.50 abc
	30	31.00 cde	38.25 cd	39.25 cd	41.00 efgh	5.75 fgh	14.00 abcde
	40	34.75 cde	40.75 bc	41.50 cde	35.50 fgh	8.75 cdefg	14.25 abc
50	10	42.00 a	46.50 ab	47.00 def	33.00 fghi	6.25 fgh	13.75 abc
	20	32.25 cde	39.00 cd	40.25 defg	37.75 efgh	7.75 efgh	14.25 abcd
	30	27.25 de	37.50 cd	38.75 defg	42.50 def	10.50 abcde	8.25 de

Trial and hot water temp. (ºC)	Duration (min)	Germination assay (% of seed)[b]					
		Germinated					
		4 days	7 days	Total	Non-germinated	Abnormal	Rotten
	40	24.00 ef	34.75 de	36.25 defg	39.75 defg	14.00 a	10.00 bcde
55	10	35.00 bcde	44.25 abc	45.00 efg	33.00 ghi	10.75 abcde	11.25 bcde
	20	12.75 fg	28.50 def	30.75 efg	47.00 cde	11.75 abcd	10.50 cde
	30	2.25 gh	21.50 efg	24.75 efg	51.25 bcd	14.75 abc	9.25 cde
	40	0.00 h	6.50 fgh	11.50 fgh	68.75 abc	8.50 bcdef	11.25 abcd
60	10	7.50 fgh	21.50 efgh	24.75 fgh	53.25 abcd	12.00 ab	10.00 bcde
	20	0.00 h	1.00 gh	2.75 gh	84.25 ab	3.50 hi	9.50 cde
	30	0.00 h	0.00 h	0.50 gh	89.00 ab	0.25 i	10.25 bcde
	40	0.00 h	0.00 h	0.00 h	93.50 a	0.00 i	6.50 e
Trial 3							
Control		37.50 a	40.75 a	40.75 a	34.50 i	6.00 cde	18.75 a
40	10	34.00 abc	35.50 abc	36.00 ab	42.25 hi	7.00 bcd	14.75 abcd
	20	35.75 abc	37.50 ab	37.75 ab	39.25 hi	5.00 cdef	18.00 ab
	30	29.75 bcd	33.25 abc	33.50 abc	46.75 efgh	7.25 bcd	12.50 abcd
	40	34.75 ab	36.75 ab	36.75 ab	44.75 ghi	6.50 bcde	12.00 abcd
45	10	29.00 bcde	30.50 bcd	30.50 bcd	52.00 defg	6.50 bcde	11.00 abcd
	20	29.25 bcd	33.25 abc	33.25 abc	45.50 ghi	6.00 def	15.25 abcd
	30	32.25 abc	35.75 ab	36.50 ab	45.50 fghi	6.75 bcd	11.25 abcd
	40	29.25 abcd	32.25 abc	32.50 abc	41.75 hi	10.00 ab	15.75 abcd
50	10	28.25 cde	32.00 abc	32.25 abc	49.00 defgh	8.25 abcd	10.50 abcd
	20	31.00 bcd	35.25 abc	35.25 abc	44.50 hi	9.50 abc	10.75 abcd
	30	18.75 ef	23.50 de	24.00 de	50.50 defgh	8.25 bcd	17.25 abcd
	40	19.00 ef	23.00 de	23.00 de	58.50 bcde	9.00 bcd	9.50 abcd
55	10	23.75 de	26.75 cd	27.00 cd	54.75 cdef	7.50 bcd	10.75 abcd
	20	11.25 fg	22.75 de	22.75 def	52.75 cdefg	14.00 a	10.50 abcd
	30	2.50 gh	16.25 ef	18.25 efg	61.00 bcd	9.25 bcd	11.50 abcd
	40	0.00 h	8.50 ef	10.25 efg	69.00 abc	7.00 bcd	13.75 abcd
60	10	9.75 fgh	24.00 de	24.75 de	49.50 defgh	10.50 ab	15.25 abc
	20	0.00 h	1.75 f	4.25 fg	84.00 ab	2.75 efg	9.00 bcd
	30	0.00 h	0.25 f	1.00 g	90.50 a	0.75 fg	7.75 d
	40	0.00 h	0.00 f	0.25 g	91.00 a	0.00 g	8.75 cd

[a] Seed were subjected to hot water treatments using a split plot randomized complete block design with four replications for each trial. Seed lots 03-409, 04-305, and 04-407 were used in trials 1, 2, and 3, respectively. Original means are presented for all variables, but means separations were calculated as a percentage of

the control treatment of each replication for parametric statistical analyses. Except for % abnormal germination, rotten seed, and other *Cladosporium* spp. in trial 1, and % total germination in trial 2, the dependent variables were analyzed using Friedman's non-parametric rank test because of non-normal data. Therefore, although original means are presented, the letters representing means separation do not always correspond to the original means shown. Square root transformations were used for % rotten seed in trial 1 and total germination in trial 2, and arcsin transformation was used for % other *Cladosporium* spp. detected in trial 1, to generate homogeneous variances and/or normal residuals. Temp. = temperature.

[b] Seed were subjected to a blotter germination assay (Yaklich, 1985).

[c] Means followed by different letters within a column are significantly different based on Fisher's protected least significance different ($P \leq 0.05$) or Friedman's non-parametric rank test.

Table 5. Efficacy of hot water seed treatments for eradication of *Cladosporium variabile*, *Stemphylium botryosum*,*Verticillium* spp., and other fungi from spinach seed

Trial and hot water temperature (ºC)	Duration (min)	Seed health assay (% of seed infected)[c]					
		C. variabile[b]	*S. botryosum*	*Verticillium* spp.	Other *Cladosporium* spp.	*Fusarium* spp.	*Alternaria* spp.
Trial 1							
		28.25 a[d]	4.75 a	39.75 a	52.25 a[c]	2.50 a	60.25 a
40	10	0.00 b	0.75 cdefg	50.50 a	1.75 b	2.00 ab	22.00 ab
	20	0.00 b	2.25 bcdef	17.00 bc	0.75 b	21.75 a	13.25 bcd
	30	0.00 b	1.75 abc	46.75 a	0.50 b	1.25 ab	13.75 bc
	40	0.00 b	1.75 bcd	36.00 ab	0.50 b	1.50 ab	10.25 bcd
45	10	0.00 b	3.25 ab	2.75 c	1.25 b	1.00 bc	12.25 bcd
	20	0.00 b	1.75 abcd	3.25 c	1.25 b	0.25 cd	9.00 cd
	30	0.00 b	1.75 abcd	1.00 d	3.00 b	0.50 cd	6.00 d
	40	0.00 b	2.25 abcd	0.25 e	3.50 b	0.00 d	9.00 cd
50	10	0.00 b	1.50 bcde	0.00 e	1.00 b	0.00 d	6.00 d
	20	0.00 b	0.50 defg	0.00 e	1.00 b	0.00 d	2.25 e
	30	0.00 b	0.75 cdefg	0.00 e	1.25 b	0.00 d	1.25 ef
	40	0.00 b	0.25 efg	0.00 e	2.25 b	0.00 d	0.50 efg
55	10	0.00 b	0.25 fg	0.00 e	0.50 b	0.25 cd	0.75 efg
	20	0.00 b	0.00 g	0.00 e	0.00 b	0.00 d	0.00 g
	30	0.00 b	0.00 g	0.00 e	1.00 b	0.00 d	0.00 g
	40	0.00 b	0.00 g	0.00 e	2.50 b	0.00 d	0.00 g
60	10	0.00 b	0.00 g	0.00 e	0.00 b	0.25 cd	0.25 fg
	20	0.00 b	0.00 g	0.00 e	0.25 b	0.00 d	0.00 g
	30	0.00 b	0.00 g	0.00 e	0.50 b	0.00 d	0.00 g
	40	0.00 b	0.00 g	0.00 e	2.00 b	0.00 d	0.50 fg
Trial 2							
Control		1.25 a	65.75 a	23.00 a	60.50 a	52.75 a	75.50 a
40	10	0.00 c	53.25 a	13.00 ab	7.75 a	41.50 ab	73.75 a
	20	0.00 c	56.50 a	11.25 bc	4.00 ab	38.25 bc	69.50 ab
	30	0.00 c	52.00 ab	13.25 ab	2.00 abc	34.25 cd	62.00 bc
	40	0.00 c	43.75 bc	13.75 bc	0.75 cde	34.00 bc	57.25 c
45	10	0.00 c	33.75 cde	9.75 b	1.00 bcd	26.00 de	28.00 d
	20	0.00 c	37.50 cd	5.50 cd	1.00 bcd	16.75 ef	26.25 de
	30	0.00 c	39.25 c	5.50 cd	0.50 de	14.25 ef	22.00 de
	40	0.00 c	33.50 cde	5.00 d	0.00 e	16.75 fg	18.75 e

Trial and hot water temperature (°C)	Duration (min)	Seed health assay (% of seed infected)[c]					
				Other			
		C. variabile[b]	*S. botryosum*	*Verticillium* spp.	*Cladosporium* spp.	*Fusarium* spp.	*Alternaria* spp.
50	10	0.00 c	35.50 cd	4.75 d	1.00 bcd	6.75 hi	21.75 e
	20	0.00 c	31.00 de	1.00 e	1.00 de	6.75 gh	20.00 ef
	30	0.00 c	31.50 de	1.00 e	0.00 e	3.25 hij	14.50 fg
	40	0.25 b	26.75 e	0.00 g	0.00 e	3.25 ij	8.00 gh
55	10	0.00 c	19.50 f	1.00 ef	1.00 de	1.75 j	7.75 h
	20	0.00 c	11.50 fg	0.25 fg	1.25 de	1.00 k	1.50 i
	30	0.00 c	10.00 fg	0.00 g	0.50 de	0.00 k	0.50 ijk
	40	0.00 c	8.50 g	0.00 g	0.75 de	0.50 k	0.75 ij
60	10	0.00 c	7.75 g	0.00 g	0.00 e	0.25 k	0.25 jk
	20	0.00 c	7.25 g	0.00 g	0.00 e	0.00 k	0.00 k
	30	0.00 c	8.50 g	0.00 g	0.75 de	0.00 k	0.00 k
	40	0.00 c	10.75 fg	0.00 g	0.25 de	0.25 k	0.00 k
Trial 3							
Control		1.50 a	66.75 a	38.75 a	72.50 a	60.75 a	76.25 a
40	10	0.00 b	48.00 bc	12.25 ab	14.00 a	49.00 ab	67.75 ab
	20	0.00 b	55.50 ab	8.50 bc	6.50 ab	49.00 ab	57.75 bc
	30	0.00 b	48.50 bc	7.75 bcd	2.00 bc	39.50 b	55.00 bc
	40	0.00 b	46.50 cd	5.25 cd	2.50 bcd	43.00 b	49.50 c
45	10	0.00 b	35.75 ef	8.50 abc	1.50 cde	26.50 c	21.50 de
	20	0.00 b	39.75 de	2.75 de	0.75 cde	18.75 c	18.25 def
	30	0.00 b	37.50 de	1.75 ef	0.00 e	19.00 c	20.50 d
	40	0.00 b	43.25 cd	2.00 ef	0.00 e	14.50 d	17.25 defg
50	10	0.00 b	42.50 cd	3.00 de	1.00 cde	9.00 d	16.50 efg
	20	0.00 b	39.75 de	2.75 ef	0.50 cde	6.50 de	13.50 gh
	30	0.00 b	32.00 fg	2.00 f	0.25 de	4.50 f	15.00 fg
	40	0.00 b	36.00 ef	0.50 g	0.25 e	2.00 g	11.50 h
55	10	0.00 b	29.50 fg	0.50 g	0.50 cde	4.75 ef	12.00 h
	20	0.00 b	20.75 gh	0.00 g	0.50 de	1.25 g	0.75 i
	30	0.00 b	12.75 i	0.00 g	0.00 e	0.75 gh	0.25 ij
	40	0.00 b	14.00 i	0.00 g	0.00 e	0.25 hi	0.00 j
60	10	0.00 b	13.25 hi	0.00 g	0.00 e	0.00 i	0.50 ij
	20	0.00 b	13.50 hi	0.00 g	0.00 e	0.50 hi	0.00 j
	30	0.00 b	11.75 hi	0.00 g	0.25 de	0.00 i	0.00 j
	40	0.00 b	12.00 i	0.00 g	0.00 e	0.00 i	0.00 j

[a] Seed were treated as described in the text.

[b] Original means are presented. Means separation, Friedman's non-parametric rank test, and transformations were calculated as described in the text and for Table 3. Original means are presented, but letters representing means separation do not always correspond to original means shown where Friedman's rank test was used.

[c] Freeze-blotter seed health assays were carried out as described in the text.

[d] Means followed by different letters within a column are significantly different based on Fisher's protected least significance difference ($P \leq 0.05$) or Friedman's rank test .

Chapter 4.

Seed transmission of *Stemphylium botryosum* and *Cladosporium variabile* in spinach.

4.1. INTRODUCTION

Seed transmission of a pathogen takes place when the pathogen in the seed colonizes the developing seedling (Cappelli, 2004). Environmental conditions at the time of planting and germination (e.g., temperature, moisture, light, soil pH, and host genotype) influence seed transmission of a pathogen (Maude, 1996). Spinach has epigeal germination, i.e., the cotyledons are carried up during hypocotyl growth (Agarwal and Sinclair, 1997; Peterson and Harris, 1997). Maude (1996) reported that, as a general rule, epigeal germination favors seed transmission of a pathogen to the cotyledons of the seedlings and subsequent secondary spread of the pathogen to the crop, while hypogeal germination (cotyledons remain in the soil during epicotyl growth) favors seed transmission of pathogens that cause foot and root rots of seedlings. Sometimes, in seedlings with epigeal emergence, the pericarp or seed coat remains attached to the cotyledons during germination, and transmission of the pathogen from these tissues to the cotyledons can be influenced by environmental conditions (e.g., Maude and Presly, 1977). Free moisture on the seedlings may be an important influence on this method of transmission (Maude, 1996).

Cladosporium variabile and *Stemphylium botryosum* both are reported to be seedborne in spinach (du Toit and Derie, 2003; Fuentes-Davila, 1988). Hansen et al. (1952) reported that *C. variabile* was transmitted from infected spinach seed planted in a greenhouse in Denmark. The Japanese spinach industry has also claimed that this

pathogen is seed transmitted, which affected the export of spinach seed to Japan from the USA (Fuentes-Davila, 1988). Currently, no phytosanitary restrictions referent to *C. variable* have been issued by importer countries of spinach seed produced in the USA (Phillip Brown, *personal communication*). Fuentes-Davila (1988) did not detect seed transmission of *C. variabile* in greenhouse and growth chamber trials carried out using a commercial seed lot with 94.3% incidence of *C. variabile*, even under conditions highly conducive for Cladosporium leaf spot (15 to 20^{o}C and >80% relative humidity). Although *S. botryosum* has not yet been demonstrated to be seed-transmitted in spinach, Raid (2004) 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. Similarly, the recent first reports of this disease in Arizona (L.J. du Toit, and S.T. Koike, *personal communication*), California (Koike et al., 2001), Maryland and Delaware (Everts and Armentrout, 2001), Washington (du Toit and Derie, 2001), and Oregon (M.L. Putnam and L.J. du Toit, *unpublished data*); and the detection of *S. botryosum* in all 66 commercial spinach seed lots produced in 2003 in Denmark, Holland, New Zealand and the USA that were assayed as part of Chapter 2 of this project, support the hypothesis of spread of the fungus via infected seed lots.

Because seed transmission of *C. variabile* in spinach has not been documented conclusively (Inglis et al., 1997), and seed transmission of *S. botryosum* remains to be investigated, this study was established to determine the potential for *C. variabile* and *S. botryosum* to be transmitted from infected spinach seed under conditions conducive for development of Cladosporium and Stemphylium leaf spots.

4.2. MATERIALS AND METHODS

A seed transmission trial was established in the greenhouse at the Washington State University (WSU) - NWREC in Mount Vernon, WA in the fall of 2004. Spinach seed lot 03-409, harvested from an inoculated spinach seed crop fungicide trial carried out at the WSU - NWREC in 2003, was subjected to a freeze-blotter seed health assay in September 2004, using the surface-sterilized protocol described in Chapter 2. The seed lot was determined to be infected with *S. botryosum* at 4.75% and *C. variabile* at 28.25%. Seed were planted into 10 flats (representing 10 replications), each consisting of 512 cells each measuring 14 mm (length) x 14 mm (width) x 19 mm (height) (Steuber Distributing Co., Snohomish, WA). Spinach seed lot B5 (originating from Denmark) was also subjected to a freeze-blotter seed health assay in November 2004, and proved to be infected with *S. botryosum* at 88.50% but was not infected with *C. variabile*. Therefore, this seed lot was used to assess seed transmission of *S. botryosum* to compare with 03-409 which had a much lower incidence of *S. botryosum* and a relatively high incidence of *C. variabile*. Seed of lot B5 were planted into five flats (representing five replications). A higher number of flats (replications) was planted for lot 03-409 than lot B5 because of the low incidence of seedborne *S. botryosum* in lot 03-409.

For lot B5, each flat consisted of 200 cells, and each cell measured 33 mm (length) x 33 mm (width) x 37 mm (height). For each seed lot, one seed was planted in each cell into Terra-Lite Redi-Earth planting medium (Grace-Sierra Horticultural Co., Onancock, VA). Flats were fertigated by hand twice a day with 20-20-20 (N-K-P, Plant Marvel Laboratories, Inc., Chicago Heights, IL) using a hose and misting nozzle (Fogg-it Nozzle Co., San Francisco, CA) until the first sign of emergence (four to five days after

planting). For each seed lot, the flats were then moved to a bench on which two misting sprinklers, staked about 50 cm above the flats, were used to water the seedlings based on a precision timer set to mist the flats for 10 s every 30 min for the first 4 days after emergence, and for 10 s every 45 min for the next 30 days. The seedlings were exposed to supplemental lighting (1,000 w) from a metal halide lamp from 8:00 am to 6:00 pm daily.

The percentage seed germination and the date of emergence of each seedling in each flat were recorded. Each seedling that emerged with the pericarp attached to the cotyledons was tagged with a yellow pin placed in the cell. Seedlings that developed lesions on the cotyledon tips, cotyledon blade, or first set of true leaves were identified with orange pins placed in the cells. The approximate number of days after planting on which lesions appeared was also recorded. However, for lot 03-409 in trial 1, only the range in days (first and last) of appearance of lesions after emergence on the cotyledon tips and cotyledon blades were recorded. Seedlings with lesions, and seedlings with the pericarp remaining attached to the cotyledons (with or without lesions on the cotyledons) were transplanted into Sunshine Mix No. 1 (SunGro Horticulture, Inc., Bellevue, WA) in 70 mm (length) x 70 mm (width) x 70 mm (height) pots (McLean Bulb Farms, Puyallup, WA), and maintained under sprinklers on the benches to monitor development of lesions. The presence or absence of pseudothecia of *P. herbarum*, the teleomorph of *S. botryosum*, developing on the pericarp of those seedlings on which the pericarp remained attached to the cotyledons after emergence, was recorded. Temperature and relative humidity were recorded hourly from 17 October to 11 November 2004 using a

WatchDog™ datalogger (Model 425, Spectrum Technologies, Inc., Plainfield, IL) placed inside a radiation shield (Spectrum Technologies, Inc.).

Isolations were carried out from the cotyledon tips, cotyledon blades, or the first pair of true leaves of a sample of seedlings from each of five flats planted with seed lot 03-409 and five flats planted with lot B5 (mean number of seedlings sampled per flat is shown in Table 1). A section of symptomatic tissue (5 mm x 5 mm) was cut from each seedling using a scalpel, surface-sterilized in 0.6% NaOCl for 15 s, triple-rinsed in sterile deionized water, dried on sterile paper towel in a laminar flow hood, and placed onto V8 agar medium (prepared as explained in Chapter 2). Isolations from asymptomatic cotyledons (mean of two seedlings per flat for each of lot 03-409 and B5, respectively) and true leaves (mean of two seedlings per flat for lot 03-409 and one seedling per flat for lot B5) were also carried out to serve as control treatments.

For each leaf spot pathogen, the incidence of seedlings infected on the cotyledon tips, cotyledons blades, or the first true leaves was calculated as a percentage of the seedlings emerged in each flat. The percentage seed transmission (calculated considering lesions on the cotyledon tips because they first appeared compared to lesions on the cotyledon blades or the first true leaves) of each pathogen in each flat was calculated as: (percentage seedlings infected on the cotyledon tips) / (percentage of the planted seed infected) * 100. Secondary spread of each pathogen in each flat was calculated as the percentage seedlings infected on the cotyledon blades or the first true leaves.

A completely randomized block design with three replications (one spinach plant per replication) was carried out in the greenhouse to test pathogenicity on spinach of isolates of *S. botryosum* and *C. variabile* obtained from lesions on the seedlings, using the

procedure described in Chapter 2. Two-week-old cultures of *S. botryosum* isolated from lesions on B5 seedlings, and of *C. variabile* isolated from lesions on 03-409 seedlings, were used to prepare spore suspensions (approximately 1.0 x 10^5spores/ml, except for two isolates of *S. botryosum* inoculated at 1.8 x 10^4 and 6.7 x 10^4 spores/ml in trial 1, because the cultures of these isolates did not produce sufficient spores). Each isolate was inoculated onto spinach plants of a proprietary, smooth leaf, medium-long standing spinach inbred line (20 ml/three plants). For *S. botryosum*, pathogenicity was tested for six isolates obtained from lesions on the cotyledon tips and one isolate from lesions on true leaves. A known pathogenic isolate of *S. botryosum*, 'Cheetah A', was used as the control isolate. For *C. variabile*, pathogenicity was tested for two isolates obtained from lesions on each of the cotyledon tips, cotyledon blades, and the first true leaves (six isolates in total). A known pathogenic isolate of *C. variabile*, '00-304', was used as the positive control isolate. In addition, one plant was atomized with sterile deionized water as a control treatment for each replication. Pathogenicity tests were also carried out for two isolates of *Alternaria* spp. obtained from lesions on the cotyledon tips of B5 seedlings, and four isolates of *Alternaria* spp. from 03-409 seedlings (two from lesions on the cotyledon tips and two from lesions on the cotyledon blades).

For each fungal isolate inoculated, isolations were made from lesions that developed on inoculated leaves (and from asymptomatic leaves for the negative control plants) as explained in Chapter 2. In addition, a single symptomatic leaf from each treatment (and an asymptomatic leaf from the negative control treatment) was placed on moist paper towel in a plastic Petri plate. The leaf sections plated onto agar media and the leaves

placed in moist chambers were examined microscopically three days later for development of conidia of *S. botryosum, C. variabile*, or *Alternaria* spp.

The experiment was repeated in the winter of 2004-05 with five 200-cell flats (each representing a replication) planted per seed lot. The emerged seedlings were misted under sprinklers for 10 s every 60 min for the first 4 days after emergence, and for 10 s every 45 min for the subsequent 30 days. Seedlings with lesions on cotyledon tips, cotyledon blades, and/or first true leaves were transplanted as described above. However, in this trial, the transplanted seedlings were moved to a different bay in the greenhouse and placed on benches without misting nozzles, to minimize the possibility of secondary spread from the lesions on these plants to seedlings remaining in the flats under the misters.

Leaf wetness was recorded with a leaf wetness sensor (Spectrum Technologies, Inc.). Temperature, relative humidity, and leaf wetness data were recorded hourly from 6 to 12 January 2005. Subsequent recordings were lost because of problems with the datalogger and leaf wetness sensor. Isolations from the cotyledon tips, cotyledon blades, and the first true leaves were carried out for each seed lot as described above (Table 1). Isolations from asymptomatic cotyledons (mean of 2.8 and 2.6 per flat for lots 03-409 and B5, respectively), and true leaves (mean of 1.6 and 1.0 per flat for lots 03-409 and B5, respectively) were also carried out to serve as controls.

The pathogenicity of three isolates of *S. botryosum* obtained from lesions on the cotyledon tips (two seedlings of lot B5 and one seedling of lot 03-409) and one isolate of *C. variabile* from lesions on each of the cotyledon tips, cotyledon blade, and first true leaves of 03-409 seedlings was tested in the greenhouse as decribed above. Pathogenicity

tests were also carried out for two isolates of an *Alternaria* sp. and one isolate of a *Colletotrichum* sp. obtained from lesions on the cotyledon tips of seedlings of lot B5.

4.3. RESULTS

Lesions were observed on the cotyledon tips of seedlings that developed from spinach seed lots 03-409 and B5 (Figs. 1A and B). Lesions typical of Cladosporium leaf spot (2 to 3 mm in diameter, sunken, discrete, tan, circular lesions developing a darker margin after 5 to 7 days) (du Toit and Derie, 2001) were detected on the cotyledon blades of lot 03-409 and on the first true leaves of lot B5 (Fig. 1C and D, respectively). Similarly, lesions typical of Stemphylium leaf spot (rapidly expanding lesions with a diffuse border that become tan after 5 to 7 days, surrounded by a water-soaked margin under moist conditions) (du Toit and Derie, 2001) developed on the first true leaves of lots 03-409 and B5 (Fig. 1E).

Emergence averaged 88.4 and 87.5% for seed lot 03-409 in trials 1 and 2, respectively; and 83.5 and 83.0% for lot B5 in trials 1 and 2, respectively. For lot 03-409, 16.9% of the seedlings developed lesions in trial 1, with a mean of 8.8, 3.7, and 8.0% of the seedlings developing lesions on the cotyledon tips, cotyledon blades, and first true leaves, respectively. In trial 2, 8.1% of the seedlings of lot 03-409 developed lesions, with a mean of 4.3, 0.3, and 3.4% of the seedlings developing lesions on the cotyledon tips, cotyledon blades, and the first pair of true leaves, respectively. For lot B5, 23.3% of the seedlings developed lesions in trial 1, with 23.3 and 7.7% of the seedlings developing lesions on the cotyledon tips and first set of true leaves, respectively. In trial 2, 25.2% of the seedlings of lot B5 developed lesions, with a mean of 7.8 and 17.4% of the seedlings

developing lesions on the cotyledon tips and the first pair of true leaves, respectively. Lesions were not observed on the cotyledon blades for lot B5 in either trial.

For seed lot 03-409, the number of days after emergence that lesions were observed on cotyledon tips, cotyledon blades, and the first set of true leaves ranged from 0 to 17 (mean of 8.9), 16 to 32, and 17 to 32, respectively, in trial 1. In trial 2, lesions appeared on the cotyledon tips and first true leaves of 03-409 seedlings at a range of 0 to 25 (mean of 9.3) and 19 to 27 (mean of 25.6) days after emergence, respectively. Only three seedlings of lot 03-409 developed lesions on the cotyledon blades in trial 2, at 16, 22, and 25 days after emergence (mean of 21 days). For seed lot B5, lesions were observed on the cotyledon tips and first set of of true leaves at a range of 0 to 15 (mean of 4.0) and 12 to 23 (mean of 18.9) days after emergence, respectively, in trial 1. In trial 2, lesions appeared on cotyledon tips and the first true leaves anywhere from 0 to 22 (mean of 7.7) and 23 to 31 (mean of 28.1) days after emergence, respectively. Lesions were not detected on cotyledon blades for lot B5.

In trial 1, *C. variabile* was isolated from lesions on a mean of 18.2 of 26.2 symptomatic seedlings sampled per flat of lot 03-409. Based on the number of symptomatic seedlings per flat, a mean of 15.3% of the 03-409 seedlings were infected with *C. variabile* in trial 1. *Cladosporium variabile* was isolated from a mean of 4.0 of 7.2 seedlings sampled per flat that developed lesions on the cotyledon tips (Table 1). Therefore, 5.1% of the 03-409 seedlings were infected by this fungus on the cotyledon tips. Based on an initial incidence of 28.25% *C. variabile* in seed lot 03-409, seed transmission occurred at a rate of 18.1% from the infected seed planted (Table 1). *Cladosporium variabile* was isolated from a mean of 6.0 of 6.8 seedlings sampled per flat

that developed lesions on the cotyledon blades. Therefore, 3.7% of the 03-409 seedlings were infected with *C. variabile* on the cotyledon blades (Table 1). The pathogen was also isolated from a mean of 8.2 of 12.2 seedlings sampled per flat that developed lesions on the first set of true leaves, i.e., a mean of 7.9% of the 03-409 seedlings were infected with *C. variabile* on the first true leaves (Table 1). Secondary spread of *C. variabile*, calculated as the mean percentage seedlings infected on the cotyledon blades or the first true leaves, was detected at a rate of 12.2% of the seedlings in trial 1 for lot 03-409.

Cladosporium variabile was isolated from lesions of only 1.0 out of 13.4 symptomatic seedlings of lot 03-409 sampled per flat in trial 2. Therefore, a mean of 0.6% of the 03-409 seedlings in trial 2 were infected with *C. variabile*. In this trial, *C. variabile* was isolated from a mean of 0.2 of 7.6 seedlings sampled per flat that developed lesions on the cotyledon tips (Table 1), which represented 0.1% of the 03-409 seedlings infected with this pathogen on the cotyledon tips, and only 0.4% transmission from the infected seed planted. *Cladosporium variabile* was isolated from all of the seedlings sampled that had lesions on the cotyledon blades (mean of 0.2 sampled per flat in trial 2). Therefore, 0.1% of the seedlings were infected with *C. variabile* on the cotyledon blades (Table 1). The pathogen was also isolated from a mean of 0.6 of 5.6 seedlings sampled per flat that developed lesions on the first true leaves. Therefore, 0.3% of the seedlings in each flat were infected with *C. variabile* on the first true leaves (Table 1). Secondary spread of *C. variabile* was detected at a rate of 0.5% of the 03-409 seedlings in trial 2.

For seed lot B5, *C. variabile* was isolated from a mean of 1.6 of 9.4 symptomatic seedlings that developed lesions on the first true leaves in trial 1, representing a mean of

1.3% of the seedlings infected with this pathogen (Table 1). Secondary spread of *C. variabile* was observed at a mean rate of 1.3% of the B5 seedlings in trial 1 (Table 1). The fungus was not isolated from any of the cotyledon tip lesions on B5 seedlings in trial 1, nor from any of the B5 seedlings that developed lesions in trial 2 (Table 1).

Stemphylium botryosum was isolated from a mean of 9.6 of 19.0 symptomatic B5 seedlings sampled in trial 1. Based on the mean number of symptomatic seedlings per flat, a mean of 10.9% of the B5 seedlings were infected with *S. botryosum* in trial 1. In this trial, *S. botryosum* was isolated from a mean of 4.0 of 9.6 symptomatic B5 seedlings sampled that developed lesions on the cotyledon tips (Table 1), i.e., 9.2% of the B5 seedlings were infected on the cotyledon tips with this pathogen, representing 10.3% transmission from infected seed of lot B5. *Stemphylium botryosum* was isolated from a mean of 5.6 of 9.4 symptomatic seedlings sampled that developed lesions on the first true leaves, i.e., 4.6% of the seedlings were infected with *S. botryosum* on the first true leaves. Lesions were not observed on the cotyledon blades, so 4.6% was the incidence of secondary spread of *S. botryosum* from lot B5 in trial 1 (Table 1).

For lot B5 in trial 2, *S. botryosum* was isolated only from lesions on the cotyledon tips from a mean of 5.4 of 12.8 symptomatic seedlings sampled, i.e., 3.3% of the B5 seedlings were infected with this pathogen in trial 2, and 3.7% transmission occurred from infected seed of lot B5.

For lot 03-409 in trial l, *S. botryosum* was isolated from a mean of 1.0 of 12.2 symptomatic seedlings sampled that developed lesions on the first true leaves, i.e., 0.9% of the 03-409 seedlings were infected with this pathogen in trial 1, with secondary spread from the cotyledons to the first true leaves of 0.8% of the seedlings. The fungus was not

isolated from cotyledon tips or cotyledon blades of lot 03-409 in this trial. In trial 2, *S. botryosum* was isolated from a mean of 0.2 of 7.6 seedlings of 03-409 that developed lesions on the cotyledon tips, i.e., 0.1% of the seedlings were infected on the cotyledon tips with 2.4% seed transmission. Secondary spread of *S. botryosum* to cotyledon blades and first true leaves was not detected in trial 2 for lot 03-409.

Alternaria spp. were isolated from lesions on the cotyledon tips, cotyledon blades, and first true leaves of lot 03-409 in trial 1, and from lesions on the cotyledon tips and first true leaves of lot 03-409 in trial 2. Isolates of this genus were also obtained from lesions on the cotyledon tips and the first true leaves of lot B5 in trial 1, and from lesions on the cotyledon tips of lot B5 in trial 2. No pathogenic fungi were isolated from asymptomatic plants of lots 03-409 or B5 in either of the trials.

In trial 1, a mean incidence of 0.8 of the 9.5 B5 seedlings sampled that had lesions on the cotyledon tips also had the pericarp attached to the symptomatic cotyledons (Fig. 1B). Although pseudothecia were observed on all of these pericarps, *S. botryosum* was isolated from only 0.2 (25%) of these cotyledon tip lesions. In trial 2, 2.8 of the 12.8 B5 seedlings sampled per flat that had the pericarp attached to the cotyledons, also had lesions on the cotyledon tips. Pseudothecia were observed on a mean of 2.0 of these pericarps, and *S. botryosum* was isolated from only 1.8 (64%) of these cotyledon tip lesions. For lot 03-409 in trial 1, *C. variabile* was isolated from a mean of 1.0 of the cotyledon tip lesions from 3.0 of the sampled seedlings that had the pericarp attached to the symptomatic cotyledons. Although a mean of 0.6 of these pericarps developed pseudothecia, *S. botryosum* was not isolated from the cotyledon tips to which the pseudothecia were attached. For lot 03-409 in trial 2, *C. variabile* and *S. botryosum* were

not isolated from any of the cotyledon tip lesions from 0.6 sampled seedlings that still had the pericarp attached to the symptomatic cotyledons.

All isolates of *C. variabile* and *S. botryosum* obtained from symptomatic seedlings in the seed transmission trials were pathogenic on spinach. None of the isolates of *Alternaria* spp. proved pathogenic on spinach in the greenhouse. A *Colletotrichum* sp. was isolated from each of two B5 seedlings in trial 2 that developed lesions on the cotyledon tips to which the pericarps remained attached. Acervuli developed on the pericarp attached to the cotyledon tips of one of these seedlings, and the *Colletotrichum* sp. from this seedling was pathogenic on spinach.

In trial 1, the mean day temperature in the greenhouse was 21.4^{o}C compared to a mean night temperature of 15.0^{o}C (Table 2). The mean relative humidity during the day was 72.8% compared to 95.4% at night (Table 2). In trial 2, carried out two months later (after the greenhouse heating system had been turned on), the mean temperature during the day was 17.3^{o}C compared to 14.4^{o}C at night, and the mean relative humidity during the day was 74.5% compared to 74.5% by night (Table 2). In trial 2, the mean leaf wetness during the day was 12.5 min/h compared to 12.7 min/h by night (Table 2).

4.4. DISCUSSION

Cladosporium variabile and *S. botryosum* were transmitted from spinach seed to seedlings in these greenhouse trials in which mean temperatures ranged from 14.4 to 21.4^{o}C and mean relative humidity ranged from 74.5 to 95.4%. Lesions on seedlings first appeared on cotyledon tips, followed by the cotyledon blades and the first two true leaves, suggesting that lesions on the cotyledon tips were a direct result of seed

transmission, and lesions on the cotyledon blades and first true leaves were probably caused by secondary spread of *C. variabile* and *S. botryosum* in the greenhouse. The misting system in the greenhouse was used to create a favorable environment for *C. variabile* and *S. botryosum* by raising the relative humidity, just as rain and overhead irrigation enhance the development of Stemphylium leaf spot in field crops (Koike et al., 2001). The fan operating in the greenhouse for temperature control may have promoted secondary spread of these fungi, as the spores of both pathogens are readily airborne and wind dispersed (Inglish et al., 1997; Koike et al., 2001).

Seed transmission of *C. variabile* for lot 03-409 averaged 18.1%. This was 43.1% higher than that of *S. botryosum* from lot B5 (mean of 10.3%) in trial 1, in which temperatures averaged 18.0^{o}C and relative humidity averaged 84.9%. However, seed transmission of *C. variabile* for lot 03-409 decreased to 0.4% in trial 2 compared to a decrease in seed transmission of *S. botryosum* from 10.3% in trial 1 to 3.7% in trial 2 for lot B5. The greatest difference in growing conditions between trials 1 and 2 was the relative humidity at night (95.4 vs. 74.5%, respectively), although day temperatures also differed by 4.1^{o}C (21.4 vs. 17.3^{o}C, respectively). The higher humidity in trial 1 vs. trial 2 probably favored seed transmission of both fungi. Baby leaf spinach crops are planted at high density (>2 million seed/acre) which promotes similar humid conditions in the crop and may favor seed transmission of these two leaf spot pathogens (Koike et al., 2001). Similarly, fresh market spinach crops are typically irrigated using overhead sprinklers, which favors high humidity and extended periods of leaf wetness (Koike et. al., 2001).

Hansen et al. (1952) did not document the conditions under which *C. variabile* was transmitted from spinach seed in a greenhouse in Denmark. Fuentes-Davila (1988)

reported that the Japanese seed industry has claimed that this pathogen is seed transmitted in spinach, although he did not cite research demonstrating this fact. The results of this greenhouse study concur with these reports. However, Fuentes-Davila (1988) did not detect seed transmission of *C. variabile* in spinach, even at 15 to 20°C and >80% relative humidity, conditions considered conducive for infection of spinach by *C. variabile*. In that study, misting sprinklers were not used to mantain leaf wetness on the seedlings. In contrast, it appears that the combination of leaf wetness and high relative humidity achieved using misters in this greenhouse study may have favored seed transmission of *C. variabile* and *S. botryosum*, but specific durations of leaf wetness required for infection of spinach by either fungus have not been investigated.

Seed transmission of *C. variabile* and *S. botryosum* was detected on seedlings on which the pericarp remained attached to the cotyledon tips after emergence, but was also observed on seedlings that did not have the pericarp attached to the cotyledons at emergence. Therefore, seed transmission of these pathogens does not appear to depend on attachment of the pericarp to the cotyledon tips after emergence. However, the epigeal nature of spinach germination probably favors seed transmission of these two pathogens because the cotyledons are carried up during hypocotyls growth and the pericarp of the seed could remain attached to the cotyledons increasing the time of contact of the pericarp and cotyledons and incrementing the possibility of transmission of the pathogens from the pericarp to the cotyledon tips of the seedlings. Similar results have been reported in other vegetable species with epigeal germination: seed transmission of *Botrytis allii* Munn, causal agent of neck rot of bulb onions (*Allium cepa* L.) (Maude and Presly, 1977); seed transmission of *Didymella lycopersici* Kleb. in tomato (Maude,

1962); and seed transmission of *Aspergillus niger* Tiegh., causal agent of black mold of onion (Hayden and Maude, 1992). Shrestha et al., (2000) also reported seed transmission of *Alternaria brassicae* in rape seed (*Brassica campestris* L.) and mustard (*Brassica juncea* L.) in field conditions. The authors did not report whether seed transmission of *A. brassicae* was observed primarily as lesions on the cotyledon tips, or if attachment of the pericarp to the cotyledons favored seed transmission. Evidence of secondary spread on first true leaves was observed after 35 days of emergence.

Alternaria spp. have been reported to be pathogens of spinach (Correll et al., 1994). The results of this study suggest that *Alternaria* spp. may be weak pathogens on spinach seedlings, but the *Alternaria* spp. isolated from lesions on seedlings did not infect mature spinach plants. Kobmoto et al., (1979) and Whiteside (1976) reported that production of host-specific toxins of *A. citri* Ellis & N. Pierce were responsible for the host specificity of the pathogen to rough lemon (*Citrus jambhiri* Lush.) and Dancy tangerine (*C. reticulate* Blanco). Similarly, *Alternaria* spp. could be producing host-specific toxins that affect spinach seedlings but not the mature spinach plants. More research is needed to clarify the possible role of *Alternaria* species pathogens of spinach.

Colletotrichum was not detected in a freeze-blotter seed assay of 400 seed of lot B5 carried out each of three times before this seed transmission study. However, development of acervuli of a *Colletotrichum* species on the pericarp attached to the cotyledons of one of the seedlings, from which a *Colletotrichum* species was isolated that was pathogenic on more mature spinach plants, demonstrated that this fungus was present at a very low incidence in seed lot B5. A total of about 2000 seed of lot B5 were planted during the two seed transmission trials and seed transmission was detected for one

seedling, suggesting a transmission rate of 0.05% under the conditions of these trials. To our knowledge, this might be the first demonstration of seed transmission of a *Colletotrichum* species of spinach.

The only report about a seedborne transmitted pathogen in spinach was documented by Inaba et al. (1983). The authors detected *Peronospora effusa* on the cotyledons of spinach seedlings developing in a growth chamber at 15^{o}C, 21 days after planting the seed. The authors did not report whether seed transmission of *P. effusa* was observed primarily as lesions on the cotyledon tips, if attachment of the pericarp to the cotyledons favored seed transmission, or evidence of secondary spread of downy mildew under the conditions of that study.

If a fresh market or processing spinach crop develops >5% leaf spot symptoms, growers may be forced to abandon the crop because of expenses incurred for hand-sorting symptomatic leaves (J. Schafer, *personal communication*). Baby leaf spinach crops are typically planted at high densities (>10 million seed/ha) under sprinkler irrigation, which favors high humidity and extended periods of leaf wetness in the canopy (Raid, 2004). Under such conditions, *C. variabile* and *S. botryosum* are likely to be transmitted from infected spinach seed. In this study, *C. variabile* was transmitted from spinach seed at a rate of 0.4 to 18.1% and *S. botryosum* at a rate of 3.7 to 10.3%. These results suggest that for 10 million seed planted/ha, the incidence of infected seedlings expected in the field under conducive conditions for transmission would be 0.04 to 1.81 million seedlings/ha for *C. variabile* for lot 03-409, and 0.37 to 1.03 million seedlings/ha for *S. botryosum* for lot B5. Further research on seed transmission of these spinach pathogens under field conditions is needed.

4.5. LITERATURE CITED

1. Agarwal, V.K., and Sinclair, J.B. 1997. Control of seedborne pathogens. D. Mechanical methods. Pages 453-459, In: Principles of Seed Pathology, 2nd Edition. CRC Press, Inc., Boca Raton, FL.
2. Cappelli, C. 2004. Seeds: Pathogen transmission through. Pages 1142-1147, In: Encyclopedia of Plant and Crop Sciences. Marcel Dekker, Inc., New York, NY.
3. Correll, J.C., Morelock, T.E., Black, M.C., Koike, S.T., Brandenberger, L.P., and Dainello, F.J. 1994. Economically important diseases of spinach. Plant Dis. 78:653-660.
4. du Toit, L.J., and Derie, M.L. 2001. *Stemphylium botryosum* pathogenic on spinach seed crops in Washington. Plant Dis. 85:920.
5. du Toit, L.J., and Derie, M.L. 2003. Inoculum sources of *Stemphylium botryosum* and *Cladosporium variabile* in spinach seed crops. Phytopathology 93:S22.
6. Everts, K.L., and Armentrout, D.K. 2001. Report of leaf spot of spinach caused by *Stemphylium botryosum* in Maryland and Delaware. Plant Dis. 85:1209.
7. Fuentes-Davila, G. 1988. Studies on a Leaf Spot Disease of Spinach Caused by *Cladosporium variable* (Cooke) de Vries. Ph.D. thesis, Washington State University, Pullman, WA. 49 pp.
8. Hansen, H.R., Weber, A., and Kristensen, H.R. 1952. Plant diseases in Denmark in 1950. Annual survey compiled by the State Phytopathological Experiment Station. Tidsskr. Planteavl. 56:1-59. (Translated abstract from Rev. Appl. Mycol. 32:234).
9. Hayden, N.J., and Maude, R.B. 1992. The role of seedborne *Aspergillus niger* in transmission of black mould of onion. Plant Pathology 41:573-581.

10. Inaba, T., Takahashi, K., and Morinaka, T. 1983. Seed transmission of spinach downy mildew. Plant Dis. 67:1139-1141.
11. Inglis, D.A., Derie, M.L., and Gabrielson, R.L. 1997. Cladosporium leaf spot on spinach seed crops and control measures. Washington State University Extension Bulletin No. 865. Pullman, WA.
12. Kohmoto, K, Scheffer, R.P., and Whiteside, J.O. 1979. Host-selective toxins from *Alternaria citri.* Phytopathology 69:667-671.
13. Koike, S.T., Henderson, D.M., and Butler, E.E. 2001. Leaf spot disease of spinach in California caused by *Stemphylium botryosum*. Plant Dis. 85:126-130.
14. Maude, R.B. 1962. Seed transmission of Didymella stem-rot of tomatoes. Ann. Appl. Biol. 50:105-111.
15. Maude, R.B. 1996. Seed transmission of diseases. Pages 44-69, In: Seedborne Diseases and Their Control. CAB International, Oxon, UK.
16. Maude, R.B., and Presly, A.H. 1977. Neck rot (*Botrytis allii*) of bulb onions. I. Seed-borne infection and its relationship to the disease in the onion crop. Ann. Appl. Biol. 86:163-180.
17. Peterson, P.S., and Harris, E. 1997. Notes on seed physiology, germination, and quality assessment. Page 67, In: Workshop of California Seed Analysts and Researchers, 26-28 March 1997, Fresno, CA.
18. Raid, R.N. 2004. Management of Stemphylium leaf spot on baby spinach harvested for spring mix production in the Everglades agricultural area of Florida. Page 24, In: 17th International Lettuce and Leafy Vegetable Conference, 28-31 August 2004, Quebec, Canada.

19. Shrestha, S.K., Mathur, S.B., and Munk, L. 2000. Alternaria brassicae in seeds of rape seed and mustard, its location in seeds, transmission from seeds to seedlings and control. Seed Sci. Technol. 28:75-84.
20. Whiteside, J.O. 1976. A newly recorded Alternaria-induced brown spot disease on Dancy tangerines in Florida. Plant Dis. 60:326-329.

Table 1. Seed transmission and secondary spread of *Stemphylium botryosum* and *Cladosporium variabile* in spinach[a]

Seed lot, pathogen, and trial[b]	Location of lesions on seedlings: Cotyledon tip — No. of seedlings infected (no. sampled)[c]	Cotyledon tip — % Seedlings infected	Cotyledon tip — % Transmission from infected seed[d]	Cotyledon blade — No. of seedlings infected (no. sampled)[c]	Cotyledon blade — % Seedlings infected	First two true leaves — No. of seedlings infected (no. sampled)[c]	First two true leaves — % Seedlings infected	% Secondary spread[e]
03-409								
C. variabile								
Trial 1	4.0 ± 1.2 (7.2 ± 0.4)[f]	5.1 ± 2.2	18.1 ± 7.8	6.0 ± 0.7 (6.8 ± 0.4)	3.7 ± 1.8	8.2 ± 1.8 (12.2 ± 0.4)	7.9 ± 3.6	12.2 ± 5.4
Trial 2	0.2 ± 0.4 (7.6 ± 3.0)	0.1 ± 0.2	0.4 ± 0.9	0.2 ± 0.4 (0.2 ± 0.4)	0.1 ± 0.3	0.6 ± 1.3 (5.6 ± 0.6)	0.3± 0.8	0.5 ± 0.7
S. botryosum								
Trial 1	0.0 ± 0.0 (7.2 ± 0.4)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0 (6.8 ± 0.4)	0.0 ± 0.0	1.0 ± 1.7 (12.2 ± 0.4)	0.9 ± 1.5	0.8 ± 1.2
Trial 2	0.2 ± 0.4 (7.6 ± 3.0)	0.1 ± 0.3	2.4 ± 5.5	0.0 ± 0.0 (0.2 ± 0.4)	0.0 ± 0.0	0.0 ± 0.0 (5.6 ± 0.6)	0.0 ± 0.0	0.00 ± 0.00
B5								
C. variabile								
Trial 1	0.0 ± 0.0 (9.6 ± 3.8)	0.0 ± 0.0	0.0 ± 0.0	-	-	1.6 ± 0.9 (9.4 ± 2.4)	1.3 ± 0.6	1.3 ± 0.6
Trial 2	0.0 ± 0.0 (12.8 ± 2.8)	0.0 ± 0.0	0.0 ± 0.0	-	-	0.0 ± 0.0 (29.4 ± 14.0)	0.0 ± 0.0	0.0 ± 0.0
S. botryosum								
Trial 1	4.0 ± 3.0 (9.6 ± 3.8)	9.2 ± 5.3	10.3 ± 6.0	-	-	5.6 ± 2.6 (9.4 ± 2.4)	4.6 ± 2.5	4.6 ± 2.5
Trial 2	5.4 ± 0.9 (12.8 ± 2.8)	3.3 ± 0.6	3.7 ± 0.6	-	-	0.0 ± 0.0 (29.4 ± 14.0)	0.0 ± 0.0	0.0 ± 0.0

[a] Seed of each lot were planted into five flats (replications) in the greenhouse. Each flat consisted of 200 cells, except for the flats planted with lot 03-409 in trial 1, for which ten 512-cell flats were used. Flats were watered using misting sprinklers set on a timer, as described in the text.

[b] The mean incidence of seedborne *C. variabile* and *S. botryosum* was 28.25 and 4.75%, respectively, for lot 03-409; and 88.50% *S. botryosum* and no *C. variabile* for lot B5 (determined by freeze-blotter seed health assay as described in Chapter 2).

[c] % Seedlings infected = (number of symptomatic seedlings from which the pathogen was isolated) / (number of symptomatic seedlings sampled) * (% symptomatic seedlings in the flat).

[d] % Seed transmission = (% seedlings infected on the cotyledon tips) / (% planted seed infected with the pathogen) * 100.

[e] % Secondary spread = % seedlings infected on the cotyledon blades or the first true leaves.

[f] Mean ± standard deviation for each variable.

Table 2. Temperature, relative humidity, and leaf wetness in greenhouse seed transmission trials for *Stemphylium botryosum* and *Cladosporium variabile* in spinach[a]

	Temperature (°C)[b]			Relative humidity (%)			Leaf wetness (min/h)[c]		
Trial[b]	**24 h**	**Day**	**Night**	**24 h**	**Day**	**Night**	**24 h**	**Day**	**Night**
1	18.0 ± 4.2 [d]	21.4 ± 3.3	15.0 ± 2.1	84.9 ± 15.6	72.8 ± 15.1	95.4 ± 4.6	-	-	-
2	15.7 ± 1.8	17.3 ± 1.4	14.4 ± 1.0	74.5 ± 4.3	74.5 ± 4.4	74.5 ± 4.3	12.6 ± 1.2	12.5 ± 1.8	12.7 ± 0.4

[a] Seed transmission trials were carried out in the greenhouse as described in the text.

[b] In trial 1, temperature and relative humidity were recorded hourly using the WatchDog™ datalogger (Model 425, Spectrum Technologies, Inc., Plainfield, IL) from 17 October to 11 November 2004. In trial 2, temperature, relative humidity, and leaf wetness (latter using a leaf wetness sensor from Spectrum Technologies, Inc.) were recorded hourly from 6 to 12 January 2005.

[c] Day = 8:00 am to 6:00 pm (when lights were on in the greenhouse). Night = 7:00 pm to 7:00 am (lights off).

[d] Mean ± standard deviation.

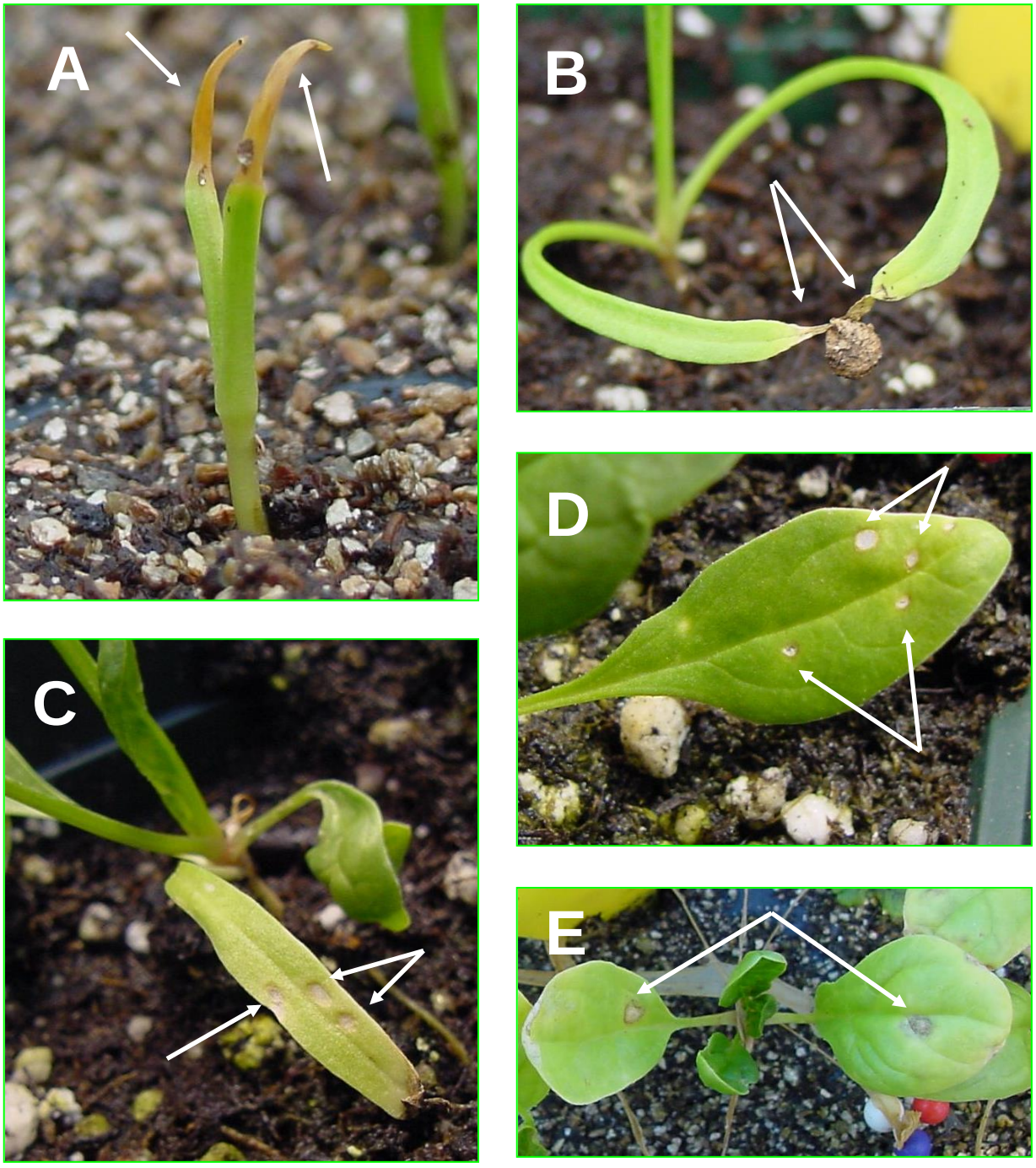

Fig. 1. Lesions resulting from seed transmission of *Cladosporium variabile* and *Stemphylium botryosum* on spinach. A) Lesions caused by *S. botryosum* on cotyledon tips. B) Lesions caused by *S. botryosum* on cotyledon tips with the pericarp attached. C) Lesions caused by *C. variabile* on a cotyledon blade. D) Lesions caused by *C. variabile* on a true leaf. E) Lesions caused by *S. botryosum* on the first pair of true leaves.