LIGHT LEAF SPOT AND WHITE LEAF SPOT OF BRASSICACEAE IN WASHINGTON STATE

By

SHANNON MARIE CARMODY

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN PLANT PATHOLOGY

WASHINGTON STATE UNIVERSITY
Department of Plant Pathology

JULY 2017

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of SHANNON MARIE CARMODY find it satisfactory and recommend that it be accepted.

_____

Lindsey J. du Toit, Ph.D., Chair

_____

Lori M. Carris, Ph.D.

_____

Timothy C. Paulitz, Ph.D.

_____

Cynthia M. Ocamb, Ph.D.

ACKNOWLEDGMENT

I would like to thank my major advisor Dr. Lindsey du Toit for her tireless mentorship, passion, and enthusiasm. I wish to thanks my committee members Dr. Lori Carris, Dr. Cynthia Ocamb, and Dr. Timothy Paulitz who welcomed me into their labs in Pullman, WA and when visiting in Corvallis, OR. This work would not have been possible without the financial support of the Clif Bar Family Foundation Seed Matters Initiative and the Western Sustainable Agriculture Research and Education Fellowship.

Thank you to all of the faculty, students, and staff of WSU Mount Vernon and WSU Pullman who have generously shared time, support, knowledge, tulips, equipment, and humor. As was noted in my hospital chart, you all made sure I was “emotionally, financially, and botanically supported” which is more than I could have ever asked for. None of my research would have been possible without the members of the Vegetable Seed Pathology Lab. In particular, I’d like to thank Mike Derie for sharing his knowledge, Barbara Holmes who is just the best, and fellow students and future colleagues John Weber and Eric Christianson for being such a positive part of this experience. I would like to thank collaborators who contributed to this research – Tom Stearns from High Mowing Seed Co., Dr. Tobin Peever from WSU, and Drs. Kevin King and Jon West from Rothamsted Research – finding people to share ideas and discoveries with in the small world of seed pathology and *P. brassicae* research was so rewarding. This process was made meaningful by the many members of the Puget Sound Seed Growers Association who shared their farms, brassica seed, knowledge, and support. And to my husband Colin Curwen-McAdams, uva uvam vivendo varia fit, I love you.

LIGHT LEAF SPOT AND WHITE LEAF SPOT OF BRASSICACEAE IN WASHINGTON STATE

Abstract

by Shannon Marie Carmody, M.S.
Washington State University
July 2017

Chair: Lindsey J. du Toit

Pyrenopeziza brassicae, cause of light leaf spot of brassicas, was first found in the USA in 2014 in the Willamette Valley of Oregon. *Neopseudocercosporella capsellae*, cause of white leaf spot of brassicas, occurred rarely in the Pacific Northwest prior to being found across the Willamette Valley in 2014. In this study, a 2016 survey of northwestern Washington, a primary region of biennial brassica vegetable seed production for the USA, revealed both diseases to be present in mustard (*Brassica juncea*) cover crops and on bird's rape mustard (*B. rapa*) weeds, but not in cabbage (*B. oleracea* var. *capitata*) seed crops. Sexual crossing tests, pathogenicity tests, and DNA phylogenetic analyses (latter of the internal transcribed spacer region of ribosomal DNA, β tubulin gene, translation elongation factor 1-α gene, and mating type genes (*MAT1-3* and *MAT1-2*); and multi-locus sequence analysis of the first three sequences) of *P. brassicae* isolates from the USA, European Union, New Zealand, and United Kingdom revealed that isolates from the USA likely represent a new species of *Pyrenopeziza*, hereafter referred to as *P.* cf. *brassicae*. *P*. cf. *brassicae* was demonstrated to be seedborne and seed transmitted on cabbage and mustard. Incubating infested seed on NP-10 agar medium at 4°C, followed by microscopic

examination of the seed revealed *P.* cf. *brassicae* to be present on 12.50 to 19.75% of a mustard seed lot and <0.50% of a cabbage seed lot. Planting the infested mustard seed in a greenhouse resulted in a seed transmission rate of 0.1 to 5.3%. Seed treatment trials revealed that chlorine (1.2% NaOCl for 10, 20, 30, and 40 minutes), hot water (50^{o}C for 15 and 30 minutes), steam (62.8, 65.6, 68.3, and 71.1^{o}C for 90 seconds), and 10 fungicide seed treatments all reduced the incidence of mustard seed infected with *P.* cf. *brassicae* to <5%, and reduced seed transmission of the fungus from 3.4% for non-treated seed to <1%. Hot water and most of the steam treatments eradicated the pathogen from seed, but the hottest steam treatment was phytotoxic. The most efficacious fungicide seed treatments contained benzimidazole, a demethylation inhibitor, and/or strobilurin active ingredients.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

For Townes

CHAPTER ONE

LITERATURE REVIEW

1.1 Brassicaceae

Brassicaceae, for which the plant members are often referred to as brassicas, crucifers, cole or rape crops, is a spectacularly diverse family of plants comprising 338 genera and 3,700 species (Koch and Mummenhoff 2006), and includes economically important genera such as *Brassica, Raphanus,* and *Sinapis* (Gomez-Campo 1980; Rakow 2004). The genus *Brassica* includes 37 species, of which six are considered economically important (Fussell 1955; Gomez-Campo 1980; Inglis et al. 2013). The multipurpose nature of Brassicaceae species grown as vegetable, fodder, oilseed, ornamental, and cover crops makes them important agricultural crops throughout the world. Brassicas were a staple crop in Europe from 1650 to 1850 (Fussell 1955). *B. rapa* and *B. juncea* have been grown as vegetable and oilseed crops in northern India and China since 1,500 BC (Rimmer et al. 2007). Brassica crop storability, in root cellars or fermented as sauerkraut and kimchi, has made the genus significant historically and essential in many cuisines. Brassicas remain an important vegetable today, with crops such as broccoli, spicy-flavored mustards, and the recently inexplicably popular kale (Martin 2014; United States Department of Agriculture (USDA) National Agricultural Statistics Service (NASS) nd). Today, China and India are the top producers of cabbage, cauliflower, broccoli, rapeseed, and other Brassicas (Food and Agriculture Organization of the United Nations Statistics Division (FAOSTAT) n.d.). In the past 40 years, there has been an increase in production of brassicas grown for oilseed, with more than 40 million metric tons worldwide annually, making this genus the next most important source of oil after soybean (Rakow 2004; Rimmer et al. 2007).

Plants in Brassicaceae are thought to have originated in the Mediterranean region, with secondary centers of diversity in Asia and throughout western and southern Europe (Rakow 2004;

Rimmer et al. 2007; Vaughan 1977). Brassicaceae plants have been observed for centuries (Gomez-Campo 1980). Pliny the Elder wrote of Brassicas in *Natural History* (77 to 79 AD), that he "knew three kinds of rapa: one flat and broad, the second round like a ball, the third wild with a long radish-like root, pointed rough leaves and acrid juice" (Fussell 1955). He was not alone in observing the hybridization of brassica crops – Theophrastus (370 to 285 BC) and the Roman Cato (234 to 149 BC) also mentioned heading cabbage and stem kales (Rakow 2004). Allogamous breeding of *B. rapa* and *B. juncea* in China, and *B. oleracea* in Europe led to an incredible diversity of morphology in cultivars (Rimmer et al. 2007). Though the three species developed in different parts of the world, parallel forms of vegetables were selected over time in each species, i.e., head (cabbage in *B. oleracea* and Chinese cabbage in *B. rapa*), leaf, stem, and root vegetables (Rimmer et al. 2007).

Genetic understanding of Brassicaceae dates back to the early 20th century (Nagaharu 1935). Woo Jang-Choon, a Korean-Japanese botanist, first proposed the Triangle of U theory in 1935, explaining the evolution and relationships among different *Brassica* species (Nagaharu 1935). Three distinct, though closely related species, *B. oleracea, B. rapa*, and *B. nigra*, can be crossed to create the remainder of six economically important species in the genus. *B. oleracea* crossed by *B. nigra* results in tetraploid *B. carinata , B. nigra* crossed by *B. rapa* forms the tetraploid *B. juncea*, and *B. rapa* crossed with *B. oleracea* forms the tetraploid *B. napus* (Rimmer et al. 2007). The Triangle of U theory was later verified by DNA and protein studies (Lysak et al. 2007).

1.1.1 Brassica diversity: *Brassica* as a genus.

All plants in the *Brassica* genus have perfect, usually yellow flowers with four petals in a cross shape that inspired the group's name, crucifer (Buttala and Siegel 2015; Gomez-Campo 1980). Though species in Brassicaceae have perfect flowers, species such as *B. oleracea* and *B. rapa* are self-incompatible and require an insect for cross pollination. Other brassicas are also insect-pollinated but

can self-pollinate, e.g., *B. juncea*. Brassicas form dry, dehiscent fruits called siliques that are heterocarpic with two separate seed-bearing cavities (Gomez-Campo 1980). The siliques on racemes are harvested when dry and brown with a mature seed color. Mature seed color can range from yellow to black (Gomez-Campo 1980). Brassica plants at full seed maturity can reach up to 1.5 meters tall, which is often remarkably different from the plant at market maturity. Some brassicas are biennial, requiring a vernalization period in order to flower and produce seed. *B. rapa*, *B. oleracea,* and *B. napus* can be biennial or annual depending on the cultivar and/or environment, while *B. juncea* is an annual (Buttala and Siegel 2015; Rimmer et al. 2007).

1.1.1.1 *Brassica carinata* A. Braun. *B. carinata* A. Braun (n = 17, where n refers to the gametic or haploid number, or the number of chromosomes in a gamete), commonly known as Abyssinian mustard, has been grown in and around Ethiopia for centuries (Rakow 2004; Rimmer et al. 2007; Vaughan 1977). A hybrid between *B. nigra* and *B. oleracea*, Ethiopian farmers grow the plant as a leafy vegetable and harvest the seed for oil (Rakow 2004; Rimmer et al. 2007). There are no known wild species of this crop type.

1.1.1.2 *Brassica juncea* (L.) Czern & Coss. Cultivars in *B. juncea* (n = 18) include head mustard (var. *capitata*), cut leaf mustard (var. *crispifolia*), Chinese mustard greens (subsp. *integrifolia*), brown mustard (subsp. *juncea*), and oilseed mustard (var. *oleifera*) (Rakow 2004; Rimmer et al. 2007; United States Department of Agriculture (USDA) Germplasm Resources Information Network (GRIN) n.d.). There is some uncertainty about the origin of *B. juncea*. Vaughan (1977) suggested that China is a major center of diversity for this species. Rimmer et al. (2007), however, offered that *B. juncea* may have developed in multiple places as a result of hybridization. In western countries, *B. juncea* has largely replaced *B. nigra* as a cultivated crop because of the tendency for *B. nigra* to shatter in the field (Buttala and Siegel 2015; Rimmer et al. 2007; Vaughan 1977). In East Asia, the plants are used culinarily as

vegetables or salad greens, often with a pungent flavor; and in India this species is used as a seed oil, or in drier regions of India for canola (Rimmer et al. 2007; Vaughan 1977). *B. juncea* plants are relatively fast growing and more adapted to drier climates than other species in this genus. These traits also have made *B. juncea* increasingly popular as a cover crop in the United States (Watts 2016).

1.1.1.3 *Brassica napus* L. *B. napus* (n = 19) is thought to be a more recent addition to the *Brassica* genus, with no naturally occurring populations (Rimmer et al. 2007). This species includes the crops rutabaga or swede (subsp. *napobrassica*), used for the roots; and canola and rape (subsp. *napus* or *oleifera*), used for oilseed (Rakow 2004; Rimmer et al. 2007; USDA GRIN n.d.; Vaughan 1977). Adapted to cool, moist climates, *B. napus* is preferred for the levels of productivity over that of *B. juncea* for canola, in regions with adequate moisture (Rimmer et al. 2007). This amphidiploid, created by crossing *B. oleracea* and *B. rapa,* has followed the same historical and culinary path as *B. juncea* (Vaughan 1977). Rapeseed canola is one of the world's 25 most important food crops, with 24 million ha planted in 2007 (Grealy et al. 2007). Furthermore, canola comprises the second largest acreage of oil crops in the world, producing 10 to 15% of the world's oil crop from 1999 to 2009 (USDA Economic Research Service (ERS) n.d.).

1.1.1.4 *Brassica nigra* (L.) Koch. *B. nigra* (L.) Koch (n = 8), commonly known as black mustard, is grown throughout central and southern Europe, and can be found as a weed in the Mediterranean region and in many places with a temperate climate (Rakow 2004; Vaughan 1977). Rimmer et al. (2007) suggested that this species has a close genetic relationship with *Sinapis*, a genus comprised of many weedy species. *B. nigra* is most widely grown for seed to make commercial table mustard (Duke 1983). However, as previously stated, this species has largely been replaced by *B. juncea* (Buttala and Siegel 2015; Vaughan 1977).

1.1.1.5 *Brassica oleracea* L. Not only is the genus *Brassica* diverse, but so is the diversity within the species *B. oleracea*. Siegel and Buttala (2015) wrote, "*Brassica oleracea* has the largest diversity of crop types among the vegetable species." *B. oleracea* (n = 9) crops can be divided into the following groups: brassicas harvested for the leaf, such as kale (var. *acephala*) and collard (var. *viridis*); heading brassicas such as cabbage (var. *capitata*), savoy-type cabbage (var. *sabauda*), and Brussels sprouts (var. *gemmifera*); inflorescence kales such as broccoli (var. *italica*), cauliflower (var. *botrytis*), and branching bush kales (var. *fruticosa*); kohlrabi (var. *gongylodes*); and Chinese kale, a leaf vegetable (var. *alboglabra*) (Rakow 2004; Rimmer et al. 2007; USDA GRIN n.d.). These cultivated groups are thought to have originated in western Europe (Rakow 2004). *B. alboglabra,* or Chinese kale, is the most popular vegetable brassica group in Europe and North America, valued for the capacity to store nutrients (Rimmer et al. 2007). These crop types require a longer and/or colder vernalization period, and are slower growing than other *Brassica* species (Buttala and Siegel 2015).

1.1.1.6 *Brassica rapa* L. Nearly as diverse as *B. oleracea*, *B. rapa* (synonymous with *B. campestris*) is divided into the following subspecies: the Mizuna group (var. *nipposinica*), the Tori group (var. *dichotoma*), Chinese cabbage (var. *pekinensis*), Pak choi (var. *chinensis*), Taatsoi (var. *narinosa*), and the turnip rape group, including var. *rapa* and var. *oleifera* (Rakow 2004; Rimmer et al. 2007; Vaughan 1977). This ancient species has been cultivated in Europe since the 13th century and, before that, the crop sarson was referenced in Indian writings from 2,000 to 1,500 B.C (Vaughan 1977). Rakow (2004) wrote that *B. rapa* was likely introduced to China as a food crop through western Asia or Mongolia. This species is important economically and culturally in East Asia, and northern China may be a center of diversity for headed Chinese cabbage (var. *pekinensis*) (Rakow 2004; Vaughan 1977). Crop types include vegetable turnip, fodder turnip, turnip rape, yellow sarson, Chinese cabbage, pak choi, mizuna, Chinese savoy, broccoli raab, canola and turnip grown for vegetables and as cover crops (Rakow 2004; Rimmer et

al. 2007; USDA GRIN n.d.) Adapted to cooler climates, *B. rapa* is relatively fast growing (Rimmer et al. 2007).

1.1.1.7 Weedy Brassicaceae genera. Rollins (1981) listed 117 weedy brassicas in North America, and defined weedy brassicas as both pestiferous, non-pestiferous, those displacing native plants, and as native brassica plants that had become weedy. *Eruca sativa* is a significant weedy species naturalized in many areas (Rimmer et al. 2007). According to Rimmer et al. (2007), *B. rapa* is not well understood as a wild or weedy plant because many naturally growing plants are suspected to have escaped cultivation. *B. napus* and *B. carinata* do not have known wild populations, and *B. nigra* is closely related to the weedy white mustard, *Sinapis arvensis*.

The study of wild brassicas is important in brassica crop breeding because of the potential value for food products and for traits that breeding programs find useful, such as cytoplasmic androsterility and disease resistance (Gomez-Campo 1980). In addition, an abundance of weedy brassicas can be problematic for commercial brassica seed production. In brassica seed production, cross-pollination between planted cultivars and weeds of the same species can affect the genetic purity of the seed produced. In some cases, brassica weeds also present disease challenges by maintaining "green bridges" of host plants year-round for survival and spread of plant pathogens spatially and temporally between cropping planting seasons.

1.1.2 Brassica production in the Pacific Northwest USA

1.1.2.1 Fresh market and processing crops. Brassicas grown as vegetables primarily belong to *B. oleracea* (Rakow 2004; Rimmer et al. 2007). In the Pacific Northwest (PNW) USA, *B. oleracea* crop types grown for both seed and vegetable crops include broccoli, Brussels sprouts, cabbage, cauliflower, Chinese kale, kale, kohlrabi, and sprouting broccoli (Inglis et al. 2013; USDA NASS

n.d.). The 2012 USDA NASS data estimated there were 606 ha of *B. oleracea* vegetable crops in Washington State that year. Other *Brassica* species grown for vegetable crop production in Washington include *B. rapa* and *B. juncea,* such as leaf mustard, leaf cabbage, and Chinese cabbage crops (Rakow 2004). In some Southeast Asian countries, Chinese kale (*B. alboglabra*) is ranked in the 10 most important market garden vegetables (Rakow 2004). The USDA NASS estimated over 40 ha of turnip and Chinese cabbage (*B. rapa),* and over 60 ha of leaf mustards (*B. juncea*) were planted for market and processing vegetable crops in the state of Washington in 2012.

1.1.2.2 Cover crops. The popularity of brassicas, and particularly the mustard types, for use as cover crops has increased because of the ability to use these crops to reduce soil erosion, suppress weeds and soilborne pests such as the Columbia root-knot nematode (*Meloidogyne chitwoodi*), reduce soil compaction, and scavenge for soil nutrients (Clark 2012). In 2015, approximately 39,500 ha of radish, 14,700 ha of turnip, 23,200 ha of rapeseed, and 3,900 ha of canola were planted as cover crops in the USA, according to a nation-wide survey, that also revealed 983 of 2,020 growers planted brassica cover crops that year (Watts 2016). According to the survey, 909 growers expected to plant more ground to brassicas in 2016, estimating 49,000 ha of radish, 22,600 ha of turnip, 24,800 ha of rapeseed, and 4,700 ha of canola. In 2012, there was an estimated 11,800 ha planted into mustard green manure crops in Washington State (McGuire 2012). Cultivars of both white mustard, *Sinapis alba,* and brown mustard, *B. juncea,* are planted as mustard cover crops in eastern and western Washington (McGuire 2012). Mustard cover crops, in particular, are popular because the glucosinolate compounds produced by these species convert to biofumigant isothiocyanates (ITCs) when the crop is mowed and incorporated into the soil (Clark 2012; Pekarek et al. n.d.). Synthetic ITCs (e.g., metam sodium) are used as a commercial means of control of weeds, nematodes, and fungal pathogens, while brassica cover crops provide a natural form of ITCs used to fumigate soil (Pekarek et al. n.d.).

1.1.2.3 Seed crops. A Mediterranean climate and abundant rivers have made the coastal PNW USA an important brassica seed crop growing region (Inglis et al, 2013). The Skagit Valley in northwestern Washington was the earliest area of vegetable seed production on the west coast of the USA, starting with cabbage seed crops in 1885 by Alvinza Gardner Tillinghast in La Conner, WA (Rackham 2002). This region, well-suited climatically for overwintering biennial cabbage seed production, produces up to 50% of the U.S. supply and up to 25% of the world cabbage seed supply annually (Inglis et al. 2013; Schreiber and Ritchie 1995). Brassica seed is a high value crop that can gross $1,500 to >$6,500 per 0.4 ha (Inglis et al. 2013). Brassica seed production is also important in the Columbia Basin of central Washington. In 2008, vegetable brassica seed production in this semi-arid region included 18 ha of collard, 45 ha of kale, 8 ha of kohlrabi, and 46 ha of turnip (Washington State University (WSU) Grant-Adams County Extension 2008). In the same year, seed production for oilseed plantings of canola and mustard were 569 and 51 ha, respectively (WSU Grant-Adams County Extension 2008).

1.1.2.4 Oilseed and biofuel crops. There are several *Brassica* species grown for oilseed in the PNW USA. High erucic acid rapeseed was used historically as a steam engine lubricant and for lamp oil (Rakow 2004). Today, there is renewed interested in using high erucic oilseed as a biodiesel (Rakow 2004). Culinary and condiment mustards are also grouped in the oilseed category. White mustard, *S. alba*, is widely grown for this purpose and accounts for 90% of condiment mustard production in North America (Rimmer et al. 2007).

B. napus includes oilseed rape and canola-quality rapeseed with zero erucic acid and low glucosinolate concentrations (Rakow 2004; Rimmer et al. 2007). Oilseed mustard (*B. juncea*), grown widely in India, has high erucic acid and high glucosinolate content and is suited for semi-arid climates (Rakow 2004; Rimmer et al. 2007). The name canola, Canadian oil low in acid, can be given to any oilseed *Brassica* sp. “in which the processed oil contains less than 2% erucic acid and the residual meal

contains less than 3 mg of glucosinolates per gram" (Rimmer et al. 2007). Cultivars of oilseed mustard grown in Canada were bred for zero erucic acid and low glucosinolate (Rakow 2004). *B. rapa*, or oilseed turnip rape, is grown less often for oilseed then *B. juncea,* but is favored in regions with short growing seasons. In 2012, there were 702,700 ha of canola grown in the USA, of which 5,900 ha were in Washington. That same year, growers in the neighboring states of Idaho and Oregon produced 14,800 ha and 2,500 ha, respectively (USDA NASS). PNW climatic conditions enable farmers to grow winter annual canola crops that are more productive than the summer annual canola crops normally grown in Canada (Rakow 2004).

1.1.3 Brassica diseases

Plants within Brassicaceae are important agricultural crops around the world. Bred for centuries to create diversity in the form, purpose, and agronomic qualities seen today, the interrelatedness of species and subspecies in this genus readily facilitates the movement of desirable traits among *Brassica* species (Rimmer et al. 2007). However, it is precisely because of the close, genetic relatedness among Brassicaceae cultivars that many are susceptible to the same diseases and pests (Rimmer et al. 2007). Significant increases in plantings of *Brassica* species as oilseed and vegetable crops around the world have led to new disease pressures, some of which previously were not important (Rimmer et al. 2007). For example, in a survey of winter oilseed rape growers in the United Kingdom (UK), Denmark, Sweden, France, and Germany, black leg (caused by *Phoma lingam)* and white mold (caused by *Sclerotinia sclerotiorum)* were damaging in all countries of production; light leaf spot (caused by *Cylindrosporium concentricum,* teleomorph *Pyrenopeziza brassicae*), was often damaging in the UK but only occasionally or locally damaging in the other countries surveyed (Garbe et al. 2000). Black leg, white mold, and light leaf spot are most damaging to winter oilseed rape production in the European Union (EU), followed by Verticillium wilt (*Verticillium dahlia*e), Alternaria leaf spot (*Alternaria brassicae* and *A. brassicicola)*,

downy mildew (*Hyalaperonospora parasitica* subsp. *brassicae*), gray mold (*Botrytis cinerea*), clubroot (*Plasmodiophora brassicae*), powdery mildew (*Erysiphe cruciferarum*), and white leaf spot (anamorph *Neopseudocercosporella capsellae,* synonym *Pseudocercosporella capsellae*, teleomorph *Mycosphaerella brassicae)*.

1.1.3.1. Brassica diseases in the Pacific Northwest USA. Growing disease-free Brassica crops in the PNW can be challenging with the diversity of cultivars grown, year-round cropping of brassicas, and weedy and volunteer brassica plants (Inglis et al. 2013; Pscheidt and Ocamb 2016a). An estimated 38 genera of Brassicaceae grow wild in Washington State (Brako et al. 1997; Inglis et al. 2013). Historically, significant brassica disease outbreaks in this region have been well-documented. Throughout the 1930s and 40s, growers in the Skagit Valley considered black leg and white mold to be the most significant diseases of cabbage (Rackham 2002). In the 1970s, the western Washington brassica seed industry was devastated by black leg outbreaks in the Midwest and northeast USA that were traced back to PNW brassica seed crops (Jenkins 2015). Neergaard (1977) estimated that in 1973 black leg and black rot (caused by the bacterial pathogen *Xanthomonas campestris* pv. *campestris*) caused estimated yield losses of 10% in the USA, valued at 20 million dollars. From the 1970s until 2011, following intensive research and implementation of strict black leg management practices by the PNW brassica seed industry and growers, the PNW was considered free of black leg (Inglis et al. 2013; Paulitz et al. 2017). Black leg was detected in northern Idaho in 2011, in eastern Washington in 2015 (Paulitz et al. 2017), and in west-central Idaho and north-central Oregon in 2015 (du Toit, *personal communication*; Paulitz et al. 2017); in addition, there was a widespread outbreak of black leg in the Willamette Valley of western Oregon in 2014 (Ocamb 2016).

There are a few economically important diseases that do not commonly impact Brassica seed crops in western Washington State, including white rust, caused by *Albugo candida* (Pscheidt and

Ocamb 2016a); and black rot, a bacterial disease caused by *Xanthomonas campestris* pv. *campestris* that is rarely found in Washington State. Williams (1980) considered black rot to be the most important vegetable brassica disease worldwide. *X. campestris* pv. *campestris* can affect all cultivated brassicas, radishes, and many Brassicaceae weeds, and can be particularly damaging for brassica seed crops because external symptoms might not always be detected when the plant is infected systemically (Williams 2007). One of the reasons brassica seed grown in the PNW is important for global brassica production is that the PNW has the reputation of being a location in which *X. campestris* pv. *campestris* rarely occurs because the relatively cool summer climate of western Oregon and western Washington is unfavorable for the bacterium (Williams 1980). In order to keep black leg and black rot out of Washington State as much as possible, Washington State Department of Agriculture (WSDA) implemented a quarantine ruling in 2006, WAC 16-301-490-580, at the request of the brassica vegetable seed growers and seed companies for protecting six counties in northwestern Washington that are the primary region for biennial brassica vegetable seed crops in that state. The black leg part of the regulation was extended in 2015 from six counties in western Washington to all counties east of the Cascade Mountains (Washington State Department of Agriculture 2015). The WSDA rule requires that any brassica seed planted in the protected area must be certified as *P. lingam*-free and be tagged for planting as having met this condition.

Potential seedborne pathogens of brassica crops are not only a concern to brassica seed producers, but also to brassica crop producers who want to ensure they purchase pathogen-free seed. Neergaard (1977) reported nine fungal pathogens that were commonly seedborne on brassicas: *A. brassicae, A. brassicicola, A. raphani, H. parasitica, M. brassicicola, P. lingam, Plasmodiophora brassicae, R. solani,* and *S. sclerotiorum* (Neergaard 1977). The most common seed transmitted pathogens on brassica crops include *A. brassicae, A. brassicicola, B. cinerea, P. lingam, S. sclerotiorum,* and *X.*

campestris pv. *campestris* (Cappelli et al. 1999; Richardson 1990). Internationally accepted seed health assays have been established by the International Seed Health Initiative (ISHI) of the International Seed Federation (ISF) for *P. lingam* and *X. campestris* pv. *campestris,* indicating that these pathogens are the most significant and potentially damaging to international seed trade of the known seedborne pathogens of brassicas (International Seed Federation 2017).

In total, Rimmer et al. (2007) cited 19 diseases of brassicas caused by fungi and oomycetes, 3 bacterial diseases, 11 diseases caused by viruses and mollicutes, and 3 plant parasitic nematodes. This literature review summarizes the foliar, fungal pathogens of brassicas in relation to two newly emerging foliar fungal diseases of brassicas in the PNW USA: light leaf spot and white leaf spot. Foliar fungal pathogens of brassicas occurring in western Washington include black spot, gray leaf spot, and pod spot *(Alternaria brassicae, A. brassicicola*, and *A. japonica,* respectively*);* black leg (*P. lingam*); Botrytis gray mold; downy mildew; white mold; ring spot (*M. brassicicola)*; and powdery mildew (Pscheidt and Ocamb 2016a; Rimmer et al. 2007).

Four species of the necrotrophic fungus *Alternaria* are pathogenic on Brassicas (Dixelius et al. 2004). *A. brassicicola* and *A. brassicae* are the most important and damaging of the four *Alternaria* species. These facultative parasites are nearly ubiquitous throughout brassica growing regions (Dixelius et al. 2004; Rimmer et al. 2007). Though all brassicas are susceptible to some degree to these *Alternaria* pathogens, *A. brassicicola* is more often associated with, and can be found on the seeds of, *B. oleracea* crops in warmer climates (Humpherson-Jones and Maude 1982; Kubota et al. 2006). *A. brassicae* is usually associated with, and can be found on, the seed of *B. napus* and *B. rapa,* as well as other species in cooler climates. Severe epidemics have affected oilseed crops in Canada, France, Germany, India, Poland, and the UK (Rimmer et al. 2007). In western Washington in 1978, 85 fields of Brussels sprouts

seed crops and cabbage seed crops were surveyed, of which 67 fields were infected with *A. brassicicola* while only 6 were infected with *A. brassicae* (Babadoost and Gabrielson 1979).

Black leg (*Leptosphaeria maculans* and *L. biglobosa,* anamorph *P. lingam*) is largely considered the most important pathogen of horticultural and oilseed Brassica crops (Tewari and Mithen 1999). The black leg fungi have a wide host range within Brassicaceae, affecting many crops and weeds, with crop failures reported in Australia, Canada, New Zealand, and the USA (Rimmer and van den Berg 2007). Sometimes confused with black leg, ring spot (*M. brassicicola)* is a well-established, but also a well-managed disease in brassica seed crops in the PNW (L. J. du Toit, *personal communication*; Inglis et al. 2013). Ring spot affects most crops in Brassicaceae, especially subspecies within *B. oleracea,* and is favored by a cool, moist environment (Rimmer et al. 2007).

B. cinerea, the cause of gray mold, is a facultative parasite with a wide host range of more than 250 plant species (Buchwaldt and Rimmer 2007). The fungus can cause a soft, brown rot of many *B. oleracea* crops both in the field and in storage; though economically, the post-harvest infection has more impact. Gray mold has been reported as a problem in Europe and the USA, and occasionally causes cankers of cabbage seed stalks in seed crops in western Washington following late spring frosts (Inglis et al. 2001). Another fungal generalist, *S. sclerotiorum,* the cause of white mold, affects many crops within Brassicaceae as well as species in numerous other plant families. The fungus affects oilseed brassicas, storability of some brassica crops, infects seed lots, and can reduce yield. Economic losses vary depending on the host plant, pathogen strain, and environment, but can reach as much as 50% in brassica head, root, or seed yields (Buchwaldt 2007).

Downy mildew, caused by *H. parasitica,* occurs anywhere brassicas are grown and affects many *Brassica* spp. (Nashaat 2007). Downy mildew can damage the market value of brassica crops but does not usually kill host plants unless infection occurs when the plant is very young. Hartill (1982) found that

seedling loss to downy mildew could be severe during times of peak production when warm rains occur in early fall. In full-grown plants, a recent assessment of yield losses caused by downy mildew on *B. juncea* found that plants were stunted, and oil content and 1000-seed weight were reduced (Meena et al. 2014). In seed crops, downy mildew on inflorescences can cause deformities called stagheads, and can infect the seed (Neergaard 1977; Pscheidt and Ocamb 2016a). Like downy mildew, powdery mildew, caused by *E. cruciferarum*, exists worldwide, but rarely causes the death of brassica plants (Shattuck 2007). Kaur et al. (2008) described powdery mildew on canola crops (*B. juncea*) in Australia as white patches that coalesced to form a white, dense, and powdery layer over infected stems and leaves. Penaud (1998) regularly observed *E. cruciferarum* on winter oilseed rape in southern France, and estimated crops losses at 0.5 T / ha.

During the time that black leg was reported in a widespread 2014 outbreak in the Willamette Valley of Oregon, two new foliar fungal diseases were detected: light leaf spot and white leaf spot (Ocamb 2014a; Ocamb 2014b). Light leaf spot had never been reported before in North America. White leaf spot had been reported sporadically in Oregon, California, and the southeastern USA (Commonwealth Mycological Institute 1986). Because these pathogens are new, or infrequent, to this region, the impacts of these diseases were not well understood in the different brassica cropping regions and production systems in the PNW USA.

1.2. Light Leaf Spot [*Cylindrosporium concentricum* (Anamorph), *Pyrenopeziza brassicae* (Teleomorph)]

1.2.1 Taxonomy. *Pyrenopeziza brassicae* is an ascomycete in the subphylum Leotiomycetes, class Leotiomycetidae, order Helotiales, and family Dermateaceae because of the dark apothecia that develop from brown, stromatic to pseudostromatic tissue (Rawlinson et al. 1978; Rakow 2004; Species Fungorum n.d.). The fungus causes light leaf spot, and is known by the anamorph *Cylindrosporium*

concentricum, which was first described in 1823 in Scotland (Greville 1823). The pathogen was briefly renamed *Gloeosporium concentricum* (Berkeley and Broome 1850), causing much confusion over the years (Rawlinson et al. 1978). The apothecial state was reported in culture in 1955 (Hickman et al. 1955), and in the field in 1965 (Kavanagh et al. 1965). The teleomorph, *P. brassicae,* was first described in 1978 (Rawlinson et al. 1978). The one name, one fungus for this organism is *P. brassicae* (P. Crous, Director of the Westerdijk Fungal Biodiversity Institute, formerly the Centraalbureau voor Schimmelcultures, in Utrecht, The Netherlands, *personal communication*).

1.2.2 Morphology and physiology. *C. concentricum* is a coelomycete that produces white, subcuticular acervuli that typically form in concentric circles (Rawlinson et al. 1978). Acervuli are each composed of pale brown to hyaline pseudoparenchyma and are approximately 100 to 200 µm in diameter (Sutton 1977). Within the acervuli, hyaline, smooth, cylindrical, aseptate conidia are produced on short (10 to 16 x 2.5 to 4 µm) conidiophores with enteroblastic/phialidic, hyaline, and smooth conidiogenous cells that have slightly tapered apices (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977). Conidia are occasionally uni-septate and usually eguttulate. The smooth, hyaline conidiophores are formed in parallel and they only branch at the base (Rawlinson et al. 1978).

The superficial apothecia of *P. brassicae* are approximately 1 mm in diameter, with or without a stipe, appearing singly or grouped, with two to three sharing a base (Cheah et al. 1980; Rawlinson et al. 1978). The black or dark gray, pruinose, cup-shaped apothecia are formed on decaying stems and leaf petioles (Lacey et al. 1987). Lacey et al. (1987) also described seeing smaller apothecia as the season progressed, without the dark outer excipulum, but with similar asci and ascospores. Asci each contain eight ascospores and are approximately the same size as the hyaline, septate paraphyses, measuring 33 to 100 µm x 4.2 to 9.5 µm (Cheah et al. 1980; Lacey et al. 1987; Rawlinson et al. 1978). Asci are clavate, unitunicate, gradually tapering at the base, and thin-walled except at the conical apex. Ascospores are

hyaline, straight or slightly curved, with rounded ends, slightly tapered to the base, sometimes uni-septate, and 8 to 15 µm long x 2 to 3 µm wide (Cheah et al. 1980; Gilles et al. 2001b; McCartney and Lacey 1990; Rawlinson et al. 1978). Ascospores are ejected forcibly (Lacey et al. 1987). In 1966, Staunton and Kavanagh (1966) first identified apothecia in a field near an infected crop of Brussels sprouts in Ireland. In the UK, Inman et al. (1992) putatively identified apothecia of the saprophyte *Unguicularia* cfr. *raripila* on oilseed rape debris, and noted that the size and shape of ascospores of *U.* cfr. *raripila* were very similar to those of *P. brassicae.* Inman et al. (1992) stated that this likely contributed to conflated perceptions of the importance of ascospores contributing to spring-time epidemics of light leaf spot in canola crops in the UK.

Distinguishing between conidia of *C. concentricum* and ascospores of *P. brassicae* by size and morphology is very difficult (Gilles et al. 2001b; Lacey et al. 1987). The season in which ascospores and conidia are observed is important to distinguish the spore types (Cheah et al. 1980). Rawlinson et al. (1978) also noted variation in ascospore size from isolates of *P. brassicae* collected in different locations throughout the UK. *P. brassicae* is heterothallic, i.e., there are two mating types (Ilott et al. 1984), and the fungus has a high level of heterogeneity, which suggests a high level of sexual reproduction (Majer et al. 1998). A sexually reproducing population has more genetic flexibility to change or evolve in response to selection pressures (McDonald 2004). As of 2001, the once rarely found sexual phase of *P. brassicae* is now considered common in the UK (Sutherland 2001). The sexually reproducing and rapidly evolving population of *P. brassicae* in the U.K. has led to the development of fungicide resistance affecting the ability to manage light leaf spot (Carter 2013; F. Ritchie, ADAS Wolverhampton, England, *personal communication* 2017).

1.2.3 History, geographic distribution, and impact. Light leaf spot is documented in Australia, Denmark, France, Germany, Ireland, Japan, Latvia, Netherlands, New Zealand, Norway, Philippines,

Portugal, Romania, Tasmania, and the UK (Hickman et al. 1955; Karolewski 2010; Rawlinson et al. 1978; Sutton 1977). Though *C. concentricum* was first described in the UK in the 19th century (Greville 1823), the teleomorph was not documented until 1978 (Rawlinson et al. 1978). *C. concentricum* was first reported on cauliflower in New Zealand in 1944 (Cunningham 1944). The first record of the teleomorph, *P. brassicae,* naturally occurring in New Zealand was in 1980 (Cheah et al. 1980). In the USA, light leaf spot was first observed and documented in the Willamette Valley of Oregon in March 2014, on canola, forage brassicas, vegetable brassicas grown for produce and for seed, wild mustard, various brassica cover crops, and volunteer black mustard (Ocamb 2014a).

Though *C. concentricum* and *P. brassicae* were well documented during the 20th century, yield losses caused by light leaf spot have increased in the last 40 to 50 years in the UK and EU. Before 1974, light leaf spot was seen as a cosmetic disease causing discoloration of brassica vegetables and resulting in reduced market value (Staunton 1967; Sutton 1977; Cheah et al. 1980). However, in the winter of 1974 to 1975, an estimated 90% of winter oilseed rape crops were lost to this disease in southern England (Rawlinson et al. 1978). In Poland, severe light leaf spot epidemics were recorded after the mild, wet winters of 1992, 1999, and 2002 (Karolewski 1999). Ireland saw the first economic losses from light leaf spot in 1964, when an outbreak caused 60% loss in a 30 ha field of Brussels sprouts (Staunton 1967). Sutherland (2001) found that a potential yield loss of 0.14 tons / ha could be expected for each 10% incidence of infection at the green bud state in winter oilseed rape crops. Historical trends tracked by scientists at Rothamsted Research in the UK showed that from 2012 to 2015, light leaf spot surpassed black leg as the number one cause of yield losses of winter oilseed rape, with >£140 million lost in 2014 (Historical trends - light leaf spot n.d.). In addition to the >£10 million spent annually on fungicides in the UK to control light leaf spot, scientists at Rothamsted Research estimated a cost of £30 to £160 million

annually in the UK caused by this disease. The potential impacts of the very recent appearance of *P. brassicae* in the maritime PNW on multiple types of brassica crops are not yet understood.

1.2.4 Host range. Light leaf spot can occur on many brassica crops such as broccoli, Brussel sprouts, cabbage, cauliflower, kale, mustard, oilseed rape, purple sprouting broccoli, swede, and turnip (Cheah et al. 1980; Hickman et al. 1955; Rawlinson et al. 1978). Karolewski (2010) tested 28 cultivars of various brassica species for susceptibility to light leaf spot and found all 22 cultivars of 4 species developed symptoms: *B. juncea, B. oleracea, B. napus, B. rapa*; the 6 cultivars tested of *R. sativus* did not develop symptoms. In 2015, the host range of *C. concentricum* was studied in the Willamette Valley of Oregon, with isolates collected from infected bird's rape mustard (*B. rapa* weedy species), black mustard (*B. juncea*), canola, *Cherianthus*, mizuna, mustard, radish, forage turnip, various cover crops, green cabbage, Chinese cabbage, turnip, wild radish, and a *Sinapis* sp. (Claassen et al. 2014). In contrast, Cheah and Hartill (1985) found that none of the following brassica weeds acted as alternative hosts to *C. concentricum*: *Barbarea intermedia*, *B. juncea*, *Capsella bursa-pastoris*, *Cardamine hirsute*, *Coronopus didymus*, *Coronopus squamatus*, *Nasturtium officinale, Raphanus maritimus*, *Sisymbrium officinale*, and *Sisymbrium orientale*. Researchers from the UK have found anecdotal support for the existence of strains of *P. brassicae* that differ in pathogenicity on different crops within Brassicaceae (F. Ritchie, ADAS Wolverhampton, England, *personal communication*).

1.2.5 Symptoms. Light leaf spot can affect all aerial parts of brassica plants, including leaves, flower buds, stems, and pods; with symptoms most commonly occurring on older leaves, leaf veins, and on both sides of the leaves (Hickman et al. 1955; Sutherland 2001). The symptoms on the veins include browning, hardening, and splitting longitudinally (Hickman et al. 1955). Reports from the EU have described foliar symptoms that may include white spots, each with a diameter <1 mm, arranged in concentric circles (Hickman et al. 1955); however, these symptoms have not been observed on plants

inoculated with USA isolates (Ocamb 2016). The concentric circles of acervuli expand and coalesce into lesions ranging from 1 to 7 cm in diameter (Sutton 1977). Mature lesions turn fawn or yellow colored in the center.

Stem lesions start as small, black speckling, each <1 mm in length, that increase in size as the plant grows, especially if stem lesions start before stem elongation (Gilles et al. 2001b). Mature stem lesions vary in length but may appear brownish purple with a bleached or light appearance in the center of each lesion (Gilles et al. 2001b). Symptoms may vary depending on the host (Karolewski 2010). Infected cauliflower inflorescences become discolored tan or brown (Cheah et al. 1980). In oilseed rape crops, infection usually appears as white pustules (acervuli) visible on the leaves, pods, and stems before any necrotic lesions are visible (Gilles et al. 2001a). Symptomatic rape leaves may appear healthy following rains or overhead irrigation, when the white pustules (acervuli) are harder to detect.

1.2.6 Disease cycle

1.2.6.1. Dispersal. The splash dispersal of conidia of *C. concentricum* and wind-dispersal of *P. brassicae* ascospores have been well-documented (Cheah et al. 1982; Fatemi and Fitt 1983; Rawlinson et al. 1978). Polycyclic light leaf spot epidemics in the UK start in autumn, with airborne ascospores acting as the primary inoculum released from apothecia located on infested crop debris and leaf litter (Cheah and Hartill 1985; Gilles et al. 2001b; Karolewski 2010). Secondary inoculum is in the form of conidia released from acervuli after a rain or irrigation event (Cheah and Hartill 1985). However, conidia can be an important source of both initial and secondary infections in agricultural production systems with short intervals between brassica crops, the presence of infected oilseed rape volunteer plants, and/or abundant infected brassica weeds (Maddock and Ingram 1981; Karolewski 2010; Sutherland 2001). Ascospores are more infective than conidia, in spite of similar morphology (Gilles et al. 2001b). During winter or early spring in the UK, if both ascospores and conidia are present, the

distribution of light leaf spot in a field will change from an initial random pattern associated with aerially dispersed ascospores to an aggregated pattern associated with localized splash dispersal of conidia (Gilles et al. 2001b). In the UK, the release of ascospores from infested leaf debris and splash dispersal of conidia from acervuli in late spring and early summer contribute to stem and pod infections (Gilles et al. 2001b). Based on when new field infections occurred, Maddock and Ingram (1981) suspected that *P. brassicae* was dispersed on infected seed or infested debris based on the patchy distribution of individual infected plants in fields.

If the teleomorph, *P. brassicae*, has not developed in a region of brassica crop production, primary infection usually is caused by splash dispersal from the mucilaginous masses of conidia produced in the acervuli (Maddock and Ingram 1981). In wet weather, spore masses develop in acervuli on both sides of the leaf, and are then splash-dispersed (Sutherland 2001; Sutton 1977). Rawlinson et al. (1978) found that conidia also were dispersed by water running off the leaves and by large splash droplets. They suggested it was unlikely that air-borne conidia, aerosolized conidial suspensions, or conidia in small water droplets dispersed between fields.

1.2.6.2 Host plant infection. Cool, wet weather is important for the establishment of light leaf spot, with conditions usually most favorable during the overwintering of brassica crops in regions like the UK and New Zealand (Hartill and Cheah 1984; Staunton 1967). For example, in 1974, *P. brassicae* caused significant damage in rapeseed crops during an exceptionally wet, mild winter in the UK (Rawlinson et al. 1978). During this time, scientists at Rothamsted Research recorded double the amount of average rainfall and demonstrated that water on brassica leaf surfaces in particular, non-waxy, wettable leaf surfaces, provided the ideal conditions for infection. Rawlinson et al. (1978) found that spore retention on leaves, leaf penetration by the fungus, and the emergence of conidiomata (acervuli) through the cuticle were all more likely on less waxy brassica leaves with thin cuticles.

Similarly, the older, outermost leaves were most susceptible to infection in a separate study in New Zealand on summer-grown oilseed rape and brassica vegetable crops (Cheah and Hartill 1985).

Numerous studies have found that 16℃ is the optimal temperature for conidial germination of *C. concentricum*, while 15 to 20℃ is the optimal temperature range for mycelial growth (Hartill and Cheah 1984; Staunton 1967; Rawlinson et al. 1978; Sutherland 2001). A decrease in rate of infection was observed >18℃, while no symptoms developed <10℃ (Hartill and Cheah 1984). Recent studies have established that light leaf spot symptoms can develop at temperatures ranging from 4 to 24℃, and are most favored at 16℃ and 80% relative humidity for 24 to 48 h (Gilles et al. 2001a; Karolewski et al. 2002). The average incubation period required for light leaf spot symptoms is three weeks (Maddock and Ingram 1981). In summary, cool and wet conditions are ideal for *P. brassicae* to infect host tissues.

1.2.6.3 Survival and seed transmission. Though the light leaf spot pathogen thrives in wet and cool conditions, inoculum can survive periods of heat, desiccation, and freezing (Cheah and Hartill 1985). Naturally infected leaves remained a viable source of inoculum for at least three months when leaves were desiccated and stored at -20°C (Rawlinson et al. 1978). Persistence of the fungus on infected plant debris in fields appears to be an effective method of survival (Cheah et al. 1980). Maddock and Ingram (1981) found conidia from infected leaf debris of oilseed rape crops to be infective for at least 10 months, and Cheah et al. (1980) were able to get conidia to germinate 19 months after desiccation. Apothecia of *P. brassicae* on infested debris typically can survive summer conditions in New Zealand (Cheah and Hartill 1985). Maddock and Ingram (1981) noted that infested field debris facilitates survival of *P. brassicae*. When colonized crop residues were incorporated into the soil, apothecia decomposed (Cheah and Hartill 1985). However, when residues were left on the soil surface, apothecia remained viable and resisted desiccation for up to 8 weeks (Cheah and Hartill 1985). Numerous studies have found that brassica weeds are not usually significant sources of inoculum for light leaf spot

outbreaks in New Zealand, the UK, and Ireland (Cheah and Hartill 1985; Staunton 1967; Maddock and Ingram 1981). However, cultivated, escaped, or volunteer brassicas infected with *P. brassicae* have the potential to serve as inoculum sources year-round, especially where brassicas can be grown every month of the year, winter oilseed rape crops are in the ground for 10 to 11 months, and summer brassica crops bridge the gap between winter brassica crops (Maddock and Ingram 1981).

Because of the introduction of light leaf spot into different regions globally, as well as within the UK, Hickman et al. (1955) suggested the potential for seedborne infection and seed transmission of *P. brassicae*. The localized distribution of light leaf spot symptoms in some fields suggests introduction of the pathogen as seedborne or debris-borne primary inoculum (Rawlinson et al. 1978). In Scotland, the incidence of light leaf spot in fields not previously planted to brassica crops indicated that seedborne transmission might be more important than previously thought (Sutherland 2001). Staunton (1967) found that seeds produced on Brussels sprouts plants inoculated with *P. brassicae* did not develop light leaf spot on the seedlings grown from that seed. Staunton surface-sterilized and plated the seed onto plates of potato dextrose agar (PDA) at 15°C, but did not observe *P. brassicae* on the seed. Staunton (1967) was only able to demonstrate seed transmission of *P. brassicae* after dipping germinated Brussels sprouts seeds into a spore suspension of *P. brassicae* for 2 minutes and then planting the seeds, which resulted in a 68% seed transmission rate in a greenhouse. When Staunton placed the inoculated seed into a vacuum chamber, he observed 71% seed transmission. These seed inoculation tests were not meant to represent seed transmission in field settings but were used to screen breeding lines or cultivars for resistance to *P. brassicae* (Staunton 1967).

Maddock and Ingram (1981) conducted a series of studies building on Staunton's work. They investigated *P. brassica*e survival on seed harvested from severely infected pods of the oilseed rape cv. Rapora, and stubble turnip cv. Appin. They tested the seed by plating surface-sterilized seed onto PDA

agar or 3% malt agar, and incubated the seed in the dark at 20°C (ideal for the growth of *P. brassicae*) or at 10°C to inhibit the growth of saprophytic, fast growing fungi. Using the same seed lots, they washed the seed to look for germinating spores, and completed a seed transmission assay but using only 40 seed of 'Rapora' oilseed rape. Approximately 1,000 non-infected oilseed rape seed were inoculated using Staunton's methods, with and without vacuum treatment, and then planted in pots in a greenhouse. *P. brassicae* was detected on 3 of 30 seeds of 'Rapora,' but light leaf spot did not develop on the seedlings of either the naturally infected 'Rapora' or the inoculated oilseed rape seed. Maddock and Ingram (1981) concluded that the seedborne phase of *P. brassicae* was more likely to play a role in introducing the fungus into new areas than at increasing inoculum levels significantly in areas with established infections.

Like Staunton, Maddock and Ingram (1981) found that when the testa was damaged or split before inoculating seed, transmission of *P. brassicae* was more likely to occur. They demonstrated that an intact testa protected the germinating seedling from infection by *P. brassicae*, but not if the embryo was already infected. Rawlinson et al. (1978) noted that hyphae of *P. brassicae* could penetrate immature seed, as well as the funicula, placentae, and repla of the pods of winter oilseed rape, but not the testa or embryos. However, this work was done mostly with inoculated seed not naturally infected seed.

Cheah and Hartill (1985) found no evidence of seed transmission when they planted 200 cauliflower seed harvested from siliques naturally infected with *P. brassicae* in a greenhouse. However, they observed *P. brassicae* on seeds surface-sterilized and plated onto PDA after harvest from infected cauliflower. They concluded that *P. brassicae* was unlikely to infect seedlings even when present on seed, and hypothesized that the high concentrations of ITCs in the leaves of some *B. oleracea* cultivar seedlings might protect the young plants from *P. brassicae,* even though cauliflower has very little ITCs.

In the UK, there are distinct geographic subpopulations of *P. brassicae,* suggesting that most disease outbreaks result not from introductions of new, virulent isolates, but from the local movement of existing populations (Majer et al. 1998). Maddock and Ingram (1981) and Cheah and Hartill (1985) showed that, although seed infection by *P. brassicae* can occur, the role of seedborne inoculum in pathogen transmission remains uncertain. However, a seed infection rate of 0.08% was demonstrated to establish light leaf spot in a field of oilseed rape in the UK, so even a low incidence of seed infection could be significant (Maddock and Ingram 1981).

1.2.7 Management

1.2.7.1 Cultural control. Cultural practices, such as crop rotation and incorporating infested crop residues, are important for managing light leaf spot effectively (Sutton 1977). More importantly, cultural control practices are the most effective methods currently available for controlling this disease in organic brassica farming systems (Ocamb 2016). Inoculum reduction can be accomplished by removing infested debris, roguing infected brassica weeds and infected brassica volunteers, and by deep plowing or flailing and incorporating infested residues and infected plants (McCartney and Doughty 2007). Since the recognition in 2014 that black leg, light leaf spot, and white leaf spot are widespread across the Willamette Valley, the Oregon Department of Agriculture (ODA) developed specific guidelines for black leg, white leaf spot, and light leaf spot management using cultural control practices. These practices include planting only seed tested to be free of *P. lingam* and treated with fungicides or hot water; rotating out of crucifers for a minimum of 3 years, with 5-year rotations recommended for brassica transplant production for seed crops; and intensive flailing and burial of crop residues with high speed tillage disks promptly following harvest (Ocamb 2016). Although there currently is no certified or standardized test to detect *P. brassicae* on seed lots, growers can purchase seed grown in regions where light leaf spot is not known to occur. Hot water seed treatments are effective at eliminating some fungal

and bacterial pathogens from brassica seed (Neergaard 1977), but research is needed on the seedborne aspects of *P. brassicae* to assess what seed treatments might be effective at eradicating the pathogen on the seed of different types of brassicas. Little work has been done to show whether or not fungicides, disinfectants, or physical seed treatments are effective for control of seedborne inoculum of the light leaf spot fungus because of the uncertainty regarding the importance of seedborne inoculum, as well as the lack of seed health assays to detect *P. brassicae* on infected seed. Rawlinson et al. (1978) wrote that breeding for resistance to light leaf spot seems possible as resistance appears to exist in some cultivars. However, research is needed to improve the understanding of phenotypic reactions of cultivars of different types of brassicas, and resistance mechanisms to *P. brassicae* in order to breed effectively for resistant cultivars (Boys et al. 2007).

Light leaf spot pressure increases when brassica crops are grown in close proximity in time and space to previously infected fields (McCartney and Doughty 2007; Rawlinson et al. 1978). Increasing row spacing or plant spacing within rows, and orienting rows into the predominant wind direction are other practices that can be used to manage light leaf spot. Greater spacing between and within rows increases air movement within the canopy, reducing relative humidity and durations of leaf wetness to be less favorable for diseases favored by wet conditions (du Toit 2014). For high value crops such as brassica seed crops, these practices are even more important, particularly for organic production (Organic Seed Alliance 2007). However, there is less flexibility to increase row spacing for brassica cover crops planted at 11.25 to 17 kg / ha, as dense plantings are required to achieve the benefits of the planting (Pekarek et al. n.d.; Clark 2012).

1.2.7.2. Chemical control. As previously discussed, early infections of plants by *P. brassicae* can be symptomless (Gilles et al. 2000). This makes the timing of fungicide applications important but difficult for effective management of light leaf spot. Fungicides should be applied before stem extension

in brassica seed crops to prevent *P. brassicae* infection moving into the upper canopy after stem extension (Rawlinson et al. 1978). In winter oilseed rape crops in the UK, it is recommended that fall fungicide applications be made before symptoms are observed in order to manage light leaf spot effectively (Sutherland 2001). Polymerase chain reaction (PCR)-based assays for pre-symptomatic diagnosis of light leaf spot have been developed to help detect early fall infections (Foster et al. 2002). Benzimidazole fungicides (Fungicide Resistance Action Committee (FRAC) Group 1) and triazole (ergosterol-inhibiting) fungicides (FRAC group 3) have been demonstrated to be highly effective for light leaf spot (McCartney and Doughty 2007). In New Zealand, Cheah et al. (1981) tested the efficacy of 23 fungicides for controlling light leaf spot in cauliflower, and found benomyl + oil, prochloraz, and fenapanil best controlled the disease. In Scotland, fungicides used against this disease include triazoles such as cyproconazole, difenoconazole, flusilazole, prochloraz, and tebuconazole, as well as the benzimidazole fungicide carbendazim (Sutherland 2001). However, *P. brassicae* isolates that are resistant to benzimidazole fungicides have been found in the UK (Carter et al. 2013). Although benomyl is no longer available for any agricultural use in the USA (Environmental Protection Agency 2002), there are other fungicides available in FRAC Group 1 for use in the USA, e.g., thiabendazole. Karolewski and Kosiada (2010) also found *P. brassicae* isolates resistant to carbendazim (FRAC Group 1). In the UK, a recent efficacy study found seven fungicides to be effective for managing light leaf spot: prothioconazole (Proline 275, Bayer CropScience, Pittsburgh, PA), prothioconazole + tebuconazole (Prosaro, Bayer CropScience), tebuconazole (Orius 20EW, Adama, Tel Aviv, Isreal), prochloraz + tebuconazole (Orius P, Adama), penthiopyrad + picoxystrobin (Refinzar, DuPont, Wilmington, DE), dimoxystrobin + boscalid (Pictor, BASF, Florham Park, NJ), and prochloraz + propiconazole (Cirkon, Adama) (Agriculture and Horticulture Development Board (AHDB) Cereals & Oilseeds Fungicide Project 2015). In that study, a previously established fungicide action threshold of applying products when 25% of oilseed rape plants in a field had light leaf spot symptoms, was changed to a 15% threshold, but even

that threshold resulted in a concomitant yield loss of 5% (AHDB Cereals & Oilseeds Fungicide Project 2015). In Scotland, applications of plant resistance elicitors, including a combination of acibenzolar-S-methyl, B-aminobutyric acid, and cis-jasmone, in fall and spring to winter oilseed crops, controlled the severity of light leaf spot when plants had just finished flowering. The level of control was as effective as applications of commercially available fungicides, metconazole and prothioconazole + tebuconazole in a 2008-2009 trial, and disease control from the resistance elicitor applications was better than that of fungicides tested in a 2009-2010 trial (Oxley and Walters 2012).

1.3. White Leaf Spot [*Neopseudocercosporella capsellae* (Anamorph) and *Mycosphaerella capsellae* (teleomorph)].

1.3.1 Taxonomy. The pathogen that causes white leaf spot of brassicas is *Neopseudocercosporella capsellae* (anamorph), with the teleomorph *Mycosphaerella capsellae* (Deighton 1973). Synonyms of *N. capsellae* include *Cercospora albo-maculans* (Ellis & Everh.), *Cylindrosporium brassicae* (Roumeguere 1891), and *Cercosporella brassicae* (Fautrey and Roumeguere Hohnel). In 2016, *N. capsellae* was assigned to the new genus, *Neopseudocercosporella* Videira & Crous, of which the white leaf spot pathogen is the type species, *N. capsellae* (Ellis & Everh.) Videira & Crous (Videira et al. 2016). In this review, the white leaf spot fungus is referred to as *N. capsellae* (P. Crous, *personal communication*). The *Cercospora* complex is composed of the genera *Cercospora*, *Cercosporella*, *Pseudocercosporella,* and others that are differentiated by morphological characteristics such as conidial scar structure, the color of conidia, and characteristics of the conidiogenous cells (Deighton 1973; Inman 1992). Inman (1992) conducted a thorough review of the literature on *N. capsellae*, an ascomycete in the subphylum Dothideomycetes, class Dothideomycetidae, order Capnoidiales, and family Mycosphaerellaceae (Species Fungorum n.d.).

1.3.2 Morphology and physiology. *N. capsellae* produces two types of mycelium: an initial mycelium that is thin and hyaline, followed by mycelium that is dematiaceous, thick, and often becomes stromatic (Crossan 1954; Deighton 1973; Inman 1992). These stromatic structures are sclerotia-like and give rise to conidiophores (Crossan 1954). The hyaline, ampulliform, smooth conidiophores are cylindrical, straight, and emerge through the stomata or directly through the host plant cuticle (Crossan 1954; Deighton 1973). Conidia are hyaline, straight or slightly curved, and 1- to 5-septate. *N. capsellae* is very slow growing, with a colony diameter of 10 to 20 mm after three weeks on PDA medium, forming white, aerial mycelium and pale gray colonies (Crossan 1954). While *N. capsellae* sporulates readily on V8 agar medium and immersed brassica leaf agar medium, the fungus does not sporulate on many commonly used media such as PDA or water agar (Crossan 1954; Inman 1992).

N. capsellae produces a red pigment in agar media that resembles the pigment associated with the phytotoxin cercosporin, which is common in species belonging to the *Cercospora* complex (Agrios 2005; Petrie and Vanterpool 1978). Gunasinghe et al. (2016b) recently confirmed that this pigment is cercosporin. Cercosporin damages the plant by causing leaf spots and blight, but is unique in that the toxin is light activated and toxic to the host by producing activated species of oxygen (Agrios 2005; Gunasinghe et al. 2016b). The amount of phytotoxin produced by a virulent isolate of *N. capsellae* was positively correlated with the degree of susceptibility of host plants of *B. napus* and *B. juncea* tested (Gunasinghe et al. 2015).

The teleomorph, *M. capsellae,* was first reported in 1991 (Inman 1991). The factors influencing the switch from asexual to sexual reproduction are not entirely understood. Light and cool temperatures might be important (Inman 1991). Inman (1991) observed proascomata on oilseed rape crops in the summer months, suggesting light quality and quantity might be important. He also observed spermagonia with spermatia, protoascomata with trichogynes, and mature ascomata producing asci and

ascospores. He described the ascomata as scattered or solitary, immersed, epiphyllous, and subepidermal, globose to subglobose, and 70 to 116 µm in diameter with an ostiole that is short, papillate and apical. He noted that the hyaline, centrally-septate ascospores are slightly curved or straight, and 15 to 23 x 3.0 to 3.5 µm. Ascospores have obtuse ends and are guttulate, smooth, and fusoid. Fasciculate asci are obpyriform, ventricose, bitunicate, and each contains eight spores, and measures 32 to 53 x 8 to 14 µm. *N. capsellae* ascospores are very similar to those of *M. brassicicola*, except one cell is larger in those of *M. brassicicola* whereas both cells are equal in size for ascospores of *N. capsellae* (Inman 1991).

1.3.3 History, geographic distribution, and impact. White leaf spot occurs in many countries that have temperate climates where brassicas are grown (Commonwealth Mycological Institution (CMI) Distribution Maps of Plant Diseases 1986). The anamorph has been reported in Algeria, Antigua, Australia, Bhutan, Belgium, Canada, Chile, Denmark, Ethiopia, France, Germany, India, Ireland, Israel, Japan, Kenya, Malaysia, Nepal, New Zealand, Norway, Romania, Russia, South Africa, Sri Lanka, Sweden, Taiwan, Turkey, the UK, and the USA (CMI Distribution Maps of Plant Diseases 1986). Following global increases in acreage of oilseed rape production, the impact of white leaf spot has become more pronounced, with 15% yield losses noted in France as a result of severe pod infection (Penaud 1987). Similarly, economically important outbreaks of white leaf spot on various brassica crops have been recorded throughout western Europe, including Germany (Sochting and Verreet 2004), France (Penaud 1987), the UK (Inman 1992), Canada (Petrie and Vanterpool 1978), and in Western Australia (Gunasinghe et al. 2016a). White leaf spot was reported on kale in Oregon in 1945, though there was no evidence the disease caused significant damage (Boyle 1945). White leaf spot was widespread across the Willamette Valley of Oregon in 2014 on various brassica crops and weeds, and has continued to be found across this region in subsequent years (Ocamb 2014b; Ocamb et al. 2015). The most significant

damage appeared to be in commercial seed fields of forage brassicas and field turnips, although the full economic impact of this disease in the PNW USA remains unknown because other diseases such as black leg and light leaf spot often have obfuscated the damage associated with any one disease in this region since 2104 (Ocamb 2014b; Ocamb et al. 2015).

1.3.4 Host range. White leaf spot has been documented on a wide range of brassica hosts including *B. rapa* (synonymous with *B. campestris*) crop types such as rape, swede, and turnip; *B. chinensis*, *B. juncea*, *B. nigra*, *B. oleracea*, *B. pekinensis*, *Capsella bursa-pastoris*, *Conringia persica*, *Goldbachia torulosa*, *Litvinovia tenuissima*, *Malcolmia africana*, *Neslia paniculata*, *Raphanus raphanistrum*, *R. sativus*, *Rapistrum perenne*, and *S. arvensis* (Deighton 1973). *N. capsellae* is more virulent on *B. napus* and *B. rapa* than *B. oleracea*, while the very similar pathogen *M. brassicicola* tends to be more virulent on *B. oleracea* hosts (Crossan 1954; Petrie and Vanterpool 1978). *B. juncea* was found to be the most susceptible to *N. capsellae* of all *Brassica* spp. evaluated by Gunasinghe et al. (2015), *B. napus* was intermediate in reaction, while *R. raphanistrum* was most resistant to white leaf spot of the species tested (Gunasinghe et al. 2015; Gunasinghe et al. 2016a). Isolates collected from one host type are commonly able to infect other brassica host types (Crossan 1954; Inman 1992). In a recent study in Australia, isolates collected from *B. rapa*, *B. juncea*, and *B. napus* were very similar genetically, while isolates collected from *R. raphanistrum* were differentiated from the rest of the isolates based on phylogenetic analysis of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequences (Gunasinghe et al. 2016a).

1.3.5 Symptoms. White leaf spot symptoms can be found on all aerial parts of brassica plants including leaves, stems, and pods (Inman 1991). Symptoms vary depending on the host type but generally are described as irregular leaf spots on both sides of the leaf, gray-whitish in color, often with a narrow brown margin, and angular (Deighton 1973). Lesions start as small brown spots, 1 to 2 mm in

diameter, expanding to 5 to 10 mm in diameter, and becoming tan with a dark margin (Inman and Fitt 2007). For severe infections, lesions may coalesce, resulting in large areas of the leaf turning grayish-white and becoming chlorotic. Lesions on the stem of oilseed rape plants become gray, each with a diffuse edge, hence the disease is known as "gray stem." These dark to gray stem lesions are superficial and do not decay the pith. On pods, like leaves, symptoms include small, brown, irregular spots (Inman 1992). Lesions can cover large areas as the pods grow, with the lesion centers becoming depressed, and the color changing to light gray.

Symptoms of white leaf spot on leaves may be similar to those of downy mildew, light leaf spot, black leg, black spot, and ringspot (Rimmer et al. 2007). Symptoms caused by *N. capsellae* are different from those caused by *M. brassicicola* on oilseed rape, as the leaf spots lack the concentric rings with spermogonia/ascomata characteristic of infection by *M. brassicicola*, have a darker margin, are usually smaller (initial lesions start at <1 mm), and mature lesions are whiter in color (Inman 1991). On stems, symptoms may be confused with light leaf spot or black leg symptoms, while on pods the symptoms can resemble those caused by *A. brassicae* (Inman 1991).

1.3.6 Disease cycle

1.3.6.1. Dispersal. *N. capsellae* is a splash-dispersed and aerially-dispersed pathogen, requiring rain or overhead irrigation and wind for dissemination (Crossan 1954; Inman 1992; Huber et al. 1997). The teleomorph, *M. capsellae,* produces ascospores that are aerially dispersed (Inman 1991; Inman et al. 1999). Splash-dispersed conidia are dispersed over short distances and unlikely to cause spread of the white leaf spot pathogen into new, previously non-infected locations (Inman 1992). Based on the patterns of disease distribution observed in two fields in the UK, Inman (1991) stated that ascospores acted as primary inoculum, initiating development of the disease in the fall, while subsequent splash-dispersal of conidia caused the disease to progress through the winter. On oilseed

rape crops in the UK, splash-dispersed conidia were reported to move vertically up the plant an estimated 12 to 16 cm, meaning infection after stem extension might not have a severe impact on pod development if conditions turn dry and less favorable for spread of inoculum up the canopy (Inman 1991).

1.3.6.2. Host plant infection. Ideal temperatures for conidial germination have been reported to range from 20 to 24°C, with germination not occurring <8° or >28°C (Crossan 1954). Reported requirements for infection to occur are a minimum leaf wetness of 8 h at 18 to 19°C and 100% humidity (Inman 1992; Mestre 1989). Spore germination can occur for each conidial cell (Petrie and Vanterpool 1978). In 1954, Crossan (1954) wrote that infection can occur through open or closed stomata, or directly when a germ tube and appressorium are formed. Gunasinghe et al. (2015) found that the host plant (*B. carinata, B. juncea,* and *B. napus*) was always penetrated through the stomata. Generally, stomatal density affected spore germination rates and, thereby, the susceptibility of a brassica host plant to infection by *N. capsellae*. For example, infections on *B. juncea* had the greatest rates of spore germination and stomatal density, while infections on *B. napus* had the least spore germination and stomatal density. A *B. carinata* cultivar that was less susceptible to *N. capsellae* in that study stimulated stomatal closure in the presence of the pathogen, hindering *N. capsellae* from completing spore germination, germ tube extension, and stomatal penetration (Gunasinghe et al. 2015).

1.3.6.3. Survival and seed transmission. The survival of *N. capsellae* between brassica crops was attributed largely to the teleomorph, *M. capsellae*, in UK outbreaks of white leaf spot (Inman 1991). When the teleomorph is not present, survival has been attributed to the stromatic tissue resembling sclerotia (Reyes 1979). Inman (1991) wrote that these stromatic tissues do not function as sclerotia, but are the primordia for the formation of ascomata and spermagonia. In the southeast USA, Crossan (1954) found that infested turnip leaf refuse had necrotic leaf areas with thick, dense mycelium

under the epidermis that could survive for up to nine months and re-infect the cotyledons of the turnip cv. Seven-top when planted into infested plots. To investigate seed transmission, Crossan (1954) planted three commercial seed lots grown in the southeast USA in a greenhouse, the collards 'Georgia' and 'Improved Heading,' and the turnip 'Seven-top.' Seedlings were placed in a moist chamber for 3 days after emergence, and 14 days later a 1% seed transmission rate was observed on the 'Improved Heading,' but seed transmission was not observed on 'Seven-top' or 'Georgia.' Petrie and Vanterpool (1978) plated seed harvested from severely infected pods of *Conringia orientalis* onto an agar medium, and found no evidence of seed infection. However, how much seed was plated and the medium used were not reported. Petrie and Vanterpool (1978) also plated turnip rape seed and rape seed on an agar medium but rarely found seed infection (although what was meant by rarely was not defined, and the quantities of seed tested were not reported). They hypothesized that seed crop residues infected with *N. capsellae* may serve as pathways for the pathogen to areas where *N. capsellae* is not present. The pathogen also can infect and survive on wild brassica plants; however, the importance of these hosts in the epidemiology of the disease is not understood (Crossan 1954).

1.3.7 Management

1.3.7.1. Cultural control. Effective cultural control methods to limit infection of turnip and oilseed rape crops by the white leaf spot pathogen have included incorporating crop residues, removing weedy and volunteer brassicas, crop rotation, and using clean seed (Crossan 1954; McCartney and Doughty 2007). Recent recommendations by the ODA (2015) for the Willamette Valley echo Crossan's (1956) advice, specifying cultural control guidelines such as: planting high quality seed, rotating out of brassica crops for a minimum of 3 years, with 5-year rotations recommended for brassica transplants used in seed beds, and quickly following harvest of crops with intensive flailing and burial of

residues using high speed tillage disks (Ocamb 2016). Cultural practices are the most effective methods currently available for control of white leaf spot in organic farming systems (Ocamb 2016).

Though there is no published, certified seed health assay to detect if *N. capsellae* has infected a brassica seed lot, growers can purchase seed grown in regions where white leaf spot does not occur. As was discussed in the light leaf spot management section, different seed treatments such as hot water, disinfectants, and fungicides, have proven effective at eliminating some fungal and bacterial pathogens from brassica seed (Neergaard 1977), but the efficacy of these seed treatments at preventing the spread of seedborne *N. capsellae* has not been studied.

Inman and Fitt (2007) wrote that breeding for resistance to *N. capsellae* has not been identified as a priority, and that there are no known *P. brassicae* resistant brassicas. However, Gunasinghe et al. (2014) observed high levels of resistance to white leaf spot in *B. carinata* ATC 94129. In this study, among the 254 different genotypes of oilseed *Brassica* germplasm evaluated, they found that genotypes of *B. oleracea* var. *acephala* were totally resistant, and those of *B. oleracea* var. *capitata* and *B. oleracea* var. *italica* plants were highly resistant.

As discussed in the light leaf spot management section, row spacing and orientation can greatly affect the potential for white leaf spot to develop. Increasing row spacing or plant spacing within rows, and orienting rows into the predominant wind direction can be used to manage white leaf spot (du Toit 2014). Depending on the cropping system, there might be more or less flexibility to implement greater spacing within and between rows. For example, this strategy could work well for high value crops, such as brassica seed crops, but is not feasible when planting brassica cover crops at 11.25 to 17 kg / ha, as dense plantings are required to achieve the benefits of the cover crop (Pekarek et al. n.d.; Clark 2012).

1.3.7.2. Chemical control. In a fungicide trial at the University of Georgia, severity of white leaf spot on turnip leafy greens was reduced by foliar applications of the following fungicide treatments: maneb + zinc, chlorothalonil, benomyl, and iprodione; with no significant differences in efficacy among the treatments (Sumner et al. 1991). In the UK, white leaf spot in oilseed rape was controlled with foliar applications benomyl, prochloraz, or flusilazole + carbendazim (Inman and Fitt 2007). However, as mentioned for light leaf spot, benomyl is no longer available for use in the USA. In the PNW USA, Pscheidt and Ocamb (2016b) recommended prothioconazole (Proline 480 SC) for use in canola crops to control Sclerotinia stem rot, and this product currently is registered for use in brassica seed crops in Oregon and Washington (Pesticide Information Center Online (PICOL) 2017). Pscheidt and Ocamb (2016b) also recommended iprodione (Rovral) to control white leaf spot, though use is limited to crucifer seed crops in Washington (PICOL 2017).

1.4. Research Needs for Light Leaf Spot and White Leaf Spot

The 2014 outbreaks of light leaf spot, white leaf spot, and black leg in western Oregon served as a warning to brassica seed producers throughout the PNW USA. In response to the black leg outbreak, measures were taken by the ODA to adopt and amend regulation OAR 603-052-0870 to protect Brassicaceae crops grown throughout Oregon State, i.e., by requiring certified *P. lingam*-free seed, mandatory crop rotations, management of susceptible brassica volunteer plants, and additional management practices in fields that become infected with black leg (ODA 2015). During this time, the WSDA took preemptive measures and extended the previous state Crucifer Quarantine regulation, WAC 16-301-490-580, from six counties in northwestern Washington to include all counties east of the Cascade Mountains [Washington State Department of Agriculture (WSDA) 2015]. The WSDA rule requires that any brassica seed lot to be planted in the protected area must be certified as *P. lingam*-free and be tagged as having been tested. Expanding the quarantine area in response to the 2014 black

leg outbreak in Oregon was possible because, this disease can be very damaging economically. Previous research on seedborne aspects of *P. lingam* was used to develop a set of valuable management recommendations that include diagnostic seed health assays, efficacious seed treatments, and regional quarantines with certified clean seed requirements designed to keep black leg from becoming established in new regions (Rimmer and van den Berg 2007). This research and the implementation of the recommendations helped to eradicate black leg from western Washington after a 1970s outbreak in Midwestern and East coast states was found to be associated with infected seed produced in the PNW.

In contrast, research on the seedborne aspects of *N. capsellae* and *P. brassicae* is limited and inconclusive, impeding the development of effective options for managing the potential seedborne phase of these two pathogens. For example, neither the ODA nor the WSDA could establish rules requiring the planting of *N. capsellae* or *P. brassicae*-free certified seed because there currently are no certified seed health assays to detect *N. capsellae* and *P. brassicae* on seed. In fact, the existing literature is inconclusive on whether *N. capsellae* and *P. brassicae* can infect brassica seed and lead to seed transmission in brassica crops. To begin to understand these aspects of these pathogens and provide growers, seed companies, and state departments of agriculture with this important information, the following research objectives were addressed for this MS thesis project:

1. Assess the potential for *P. brassicae* and *N. capsellae* to be seedborne in brassica crops, and to understand if these pathogens can be introduced into new regions on infected seed. This was addressed by:
    a. Establishing whether *P. brassicae* and/or *N. capsellae* can infest or infect brassica seed; and
    b. Establishing whether brassica seed infected with *P. brassicae* and/or *N. capsellae* can lead to seed transmission.

2. Survey brassica crops, brassica weeds, and brassica seed lots to establish if the light leaf spot pathogen and/or white leaf spot pathogen are present in the primary region of biennial brassica vegetable seed production in northwestern Washington. This entailed:
    a. Confirming pathogenicity of isolates collected from infected plants in this region, using Koch's postulates; and
    b. Determining differences between isolates collected in the PNW USA and isolates collected from other countries where *P. brassicae* and *N. capsellae* have established, such as the UK and New Zealand.
3. Identify seed treatments effective at eradicating *P. brassicae* and/or *N. capsellae* from infected brassica seed. This was accomplished by:
    a. Evaluating the efficacy of organic seed treatments such as hot water, steam, and 1.2% NaOCl;
    b. Evaluating fungicide seed treatments representing different FRAC groups to identify products effective at preventing seed transmission of *P. brassicae* and/or *N. capsellae*.

The research was aimed at developing a better understanding of the prevalence of light leaf spot and white leaf spot in northwestern Washington, and helping brassica growers and the brassica seed industry understand if the seedborne phase of either pathogens is of concern. This research is expected to provide brassica growers and the brassica seed industry with tools to detect the pathogens on infected seed, and to eradicate the pathogen from infected seed by treating infected seed lots using organic and/or conventional treatments.

1.5. Literature Cited

Agriculture and Horticulture Development Board (AHDB) Cereals & Oilseeds Fungicide Project. 2015. Fungicides for light leaf spot control in winter oilseed rape: 2015 Summary. Warwickshire, UK.

Agrios, G. 2005. Plant Pathology, Fifth Ed. Elsevier Ltd., San Diego, CA.

Babadoost, M., and Gabrielson, R. L. 1979. Pathogens causing Alternaria diseases of Brassica seed crops in western Washington. Plant Dis. Report. 63:815–820.

Berkeley, M. J., and Broome, C. E. 1850. Notices of British fungi. Ann. Mag. Nat. Hist. 2:455–466.

Boyle, L. W. 1945. Cercosporella leaf spot of kale in Oregon. Plant Dis. Rep. 29:387.

Boys, E. F., Roques, S. E., Ashby, A. M., Evans, N., Latunde-Dada, A. O., Thomas, J. E., West, J. S., and Fitt, B. D. L. 2007. Resistance to infection by stealth: *Brassica napus* (winter oilseed rape) and *Pyrenopeziza brassicae* (light leaf spot). Eur. J. Plant Pathol. 118:307–321.

Brako, L., Rossman, A.Y., and Farr, D.F. 1997. Scientific and Common Names of 7,000 Vascular Plants in the United States. American Phytopathological Society, St. Paul, MN.

Buchwaldt, L. 2007. Sclerotinia white mold (cabbage drop, stem rot, watery soft rot). Pages 43–47 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Buchwaldt, L., and Rimmer, S. R. 2007. Botrytis gray mold. Pages 24–25 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Buttala, L., and Siegel, S. 2015. The Seed Garden: The Art and Practice of Seed Saving. Seed Savers Exchange, Decorah, IA.

Cappelli, C., Buonaurio, V., and Paul, V. H. 1999. Traditional and innovative methods to detect seed-transmitted pathogens in linseed and rape. Pages 55–61 In: Integrated Control of Oilseed Crops. V.H. Paul, I. Foller, N. Evans, and I. Williams, eds. International Organisation for Biological and Integrated Control (IOBC), Prague.

Carter, H. E., Cools, H. J., West, J. S., Shaw, M. W., and Fraaije, B. A. 2013. Detection and molecular characterisation of *Pyrenopeziza brassicae* isolates resistant to methyl benzimidazole carbamates.

Pest Manag. Sci. 69:1040–1048.

Cheah, L.-H., and Hartill, W. F. T. 1985. Disease cycle in New Zealand of light leaf spot of brassicas, induced by *Pyrenopeziza brassicae*. N. Z. J. Agric. Res. 28:567–573.

Cheah, L. H., Corbin, J. B., and Hartill, W. F. T. 1981. Control of light leaf spot of brassicas (*Pyrenopeziza brassicae* Sutton & Rawlinson) with fungicides. N. Z. J. Agric. Res. 24:391–395.

Cheah, L. H., Hartill, W. F. T., and Corbin, J. B. 1982. Ascospore release in *Pyrenopeziza brassicae*. Trans. Br. Mycol. Soc. 79:536–539.

Cheah, L. H., Hartill, W. F. T., and Corbin, J. B. 1980. First report of the natural occurrence of *Pyrenopeziza brassicae* Sutton et Rawlinson, the apothecial state of *Cylindrosporium concentricum* Greville, in brassica crops in New Zealand. N. Z. J. Bot. 18:197–202.

Claassen, B. J., Serdani, M., Thomas, W. J., Putnam, M. L., and Ocamb, C. M. 2014. Pathogenicity of *Cylindrosporium concentricum* on cultivated and weedy Brassica hosts. Phytopathology 105:S4.30 (Abstr.)

Clark, A. 2012. Managing Cover Crops Profitably, Third Ed. Sustainable Agriculture Research and Education Program, College Park, MD.

Commonwealth Mycological Institute Distribution Maps of Plant Diseases. 1986. Commonwealth Mycological Institute, Wallingford.

Crossan, D. F. 1954. Cercosporella leaf spot of crucifers. North Carolina Agric. Res. Stn. Tech. Bull. 109.

Cunningham, G. H. 1944. The Gasteromycetes of Australia and New Zealand. J. McIndoe, Dunedin, Australia.

Deighton, F. C. 1973. Studies on *Cercospora* and allied genera. IV. *Cerosporella* SACC., *Pseudocercosporella* Gen. Nov. and *Pseudocercosporidium* Gen. Nov. Mycol. Papers. 133:1–62.

Dixelius, C., Bohman, S., and Wretblad, S. 2004. Disease resistance. Pages 253–271 In: Biotechnology in Agriculture and Forestry, E. C. Pua, and C. J. Douglas, eds. Springer-Verlag, Berlin.

Duke, J. A. 1983. Handbook of Energy Crops. Purdue Univ. Cent. New Crop. Plants Prod. https://www.hort.purdue.edu/newcrop/duke_energy/dukeindex.html [Accessed 28 August 2015].

du Toit, L.J. 2004. Management of diseases in seed crops. Page 675-677 In: Encyclopedia of Plant and Crop Science. Goodman, R.M., ed. CRC Press, Boca Raton, FL.

Ellis, M. B., and Ellis, J. P. 1985. Microfungi on Land Plants. Macmillan Publishing, New York.

Environmental Protection Agency. 2002. Benomyl; Proposed Revocation of Tolerances. Fed. Regist. 67:1917–1925.

Fatemi, F., and Fitt, B. D. L. 1983. Dispersal of *Pseudocercosporella herpotrichoides* and *Pyrenopeziza brassicae* spores in splash droplets. Plant Pathol. 32:401–404.

Food and Agriculture Organization of the United Nations Statistics Division (FAOSTAT). http://faostat.fao.org [Accessed 22 July 2015].

Foster, S. J., Ashby, A. M., and Fitt, B. D. L. 2002. Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape. Eur. J. Plant Pathol. 108:379–383.

Fussell, G. E. 1955. History of cole (*Brassica* sp.). Nature 176:48–51.

Garbe, V., Gladders, P., and Lane, A. 2000. Research for the adaptation of oilseed crops to the new requirements of the common agricultural policy: Crop competitivity, seed quality, and environment. International Organisation for Biological and Integrated Control (IOBC) Bull. 23. Belvaux, Luxembourg.

Gilles, T., Evans, N., Fitt, B.D., and Jeger, M. 2000. Epidemiology in relation to methods for forecasting light leaf spot (*Pyrenopeziza brassicae*) severity on winter oilseed rape *(Brassica napus*) in the UK. Eur. J. Plant Path. 106:593-605.

Gilles, T., Fitt, B. D., and Jeger, M. J. 2001a. Effects of environmental factors on development of *Pyrenopeziza brassicae* (light leaf spot) apothecia on oilseed rape debris. Phytopathology 91:392–398.

Gilles, T., Fitt, B. D. L., McCartney, H. A., Papastamati, K., and Steed, J. M. 2001b. The roles of ascospores and conidia of *Pyrenopeziza brassicae* in light leaf spot epidemics on winter oilseed rape (*Brassica napus*) in the UK. Ann. Appl. Biol. 138:141–152.

Gomez-Campo, C. 1980. Morphology and morpho-taxonomy of the tribe Brassiceae. Pages 3–31 In:

Brassica Crops and Wild Allies, Biology and Breeding, S. Tsunoda, K. Hinata, and C. Gomez-Campo, eds. Japan Scientific Societies Press, Tokyo.

Grealy, R., Bradford, K., Hall, L., Hellmich, R., Raybould, A., Wolt, J., and Zilberman, D. 2007. Implications of gene flow in the scale-up and commercial use of biotechnology-derived crops: economics and policy considerations. Pages 1-24, Council for Agricultural Sciences and Technology, Ames, IA.

Greville, R. K. 1823. Scottish cryptogamic flora, or coloured figures and descriptions of cryptogamic plants, belonging chiefly to the order Fungi; and intended to serve as a continuation of English Botany. Maclachan and Stewart, eds. Edinburgh, London.

Gunasinghe, N., You, M. P., and Barbetti, M. J. 2016a. Phenotypic and phylogenetic studies associated with the crucifer white leaf spot pathogen, *Pseudocercosporella capsellae*, in Western Australia. Plant Pathol. 65:205–217.

Gunasinghe, N., You, M. P., Cawthray, G. R., and Barbetti, M. J. 2016b. Cercosporin from *Pseudocercosporella capsellae* and its critical role in white leaf spot development. Plant Dis. 100:1521–1531.

Gunasinghe, N., You, M. P., Clode, P. L., and Barbetti, M. J. 2015. Mechanisms of resistance in *Brassica carinata, B. napus* and *B. juncea* to *Pseudocercosporella capsellae*. Plant Pathol. 65:6:888-900.

Gunasinghe, N., You, M. P., Banga, S. S., Barbetti, M. J. 2014. High level resistance to *Pseudocercosporella capsellae* offers new opportunities to deploy host resistance to effectively manage white leaf spot disease across major cruciferous crops. Eur. J. Plant Pathol. 138:873-890.

Hartill, W. F. T., and Cheah, L.-H. 1984. Some effects of climate and plant growth on the development of light leaf spot in cauliflowers. N. Z. J. Agric. Res. 27:441–449.

Hartill, W. F. T. 1982. Control of downy mildews of brassica seedlings and lettuce with systemic fungicides. N. Z. J. Exper. Agric. 10:69-72.

Hickman, C. J., Schofield, E. R., and Taylor, E. R. 1955. Light leaf spot of *Brassica*. Plant Pathol. 4:129–131.

Historical Trends - Light Leaf Spot. Rothamsted Research. http://rothamsted.ac.uk/light-leaf-spot-forecast/historical-trends-light-leaf-spot [Accessed 1 January 2017].

Huber, L., McCartney, H. A., and Fitt, B. D. L. 1997. Influence of target characteristics on the amount of water splashed by impacting drops. Agric. For. Meteorol. 87:201–211.

Humpherson-Jones, F. M., and Maude, R. B. 1982. Studies on the epidemiology of *Alternaria brassicicola* in the *Brassica oleracea* seed production crops. Ann. Appl. Biol. 100:67–71.

Ilott, T.W., Rawlinson, C.J., and Ingram, D. S. 1984. Heterothallism in *Pyrenopeziza brassicae*, cause of light leaf spot in brassicas. Trans. Br. Mycol. Soc. 82:477–483.

Inglis, D. A., Derie, M., and Hsiang, T. 2001. Stem canker of cabbage seed stalks caused by *Botrytis cinerea* in western Washington. Plant Dis. 85:559.

Inglis, D. A., du Toit, L.J., and Miller, T. W. 2013. Production of Brassica seed crops in Washington State: A case study on the complexities of coexistence. Washington State Univ. Ext. Man. EMO062E.

Inman, A. J. 1992. The Biology and Epidemiology of White Leaf Spot (*Pseudocercosporella capsellae*) on Oilseed Rape. Ph.D. Dissertation, University of London, London.

Inman, A. J. 1991. The biology of *Mycosphaerella capsellae* sp. nov., the teleomorph of *Pseudocercosporella capsellae*, cause of white leaf spot of oilseed Rape. Mycol. Res. 95:1334–1342.

Inman, A. J., and Fitt, B. D. L. 2007. White leaf spot. Pages 50–54 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Inman, A. J., Fitt, B. D. L., Evans, R. L., and End, M. 1992. A species of *Unguicularia* on oilseed rape, and its importance in studies of the epidemiology of light leaf spot (*Pyrenopeziza brassicae*). Plant Pathol. 41:646–652.

Inman, A. J., Fitt, B. D. L., Todd, A. D., and Evans, R. L. 1999. Ascospores as primary inoculum for epidemics of white leaf spot (*Mycosphaerella capsellae*) in winter oilseed rape in the UK. Plant Pathol. 48:308–319.

International Seed Federation. 2017. ISHI-Veg Protocols. http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/#protocols. (Accessed 17 November 2016).

Jenkins, D. 2015. WSDA extends battle against black leg to E. Washington. Cap. Press. 23 July 2015. http://www.capitalpress.com/Washington/20150723/wsda-extends-battle-against-black leg-to-e-

washington.

Karolewski, Z. 2010. Development of light leaf spot (*Pyrenopeziza brassicae*) on Brassicas. Phytopathologia 55:13–20.

Karolewski, Z. 1999. The occurrence of light leaf spot on winter oilseed rape in western Poland in 1991-1996 and the characteristics of *Pyrenopeziza brassicae* isolates. Phytopathol. Pol. 113–121.

Karolewski, Z., Evans, N., Fitt, B. D. L., Todd, A. D., and Baierl, A. 2002. Sporulation of *Pyrenopeziza brassicae* (light leaf spot) on oilseed rape (*Brassica napus*) leaves inoculated with ascospores or conidia at different temperatures and wetness durations. Plant Pathol. 51:654–665.

Karolewski, Z., and Kosiada, T. 2010. Sensitivity of *Pyrenopeziza brassicae* (light leaf spot) populations to fungicides recommended for the fungus control. Phytopathologia 56:59–65.

Kaur, P. Li, C.X., Barbetti, M.J., You, M.P., Li, H., Sivasithamparam, K. 2008. First report of powdery mildew caused by *Erysiphe cruciferarum* on *Brassica juncea* in Australia. Plant Dis. 92:650.

Kavanagh, T., Staunton, W. P., and Ryan, E. W. 1965. Light leaf spot of Brussels. Irish J. Agric. Res. 4:112–114.

Koch, M. A., and Mummenhoff, K. 2006. Editorial: Evolution and phylogeny of the Brassicaceae. Pl. Syst. Evol. 259:81–83.

Kubota, M., Abiko, K., Yanagisawa, Y., and Nishi, K. 2006. Frequency of *Alternaria brassicicola* in commercial cabbage seeds in Japan. J. Gen. Plant Pathol. 72:197–204.

Lacey, M. E., Rawlinson, C. J., and McCartney, H. A. 1987. First record of the natural occurrence in England of the teleomorph of *Pyrenopeziza brassicae* on oilseed rape. Trans. Br. Mycol. Soc. 89:135–140.

Lysak, M. A, Cheung, K., Kitschke, M., and Bures, P. 2007. Ancestral chromosomal blocks are triplicated in Brassiceae species with varying chromosome number and genome size. Plant Physiol. 145:402–410.

Maddock, S. E., and Ingram, D. S. 1981. Studies of survival and longevity of the light leaf spot pathogen of Brassicas, *Pyrenopeziza brassicae*. Trans. Br. Mycol. Soc. 77:153–159.

Majer, D., Lewis, B. G., and Mithen, R. 1998. Genetic variation among field isolates of *Pyrenopeziza*

brassicae. Plant Pathol. 47:22–28.

Martin, A. 2014. Boom times for farmers in the United States of kale. Bloomberg News. https://www.bloomberg.com/news/articles/2014-05-09/farmers-are-growing-a-lot-more-kale-now-a-census-reports [Accessed on 20 February 2017].

McCartney, H. A., and Doughty, K. J. 2007. Light leaf spot. Pages 31-35 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

McCartney, H. A., and Lacey, M. E. 1990. The production and release of ascospores of *Pyrenopeziza brassicae* on oilseed rape. Plant Pathol. 39:17–32.

McDonald, B.A. 2004. Population genetics of plant pathogens. The Plant Health Instructor. http://www.apsnet.org/edcenter/advanced/topics/popgenetics/Pages/default.aspx [Accessed on 20 February 2017].

McGuire, A. 2012. Mustard Types, Seed Sources, & Acreage Estimates. http://csanr.wsu.edu/mustard-green-manures/mustard-types/ [Accessed 12 August 2016].

Meena, P.D., Thomas, L., Singh, D. 2014. Assessment of yield losses in *Brassica juncea* due to downy mildew (*Hyaloperonospora brassica*). J. Oilseed Brassica 5:73-77.

Mestre, R. 1989. Disease and disease management in oilseed rape in France. Page 17 In: International Symposium on Sportak in Oilseed Rape, 3-5 December 1991, Schering, Berlin.

Nagaharu, U. 1935. Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jap. J. Bot. 7:389–452.

Neergaard, P. 1977. Seed Pathology. Halsted Press, New York.

Ocamb, C. M. 2016. A Clinic Close-Up: Black leg, light leaf spot, and white leaf spot in western Oregon. Oregon State University, Corvallis, OR. https://pnwhandbooks.org/sites/pnwhandbooks/files/insect/document/turnip-rutabaga-brassica-sp-light-leaf-spot/black-leg-light-leaf-spot-white-leaf-spot-crucifers16.pdf [Access 12 December 2016]

Ocamb, C. M., Mallory-Smith, C., Thomas, W. J., Serdani, M., Putman, M. 2015. New and re-emerging

fungal pathogens affecting Brassicaceae plants in western Oregon: black leg, light leaf spot, and white leaf spot. Phytopathology 105:S4.103.

Ocamb, C. M. 2014a. Disease Alert – Light Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/Light%20leaf%20spot%20OSU%20Disease%20Alert%20in%20crucifers%2017%20June.pdf [Accessed 12 December 2016]

Ocamb, C. M. 2014b. Disease Alert – White Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/White%20leaf%20spot%20OSU%20Disease%20Alert%20in%20Brassiceae.pdf [Accessed 12 December 2016]

Oregon Department of Agriculture. 2015. FAQ: Permanent Rule for Black Leg Disease Prevention in the Willamette Valley. https://www.oregon.gov/ODA/shared/Documents/Publications/MarketAccess/Black legFAQ.pdf [Accessed 1 January 2017].

Organic Seed Alliance. 2007. Principles and Practices of Organic Radish Seed Production in the Pacific Northwest. Port Townsend, WA.

Oxley, S. J. P. P., and Walters, D. R. 2012. Control of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) with resistance elicitors. Crop Prot. 40:59–62.

Paulitz, T. C., Knerr, A. J., Carmody, S., Schlatter, D., Sowers, K., Derie, M., and du Toit, L. J. 2017. First report of *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, causal agents of black leg, on canola in Washington State. Plant Dis. 101: 504.

Pekarek, R., Hoyt, G., Monks, D., and Jennings, K. Biomass Production of Biofumigant Cover Crops – “Caliente” Mustard and Oilseed Radish. North Carolina Coop. Ext. 14-CALS-4060.

Penaud, A. 1999. Chemical control and yield losses caused by *Erysiphe cruciferarum* on oilseed rape in France. In Proceedings of the 10th International Rapeseed Congress N. Wratten PA. Salisbury [eds.], the Regional Institute Ltd., Canberra, Australia. September.

Penaud, A. 1987. La maladie des taches blanches du colza. Phytoma 95:23–26.

Pesticide Information Center Online. 2017. http://cru66.cahe.wsu.edu/labels/Labels.php?SrchType= accessed 4 Feb 2017. Washington State University, Pullman, WA.

Petrie, G. A., and Vanterpool, T. C. 1978. *Pseudocercosporella capsellae*, the cause of white leaf spot and grey stem of Cruciferae in western Canada. Canad. Plant Dis. Surv. 58:69–72.

Pscheidt, J. W., and Ocamb, C. M. 2016a. Pacific Northwest Plant Disease Management Handbook. http://pnwhandbooks.org/plantdisease. Oregon State University, Corvallis, OR.

Pscheidt, J. W., and Ocamb, C. M. 2016b. Turnip and rutabaga (*Brassica* spp.) - white leaf spot and gray stem. In: Pacific Northwest Plant Disease Management Handbook. https://pnwhandbooks.org/plantdisease/host-disease/turnip-rutabaga-brassica-sp-white-leaf-spot-gray-stem2016. [Accessed 1 January 2017]. Oregon State University, Corvallis, OR.

Rackham, R. 2002. History of Specialty Seed Crop Production in the Pacific Northwest. Willamette Valley Specialty Seed Association, Corvallis, OR.

Rakow, G. 2004. Species origin and economic importance of *Brassica*. Biotechnol. Agric. For. 54:1–5.

Rawlinson, C. J., Sutton, B. C., and Muthyalu, G. 1978. Taxonomy and biology of *Pyrenopeziza brassicae* sp. nov. (*Cylindrosporium concentricum*), a pathogen of winter oilseed rape (*Brassica napus* spp. *oleifera*). Trans. Br. Mycol. Soc. 71:425-4-39.

Reyes, A. A. 1979. First occurrence of a severe white leaf spot on Chinese mustard in Canada. Can. Plant Dis. Surv. 59:1-2.

Richardson, M. L. 1990. An Annotated List of Seed-Borne Diseases, 4th Ed. International Seed Testing Assoc., Zurich.

Rimmer, S. R., and van den Berg, C. G. J. 2007. Black leg (Phoma stem canker). Pages 19-22 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Rimmer, S. R., Shattuck, V. I., and Buchwaldt, L. 2007. Compendium of Brassica Diseases. American Phytopathological Society, St. Paul, MN.

Rollins, R. C. 1981. Weeds of the Cruciferae (Brassicaceae) in North America. J. Arnold Arbor. 62:517-540.

Roumeguere, C. 1891. Fungi exsiccati precipue Gallici: *Cylindrosporium brassicae.* Rev. Mycol. 13:73–83.

Schreiber, A., and Ritchie, L. 1995. Washington Minor Crops. Food & Environmental Quality Lab, Washington State University Tri-Cities, Richland, WA.

Shattuck, V. I. 2007. Powdery mildew. Pages 37-39 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Sochting, H. P., and Verreet, J. A. 2004. Effects of different cultivation systems (soil management, nitrogen fertilization) on the epidemics of fungal diseases in oilseed rape (*Brassica napus* L . var. *napus*). J. Plant Dis. Prot. 111:1–29.

Species Fungorum. http://www.speciesfungorum.org/ [Accessed 31 December 2015].

Staunton, W. P. 1967. Studies on light leaf spot (*Gloeosporium concentricum*) of Brassicae. Irish J. Agric. Res. 6:203–211.

Staunton, W. P., and Kavanagh, T. 1966. Natural occurrence of the perfect stage of *Gloeosporium concenctricum* (Grev.) Berk. & Br. Irish J. Agric. Res. 5:140-141.

Sumner, D. R., Bell, D. K., Lillard, D. A., Hurst, W. C., Bush, P. A., and Brenneman, T. B. 1991. Leaf spot control, maneb, and ethylenethiourea residues in processed turnip greens treated with maneb. Plant Dis. 75:878-882.

Sutherland, K. G. 2001. Technical note: light leaf spot in winter oilseed rape. The Scottish Agricultural College, Edinburgh.

Sutton. 1977. *Cylindrosporium concentricum.* CMI Descr. Fungi Bact. No. 536. Commonwealth Mycological Institute, Wallingford.

Tewari, J. P., and Mithen, R. F. 1999. Diseases. Pages 375-411 In: Biology of Brassica Coenospecies. C. Gomez-Campo, ed. Elsevier, New York.

United States Department of Agriculture Economic Research Service. http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx. [Accessed 23 July 2015].

United States Department of Agriculture Germplasm Resources Information Network. http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?311416 [Accessed 31 July 2015].

United States Department of Agriculture National Agricultural Statistics Service. http://quickstats.nass.usda.gov/. [Accessed 15 July 2015 and 19 Feb 2017].

Vaughan, J. G. 1977. A multidisciplinary study of the taxonomy and origin of “Brassica” crops. Bioscience 27:35–40.

Videira, S. I. R., Groenewald, J. Z., Braun, U., Shin, H. D., and Crous, P. W. 2016. All that glitters is not *Ramularia*. Stud. Mycol. 83:49–163.

Washington State Department of Agriculture. 2015. Crucifer Seed Quarantine. http://agr.wa.gov/Inspection/SeedInspection/docs/478-CruciferSeedQuarantineFactSheet-Web.pdf [Accessed 1 January 2017].

Washington State University Grant-Adams County Extension. 2008. 2008 Columbia Basin Vegetable Seed Acreage and Yield Estimate. Moses Lake, WA. http://extension.wsu.edu/grant-adams/wp-content/uploads/sites/39/2014/01/2008ColBasinVegSeedAcreageandYieldEstimateReport11.pdf [Access 1 January 2017].

Watts, C. 2016. Annual Report. 2015-2016 Cover Crop Survey. Sustainable Agriculture Research and Education/Conservation Technology Information Center, College Park, MD.

Williams, P. H. 2007. Black rot. Pages 60-62 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Williams, P. H. 1980. Black rot: a continuing threat to world crucifers. Plant Dis. 64:736–742.

CHAPTER 2

EVALUATION OF SEEDBORNE ASPECTS OF *PYRENOPEZIZA BRASSICAE* AND *NEOPSEUDOCERCOSPORELLA CAPSELLAE* ON BRASSICAS

2.1 Introduction

In 2014, two foliar fungal diseases of brassicas, light leaf spot (caused by *Pyrenopeziza brassicae)* and white leaf spot (caused by *Neopseudocercosporella capsellae)*, were detected in multiple types of brassica crops and on Brassicaceae weeds in the Willamette Valley of Oregon (Ocamb 2014a; Ocamb 2014b). Light leaf spot had never been reported before in North America (Ocamb 2014a; Farr and Rossman 2017). White leaf spot had been reported infrequently in Oregon and California but was common in the southeastern USA [Boyle 1945; Commonwealth Mycological Institute (CMI) Distribution Maps of Plant Diseases 1986; Crossan 1956]. The pathogens have since persisted and become widespread in the Willamette Valley (C.M Ocamb, Oregon State University, *personal communication*). In the spring of 2016, *P. brassicae* and *N. capsellae* were detected in *Brassica juncea* cover crops and *B. rapa* weeds (bird's rape mustard) in northwestern Washington (see Chapter 3). In 2017, a Willamette Valley grower reported nearly 100% incidence of infection by *P. brassicae* in each of a *B. juncea* seed crop and a *B. rapa* seed crop, estimating >50% yield losses (L.J. du Toit, Washington State University, *personal communication*). Interestingly, adjacent *B. napus* and *B. oleracea* seed crops on that farm had very little infection. Because these two pathogens are considered new to the Pacific Northwest USA, the impacts of these diseases are not yet well understood in the context of the diverse brassica crop production systems in this region (Inglis et al. 2013).

Of these two pathogens, *N. capsellae* is more widespread geographically on a global scale than *P. brassicae* (CMI Distribution Maps of Plant Diseases 1986; Rawlinson et al. 1978). White leaf spot has been found in many countries where brassicas are grown in temperate climates (CMI Distribution

Maps of Plant Diseases 1986). The anamorph has been reported in Algeria, Antigua, Australia, Bhutan, Belgium, Canada, Chile, Denmark, Ethiopia, France, Germany, India, Ireland, Israel, Japan, Kenya, Malaysia, Nepal, New Zealand, Norway, Romania, Russia, South Africa, Sri Lanka, Sweden, Taiwan, Turkey, the UK, and the USA. Prior to detection in the USA, the light leaf spot pathogen, *P. brassicae*, had been found in Australia, Denmark, France, Germany, Ireland, Japan, Latvia, Netherlands, New Zealand, Norway, Philippines, Portugal, Romania, Tasmania, and the UK (Hickman et al. 1955; Karolewski 2010; Rawlinson et al. 1978; Sutton 1977). The anamorph, *Cylindrosporium concentricum*, was first described in the UK in the 19th century (Greville 1823); and the teleomorph, *P. brassicae*, was first described in 1978 (Rawlinson et al. 1978).

Ingold (1953) defined seedborne fungi as fungi that are disseminated in association with a dispersal unit of the host. However, not all seedborne fungi are plant pathogens (Munkvold 2009). It was not until the 1970s that disease management and epidemiological concepts were integrated comprehensively into the field of seed pathology (Munkvold 2009). Neergaard (1977) discussed two main categories of seedborne fungal plant pathogens: those for which infected seed is typically the primary inoculum for disease outbreaks, and those for which infected seed is not usually the primary inoculum source. For both types of seedborne pathogens, however, seed transmission can play a significant role in the spread of virulent strains of the pathogens or races to new areas (Neergaard 1977). Plant pathologists have noted that the increasingly global movement of seed has provided an increasingly common pathway for the dispersion of many seedborne crop pathogens (Maude 1996; Neergaard 1977).

Previous research on the potential seedborne aspects of *P. brassicae* and *N. capsellae* has been very limited. Crossan (1954) investigated seed transmission of *N. capsellae* by planting in a greenhouse three commercial seed lots that had been grown in the southeastern USA: the collards (*B. oleracea* var.

acephala) 'Georgia' and 'Improved Heading,' and the turnip (*B. rapa*) 'Seven-top.' The seedlings were placed in a moist chamber for 3 days after emergence. After another 14 days, a 1% seed transmission rate was calculated for the 'Improved Heading,' plants, but seed transmission was not observed for the 'Seven-top' or 'Georgia' plants. Petrie and Vanterpool (1978) harvested seed from pods of *Conringia orientalis* (hare's ear mustard) that were infected severely with *N. capsellae,* and plated the seed onto an agar medium. They found no evidence of seed infection by this fungus. However, the amount of seed plated and the medium used were not reported in that study. Petrie and Vanterpool (1978) also plated turnip rape (*B. campestris*) seed and rape (*B. napus*) seed on an agar medium, and reported rarely finding seed infection, although they did not define what was meant by "rarely," and the quantities of seed tested for each lot were not reported. They hypothesized that seed crop residues infected with *N. capsellae* and present in seed lots may serve as pathways for the pathogen to areas where *N. capsellae* is not present.

Hickman et al. (1955) suggested the potential for seed infection and seed transmission of *P. brassicae* because of the introduction of light leaf spot into different regions globally, as well as different regions of the UK. In addition, the localized distribution of light leaf spot symptoms in some fields has been interpreted to suggest the pathogen can be introduced as seedborne or debris-borne primary inoculum (Rawlinson et al. 1978). In the UK, distinct geographic subpopulations of *P. brassicae* have been documented*,* suggesting that most disease outbreaks result not from introductions of new, virulent isolates, but from the local movement of existing populations of the fungus (Majer et al. 1998). However, the presence of light leaf spot in fields in Scotland that previously had not been planted to brassica crops indicated that seedborne transmission might be more important than previously thought (Sutherland 2001).

Staunton (1967) found that seeds produced on Brussels sprouts (*B. oleracea* var. *gemmifera*) plants inoculated with *P. brassicae* did not develop light leaf spot on the seedlings grown from that seed. Staunton surface-sterilized the seed (dipped in 0.85% sodium hypochlorite for 1 min, and then dried on sterilized filter paper) and then plated the seed onto potato dextrose agar (PDA) at 15°C, but did not observe *P. brassicae* on the seed. Maddock and Ingram (1981) conducted a series of studies building on Staunton's work. They investigated *P. brassica*e survival on seed harvested from severely infected pods of the oilseed rape (*B. napus*) cv. Rapora and stubble turnip (*B. campestris* ssp. *rapifera*) cv. Appin. They surface-sterilized the seed using a 10 min soak in 10% sodium hypochlorite, triple-rinsed the seed in sterilized, distilled water, blotted the seed dry between layers of sterilized filter paper, plated the seed onto PDA and 3% malt agar, and incubated the seed in the dark at 20°C (ideal for the growth of *P. brassicae*) and at 10°C to inhibit the growth of saprophytic, fast-growing fungi. They also washed samples of the same seed lots to look for germinating spores of *P. brassicae,* and grew plants of 'Rapora' from 40 seed harvested from infected pods in a greenhouse. In addition, they inoculated approximately 1,000 non-infected oilseed rape seed with a *P. brassicae* spore suspension, with and without a vacuum treatment, and then planted the seed in pots in a greenhouse. *P. brassicae* was detected on 3 of 30 seeds of the oilseed rape cv. Rapora harvested from severely infected pods, but light leaf spot did not develop on the seedlings that grew from either the naturally infected Rapora seed lot or the 1,000 inoculated oilseed rape seed. Maddock and Ingram (1981) concluded that the seedborne phase of *P. brassicae* was more likely to play a significant role in introducing the fungus into new areas than increasing inoculum levels significantly in areas with established infections.

In New Zealand, Cheah and Hartill (1985) observed *P. brassicae* on 4 of 20 seeds (25% incidence of infection) that were surface-sterilized (immersed in 1% sodium hypochlorite for 3 min, rinsed in distilled water, and dried on filter paper) and then plated onto PDA. However, they found no evidence of

seed transmission in a greenhouse when they planted 200 cauliflower (*B. oleracea* var. *botrytis*) seed harvested from siliques naturally infected with *P. brassicae*. They concluded that *P. brassicae* was unlikely to infect seedlings, even when present on seed. Maddock and Ingram (1981) and Cheah and Hartill (1985) showed that, although seed infection by *P. brassicae* can occur, the role of seedborne inoculum in pathogen transmission was uncertain. Light leaf spot developed in a field of oilseed rape in the UK that was planted with seed infected at 0.08%, demonstrating that even a small level of seed infection could result in disease establishment (Maddock and Ingram 1981).

To prevent the spread of seedborne fungal pathogens on seed, the seed industry and many regulatory agencies around the world rely on standardized seed health assays to test seed lots in support of specific quarantines, to certify seed lots, to assess if seed lots need to be treated, and to test treated seed lots to assess if the treatments eradicated target pathogens (Neergaard 1977). Seed health assays can be done in a number of ways: direct or microscopic inspections of seed, including examination of seed incubated on blotters or agar media; grow-outs of seedlings under specific conditions favorable for particular seedborne pathogens; serological tests; and molecular (RNA or DNA) assays (Munkvold 2009; Neergaard 1977). Neergaard (1977) outlined a basic set of requirements that should be common to all seed health assays: the assays must provide consistent information relevant to quarantine requirements and field performance; within statistical limits, results of seed health assays must be reproducible; economic considerations of equipment, time, and labor must be considered; and results of the assays should be available quickly, which can be challenging if time-consuming incubation of the seed samples is required.

The recent detection of *P. brassicae* and *N. capsellae* in the Pacific Northwest, a very important vegetable, cover crop, seed, and oilseed Brassicaceae growing region [Inglis et al. 2013; McGuire 2012; United States Department of Agriculture (USDA) National Agricultural Statistics Service (NASS)],

warrants investigation into the risk that these pathogens might be moved on infected seed. The introduction of these brassica pathogens into the Pacific Northwest is of concern, not only to the farmers of brassica crops but also for the brassica seed industry because of the risk of disseminating infected seed produced in this region. Given the very limited and sometimes contradictory results of studies on seedborne aspects of *P. brassicae* and *N. capsellae*, the objectives of this study were to assess the potential for *P. brassicae* and *N. capsellae* to be seedborne and seed transmitted in brassica species, in order to understand the risks of these pathogens being introduced into new regions on infected seed. This was addressed by attempting to produce brassica seed lots infected with *P. brassicae* and/or *N. capsellae*, develop a brassica seed health assay for each pathogen, and quantify the risk of seed transmission of *P. brassicae* and/or *N. capsellae* from infected brassica seed lots.

2.2 Materials and Methods

2.2.1. Production of infected seed lots. Leaves with typical symptoms of light leaf spot and white leaf spot were collected on 5 February 2015 from a biennial turnip (*B. rapa*) seed crop of the cv. Barkant grown in the Willamette Valley, OR, from 2014 to 2015. *P. brassicae* and *N. capsellae* infection was confirmed by Dr. Cynthia Ocamb at Oregon State University. The fungi were isolated by plating small pieces (up to 5 mm^2) of surface-sterilized, symptomatic leaf tissue onto chloramphenicol (100 mg/liter)-amended potato dextrose agar (cPDA) and water agar (WA). Tissue was surface-sterilized in 0.6% NaOCl for 30 s, then the pieces were each triple-rinsed in sterilized, deionized water, dried, and plated onto the agar media. Morphological characteristics used to identify isolates of *P. brassicae* included hyaline, smooth, cylindrical, and mostly aseptate and eguttulate conidia (each 10 to 16 x 3 to 4 μm) on short (10.0 to 16.0 x 2.5 to 4.0 um), non-branching conidiophores (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977). *N. capsellae* was confirmed morphologically by the presence of hyaline, straight or slightly curved conidia that were each 1- to 5-septate, and 30 to 90 μm x 2 to 3 μm (Crossan

1954; Deighton 1973). The hyaline, straight conidiophores emerging through stomata were 5 to 15 μm x 2 to 4 μm (Crossan 1954; Deighton 1973). *N. capsellae* was isolated using 10% clarified V8 agar medium [100 ml of clarified V8 juice (with the clarified V8 juice made by mixing 1.0 g of $CaCO_3$/100 ml V8 juice, centrifuging the suspension at 7,000 rpm for 10 min, and collecting the supernatant); 15 g agar, and 900 ml deionized water/liter] (Jeffers 2007), and confirmed by the development of dematiaceous, stromatic structures giving rise to the conidiophores described by Crossan (1954). In addition to *P. brassicae* and *N. capsellae*, *Alternaria brassicae, Phoma lingam, Itersonilia* spp., and *Colletotrichum* spp. were isolated from leaf lesions of the Barkant turnip seed crop (Barnett and Hunter 1998).

2.2.1.1. Species identity of *P. brassicae* isolates. In March 2015, four single-spore isolates of *P. brassicae* (Cyc001, Cyc003, Cyc005, and Cyc006) were produced from the turnip leaf samples described above by streaking conidia onto cPDA. Individual colonies that developed from the streaks were each transferred to a plate of WA on which five 1.5 cm-diameter, sterilized filter disks (VWR, Visalia, CA) had been placed. Since fungal growth was very slow, the plates were flooded after approximately 6 weeks with 50 μl of sterilized, deionized water to promote colonization of the disks. Once colonized, the filter disks were transferred into sterilized No. 1 coin envelopes (Office Depot, Boca Raton, FL), dried overnight in a laminar flow hood, and placed in an air-tight container with desiccant at -20°C for long-term storage.

The genus and species identity of isolates Cyc001, Cyc003, Cyc005, and Cyc006 were confirmed morphologically, as described above, and by sequencing both the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) and the β tubulin gene. Two DNA regions were sequenced because the ITS rDNA sequences were only 95% similar to the ITS rDNA sequence of the single isolate of *P. brassicae* in GenBank (see Results), which was not similar enough to confirm the identity of the Oregon isolates as *P. brassicae* or any other fungal species (Blaalid et al. 2013). DNA was extracted from a mycelial mat and

associated spores of each isolate grown in potato dextrose broth (PD broth) in 60-mm-diameter Petri plates (VWR), using the standard protocol for the DNeasy Plant Mini Kit (Qiagen, Germantown, MD). For each isolate, approximately 200 mg of mycelium was disrupted using a Lysing Matrix A tube to which 430 μl of AP1 buffer was added, and the mycelium macerated in a bead beater (Mini-Beadbeater, Biospec Products, Inc., Bartlesville, OK) for 45 s. The contents of the tube were then centrifuged at 20,000 g for 15 min, and the supernatant transferred to a new 1.7-ml microcentrifuge tube. RNase was added and the contents of the tube incubated for 10 min at 65°C. A QIAshredder spin column was used to create a homogenous lysate, and a DNEasy Mini spin column was used to purify nucleic acids before the nucleic acids were eluted with buffer AE. The ITS region was amplified using primers UNUP18S42 (5'-CGTAACAAGGTTTCCGTAGGTGAAC-3') and UNLO28S576B (5'GTTTCTTTTCCTCCGCTTATTAATATG-3'), as described by Bakkeren et al. (2000), in a total reaction volume of 30 μl, which included 6 μl 5x buffer (Invitrogen Life Technologies, Carlsbad, CA), 1.50 mM $MgCl_2$, 0.20 of each dNTP, 0.04 mM of each primer, 5 units/μl of *Taq* DNA polymerase (Invitrogen Life Technologies), and 2 μl of genomic DNA. PCR assays were performed in a Thermohybaid PCR Express thermocycler (ThermoFisher Scientific, Waltham, MA) using the following cycling parameters: 94°C for 3 min; 31 cycles of 92°C for 45 s, 62°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The β-tubulin gene was amplified using primers F-Btub3 (TGG GCY AAG GGT YAY TAY AC) and F-Btub2r (GGR ATC CAY TCR ACR AA) (Carter et al. 2013), with a total reaction volume of 25 μl, including 5 μl 10x buffer, 1.50 mM $MgCl_2$, 2.5 μl of each dNTP, 1.2 μl (10 um) of each primer, 0.25 μl (5 units/μl) of *Taq* DNA polymerase, and 1 μl of genomic DNA. The PCR assays were performed in a Thermohybaid PCR Express thermocycler, using the following cycling parameters: 94°C for 3 min; 35 cycles of 92°C for 45 s, 55°C for 45 s, 72°C for 1 min; and a final extension at 72°C for 10 min. In addition to the ITS rDNA and β tubulin sequences, PRC assays for the mating type idiomorphs (MAT-1 and MAT-2) were attempted using the *P. brassicae* primers and

protocol developed by Foster et al. (1999). That assay was run without a *P. brassicae* control isolate, however, because the pathogen was new to the USA and a United States Department of Agriculture (USDA) Animal and Plant Health Inspection Services Plant Protection and Quarantine (APHIS-PPQ) permit had not yet been obtained at the time this aspect of the study was completed.

Amplified DNA was run on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, MO) in 1x TBE buffer (Sigma-Aldrich) with a 100 bp DNA ladder (Invitrogen Life Technologies), and visualized using UV light. ExoSAP-IT (ThermoFisher Scientific) was used to clean each PCR product for sequencing, using 2 μl of ExoSAP-IT for every 5 μl of PCR product. Approximately 10 to 15 ng of cleaned PCR product and 8 pmole of each primer were sent to Elim Biopharmaceuticals, Inc. (Hayward, CA) for both forward and reverse sequencing of the amplified DNA of each isolate. Raw sequence data were aligned and edited using the software MEGA 7 (Kumar et al. 2015). The consensus sequence of each isolate was compared with sequences in GenBank using the National Center for Biotechnology Information (NCBI) online Basic Local Alignment Search Tool (BLAST).

2.2.1.2 Pathogenicity test of *P. brassicae* isolates. Pathogenicity tests were completed on multiple *Brassica* spp. for two isolates of *P. brassicae*. For each isolate, a V8 agar plate colonized with the *P. brassicae* isolate was flooded with 0.5 ml sterilized, deionized water and the spore suspension dispersed onto five Petri plates of 10% clarified V8 juice agar. Cauliflower plants of 'Candid Charm' (Sakata Seed, Morgan Hill, CA), kale (*B. oleracea* var. *acephala*) plants of 'Black Magic' (Osborne International Seed Co., Mount Vernon, WA), and turnip plants of 'Hakurei' (Osborne International Seed Co.) were grown in a greenhouse at the Washington State University (WSU) Mount Vernon Northwestern Washington Research and Extension Center (NWREC). Seeds were started in RediEarth Seedling Starter Mix (SunGro, Agawam, MA) in 72-cell flats (2 seed/cell, with each cell 3.8 cm diameter x 5.7 cm deep) on 5 March 2015, and the seedlings transplanted after three weeks into Sunshine Mix No.

1 (SunGro) in 10-cm-diameter pots. On 20 April 2015, when each plant had 4 to 5 true leaves, the plants were put into plastic bins (each 41.0 cm x 28.0 cm x 16.5 cm deep) with 5 replicate plants of a species/container/isolate, and each container with plants was enclosed in a large plastic bag to create a humidity chamber. A 45-cm-long bamboo stake was put into one of the pots to prevent the bag from collapsing onto the plants. The plants were incubated overnight under a greenhouse bench that was covered with two layers of Remay cloth for shading.

The next day, a spore suspension was made of each of the two *P. brassicae* isolates to inoculate the appropriate plants using an atomizer (Rescende Model 175, Badger Air-Brush Co., Franklin Park, IL) powered by a compressor (0523-V4B-G180DX, Gast Manufacturing, Benton Harbor, MI). A conidial suspension of each isolate was made by flooding each plate with 20 ml sterilized, deionized water, and gently rubbing the surface of the plate with a sterilized, bent glass rod. The spore suspension was strained through two layers of cheesecloth and quantified using a hemocytometer. Spore suspensions were each diluted to 1 x 10^5 spores/ml, and the surfactant Tween 20 was added (0.01%). The spore suspension was atomized onto both sides of each leaf of all five plants/treatment. Five plants/plant species were included as a negative control treatment by atomizing the foliage with water and Tween 20. After inoculation, plants were placed back into the plastic bags for 4 days under the greenhouse benches covered with Remay, similar to the protocol described by Fitt et al. (1998). The plants were maintained in the WSU Mount Vernon NWREC greenhouse Bay 102, set at 20 ± 3°C by day and 15 ± 3°C by night, with lights on for 12 h/day.

The pathogenicity test was set up as a 3 x 3 factorial treatment design of three plant species (cauliflower, kale, and turnip) and three inoculation treatments (two fungal isolates and a control treatment), using five replicate plants per treatment combination. Plants were arranged in a randomized complete block (RCB) experimental design when removed from the plastic bags and placed on the

greenhouse benches. Each plant was evaluated 14 days after inoculation (dai) for the percentage of the leaf area with light leaf spot symptoms for each of three leaves/plant. For plants on which limited veinal browning was the primary symptom, disease severity was rated as 1%. The plants were placed back in the plastic bags 14 dai for 48 h to promote development and sporulation of *P. brassicae* isolates. A second severity rating was completed 20 dai. Glass slide mounts made from leaf lesions were examined microscopically, and isolations completed from the lesions onto V8 agar medium to verify the causal agent of symptoms.

The mean severity rating for three leaves/plant for each replication of each treatment combination were subjected to analysis of variance (ANOVA), with replication a random effect, and plant species and inoculation treatments fixed effects in the model. The non-inoculated control plants of each species were excluded from the ANOVA because symptoms were not observed on those plants (see Results), so the data were analyzed as a 2 x 3 factorial design. Assumptions of normality and equal variance were tested for parametric analysis. Treatment means were compared using Fisher's protected least significant difference (LSD) at $P < 0.05$. The pathogenicity test of *P. brassicae* isolates Cyc001, Cyc003, Cyc005, and Cyc006 was repeated similarly in a trial that concluded on 8 August 2015.

2.2.1.3. Production of brassica seed infected with *P. brassicae.* In order to produce seed lots infected with *P. brassicae* and/or *N. capsellae,* 40 vernalized cabbage (*B. oleracea* var. *capitata*) plants of each of a proprietary male line and a proprietary female line were donated by Vikima USA (Burlington, WA) in March 2015. Using vernalized cabbage plants enabled a seed lot to be generated in 2015, with inoculation of the cabbage pods during seed development in June and July to mimic the natural mode of infection of seed on plants by *P. brassicae*. The original seedlings had been transplanted into a field in Skagit Co., WA in August 2014, and the plants vernalized through the winter of 2014-15 for a hybrid cabbage seed crop. The cabbage plants were dug up from field F8 South at the

WSU Mount Vernon NWREC on 12 March 2015, and transplanted into Sunshine Mix No. 1 in sanitized (washed, triple-rinsed, and sprayed with 70% ethyl alcohol), 18.9 liter (5 gal) buckets (one plant/bucket), with 8 holes drilled into the bottom of each bucket for drainage. The plants were placed in a screenhouse at the WSU Mount Vernon NWREC, and European honey bees (*Apis mellifera*) were used to pollinate the flowers that developed. *B. oleracea* var. *capitata* plants are self-incompatible, so using compatible male and female inbred lines enabled cross-pollination for viable seed set. A nuclear hive was provided by Belleville Pollination (Burlington, WA). Plants were kept in the screenhouse until approximately 80% of the blossoms had been pollinated and developed pods.

Two humidity chambers, each 3.9 m x 1.5 m x 2.0 m tall, were constructed out of 1.9-cm-diameter polyvinyl chloride (PVC) pipe in greenhouse Bay 102 of the WSU Mount Vernon NWREC. A pipe was installed several inches above the length of the center of the frame to provide a slope along the length of the plastic sheet that was placed over the entire frame, to prevent water from dripping directly onto the plants. Each humidity chamber was covered with 0.31-ml thick, clear construction film (Painter's Plastic, Lowe's, Burlington, WA), as well as two layers of Remay as shade cloth. Misters were set using a timer (CycleStat II Precision Repeat Cycle Timer, Green Air Products Inc., Boring, OR) to mist the plants for 30 s every 60 min at a rate of 3.79 liters/h. In each humidity chamber, there were two 4.27-m-long PVC pipes with misters attached to form the central manifold. The misters in each humidity chamber could be turned on or off via a ball valve to control relative humidity independently in each chamber. Misters were each made using 1.27-cm-diameter PVC pipe to which PVC misting tees (M6045, Charley's Greenhouse, Mount Vernon, WA) were fitted with poly misting nozzles (M6032, Charley's Greenhouse) installed every 61 cm for a total of 14 nozzles/chamber.

Symptomatic turnip leaves (approximately 10) of the cv. Hakurei infected with isolates of *P. brassicae* from the pathogenicity test described above, were soaked in 250 ml sterilized, deionized

water for 30 min. The leaves were removed, and the *P. brassicae* spore suspension quantified using a hemocytometer. Additionally, spore suspensions were made by flooding colonized plates of each of the four isolates of *P. brassicae*, Cyc001, Cyc003, Cyc005, and Cyc006, growing on clarified V8 juice agar plates. The agar plates were flooded with sterilized, deionized water, and the surface of the medium in each plate rubbed gently with a sterilized glass rod. The spore suspensions were strained through two layers of cheesecloth and quantified using a hemocytometer. The spore suspensions from the leaf wash and agar media were then combined, quantified, and diluted with deionized water to 1 x 10^5 spores/ml. Additionally, 0.5% gelatin (5 g/liter, Knox Brand, Original, Unflavored, Kraft Foods Global, Inc., Northfield, IL) was added to the spore suspension to aid adherence of the spores to the cabbage racemes and siliques (Jacobsen and Williams 1971).

The *P. brassicae* spore suspension was applied to the 40 cabbage plants (20 of each parent line) at growth stage 4.5 (Harper 1973), when approximately 80% of the flowers had senesced and pods formed, using two humidity chambers (20 plants of each cabbage parent line/chamber). Cabbage plants were sprayed until just before run-off on 2 June 2015, by applying approximately 6 liters of inoculum using a 7.6 liter Home and Garden Sprayer (Project Source, Lowes). After inoculation, the humidity chambers were covered for 3 days with a layer of plastic and two layers of Remay as shade cloth. The misters were turned on for 30 s/h for 42 h after inoculation. The plants were maintained at 15 to 20^{o}C. A Watchdog data logger (Model 425, Spectrum Technologies, Inc., Aurora, IL) was installed among the plants in each humidity chamber to record temperature and relative humidity (RH) at 1-h intervals.

The cabbage plants were uncovered on 5 June 2015. *Botrytis cinerea,* as well as saprophytic fungi such as *Cladosporium* spp., had started to grow on the bamboo stakes and twine supporting the plants, and on some of the cabbage blossoms. To prevent potential interference of these fungi with development of *P. brassicae* on the pods and seed, the fungicide iprodione (Rovral 4 Flowable, FMC

Corp., Philadelphia, PA) was applied to the base of each plant on which *B. cinerea* was observed, by applying 1 liter of suspension (6 ml product/liter) to all 40 cabbage plants using a handheld, 1 liter spray bottle (Rubbermaid, Hoboken, NJ). The insecticide imidacloprid (Leverage 2.7, Bayer CropScience LP, Research Triangle Park, NC) also was applied to the plants using a garden sprayer (Project Source) on 8 June 2015 to manage aphids (3.8 liters of suspension mixed at 1.25 ml of product/liter water). The two humidity chamber frames were covered again with plastic and Remay on 10 June 2015, and the plants inoculated a second time with *P. brassicae* the following day using the inoculation protocol previously described. After inoculation, plants were placed in the humidity chambers again for two days.

An attempt also was made to produce a mustard (*B. juncea*) seed lot infected with *P. brassicae* because of potential differences in propensity for *P. brassicae* to be seedborne and seed transmitted in different *Brassica* spp. (Karolewski 2010; Maddock and Ingram 1981). Seed of the mustard cv. Caliente 199 (High Performance Seeds, Inc., Moses Lake, WA) were planted on 7 April 2015 in RediEarth Seedling Starter Mix in 72-cell flats (2 seed/cell). The seedlings were transplanted into 3.8 liter (1 gal) pots filled with Sunshine Mix No. 1 and moved from the greenhouse to a screenhouse at the WSU Mount Vernon NWREC on 25 May 2015. Plants were repotted into 7.6-liter pots (one plant/pot) 1 week later. European honey bees in a nuclear hive were used to pollinate the mustard flowers.

Once the mustard plants had pods developing from >80% of the flowers, the plants were moved into greenhouse Bay 102 at the WSU Mount Vernon NWREC and placed in the same humidity chambers that had been used for the cabbage pod inoculations. The height of each humidity chambers was raised to 2.4 m to accommodate the tall mustard plants. On 24 June 2015, the humidity chambers were covered with plastic and two layers of Remay. A similar protocol as that described previously for the production of infected cabbage seed was followed to inoculate the developing pods on 70 mustard plants with *P. brassicae*. Inoculum of *P. brassicae* was produced using the method described above for

the cabbage pod inoculations, and the spore suspension sprayed onto the mustard plants (3.5 liters of 1 x 10^5 spores/ml applied to 70 plants). Plants were placed in the humidity chambers for four days under plastic and shade cloth, uncovered on 29 June 2015, and the misters turned on for 30 s/h the following day. Powdery mildew, caused by *Erysiphe cruciferarum*, was observed on the mustard stems, and saprophytic fungi such as *Cladosporium* spp. had started to grow on the bamboo stakes and twine supporting the plants. The inoculation was repeated on 8 July 2015 using the same spore concentration and volume of application to promote pod and seed infection by *P. brassicae*. The plants were maintained in the greenhouse at 15 to 20°C with 12 h light/day. Watchdog data loggers were used to record the temperature and RH at 1-h intervals.

2.2.1.4. Species identity of *N. capsellae* isolates. Nine single-spore isolates (Psc001 to Psc009) of *N. capsellae* were obtained from the same 2015 Willamette Valley 'Barkant' turnip seed crop from which the *P. brassicae* isolates were obtained. The single-spore isolates were each stored on filter disks at -20°C as described above for the *P. brassicae* isolates. Spores of *N. capsellae* were not observed on cPDA or WA plates, so various agar media were tested to induce sporulation of the isolates. A colonized Petri plate of cPDA of each isolate was flooded with 6 ml of sterilized, deionized water, and the hyphae dislodged using a sterilized, bent glass rod to generate a hyphal fragment suspension. A 1-ml aliquot of each hyphal suspension was spread onto Petri plates containing one of each of five agar media, as described by Inman et al. (1991), i.e., malt extract agar (33.6 g malt extract/liter water); 10% clarified V8 juice agar (prepared as described above); 20% V8 juice agar (200 ml V8 juice per liter, 2.0 g $CaCO_3$ per liter, 15 g agar, and 800 ml deionized water/liter) (Jeffers 2007); straw agar (water agar with five 5.0-cm-long pieces of sterilized straw) (Rawlinson et al. 1978); and half-strength PDA (½PDA). Conidia were observed on both clarified and unclarified V8 agar media and, to a lesser extent, on malt extract agar and straw agar, but not on ½PDA. Therefore, clarified V8 agar medium was used to produce

inoculum of *N. capsellae*. The identity of the isolates was confirmed by sequencing the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). DNA was grown, extracted, amplified, sequenced, and analyzed as described above for the *P. brassicae* isolates.

2.2.1.5. Pathogenicity test of *N. capsellae* isolates. The pathogenicity test for isolates Psc001 to Psc009 of *N. capsellae* was completed using the protocol described for the *P. brassicae* pathogenicity test, with the following exceptions: spore suspensions could only be concentrated to 1 x 10^4 spores/ml, and the limited amount of inoculum produced on the agar plates necessitated inoculating different numbers of plants with *N. capsellae*, depending on the isolate: isolate Psc001 and Psco002 were inoculated onto 9 plants, 3 of each host type; Psc003 was inoculated onto 15 plants, 5 of each host type; Psc004 to Psc008 were inoculated onto 14 plants, 4 cauliflower, 5 kale, and 5 turnip; and Psc009 was inoculated onto 5 plants, 1 cauliflower, 3 kale, and 1 turnip. The inoculum concentration used for isolate Psc009 was only 1 x 10^3 spores/ml because of the difficulty of procuring enough spores of this isolate.

The pathogenicity test was set up as a 3 x 10 factorial treatment design with three brassica host types (cauliflower, kale, and turnip) and 10 inoculation treatments (9 isolates of *N. capsellae* and a water control treatment), with replication as a random factor and plant species and fungal isolate as fixed factors in the model. Plants inoculated with *N. capsellae* or water (control treatment) were placed back into the plastic bags for 48 h post-inoculation instead of the 96 h used in the *P. brassicae* pathogenicity test described above. Plants were arranged in a RCB design on the benches of the greenhouse when removed from the plastic bags. Each plant was evaluated for the percentage of leaf area with light leaf spot symptoms by rating three leaves/plant 14 dai. A second rating was completed 20 dai. Glass slide mounts made from leaf lesions were examined microscopically, and isolations completed from the lesions onto clarified V8 agar medium to verify the identity of the fungus. The mean white leaf spot

severity rating (percentage of each leaf surface area with symptoms) for three leaves/plant for each replication of a treatment combination were subjected to ANOVA. White leaf spot symptoms were not detected on the control plants, so results for the control plants were not included in the ANOVA, i.e., the statistical analysis was run for a 3 x 9 factorial treatment design. The data were log-transformed to meet the assumptions of normality and equal variance for parametric analysis. Treatment means were compared using Fisher's protected LSD at $P < 0.05$.

2.2.1.6. Production of brassica seed infected with *N. capsellae.* Plants of the two proprietary cabbage inbred lines and the mustard cv. Caliente 199 were grown to flowering and pod set as described above, alongside the plants grown for pod inoculation with *P. brassicae*. When the plants of each species were ready to be inoculated, a conidial suspension of *N. capsellae* was made by flooding plates of all nine single-spore isolates, Psc001 to Psc009, with sterilized, deionized water, and then gently scraping the surface of the agar medium with a flame-sterilized, thin, metal spatula. Each spore suspension was strained through two layers of cheesecloth and quantified using a hemocytometer. Spore suspensions for the nine isolates were combined, quantified again with a hemocytometer, and diluted with deionized water to 1×10^4 spores/ml. As detailed above, 0.5% gelatin was added (5 g/liter) to aid adherence of the spores to the cabbage and mustard racemes and siliques. The spore suspension was applied to the plants as described for the *P. brassicae* inoculations, and the plants were maintained in humidity chambers in the greenhouse under the same conditions. Gray mold (*B. cinerea*) was observed on the *N. caspsellae*-inoculated plants, so iprodione (Rovral 4F Flowable) was applied to the base of each plant, and imidacloprid (Leverage 2.7) was applied for aphid control on the same dates and at the same rates detailed above for the *P. brassicae*-inoculated plants. The frames of the humidity chambers were covered with plastic and Remay again in preparation for inoculating the plants a second

time with *N. capsellae,* using the same protocol. The cabbage plants were inoculated on 4 June and 12 June 2015, and the Caliente 199 mustard plants were inoculated on 25 June and 8 July 2015.

2.2.1.7. Seed harvest. The cabbage and mustard plants inoculated with each fungus were harvested when the seed pods had fully matured and dried. Pods were harvested separately for each of the female and male cabbage inbred lines, and the mustard plants, for each pathogen. Each of the seed lots was cleaned, and then sized with screen sizes 3.5 to 7.0 for the four cabbage lots, and screen sizes 3.5 to 6.0 for the two mustard lots, to meet industry standards for marketable seed sizes of these species. The quality of the cabbage and mustard seed lots harvested from the *P. brassicae*-inoculated and *N. capsellae*-inoculated plants was then assessed. For the seed lots harvested from *P. brassicae*-inoculated plants, four replications of 100 seed for each seed lot were subjected to the blotter germination assay of the Association of Official Seed Analysts (AOSA) (Yaklich 1985). For the *N. capsellae*-inoculated lots, only one replication of 100 seed for each seed lot was subjected to the blotter germination assay, following results of the seed health assay (see Results). For each replicate sample of 100 seed of each lot, 50 seed were placed onto a sheet of seed germination blotter paper (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 second set of 50 seeds/replication/lot were set up similarly, for a total of 100 seeds assayed/replication/seed lot. The seeds on each blotter were covered with another germination blotter moistened with deionized water. The blotters were rolled, secured with a rubber band, and stored in an upright position in a plastic bag (10 rolls/plastic bag). Each bag was placed into a plastic bucket in an incubator (Model 130BLL, Percival Scientific, Perry, IA) set at 30°C with black (near UV) light for 8 h/day, and 20°C without light for 16 h/day. For the mustard seed lots, counts of normally germinated seed were completed after 3 and 7 days; and counts of non-germinated, abnormally germinated, and rotten seed were carried out after 7 days. For the cabbage seed lots, counts of

normally germinated seed were completed after 3 and 10 days; and counts of non-germinated, abnormally germinated, and rotten seed were done after 10 days, as detailed by the AOSA blotter seed germination assay (Yaklich 1985). Data were analyzed by calculating the means and standard errors of the percentage normally germinated, non-germinated, abnormally germinated, and rotten seed of each lot.

2.2.2. Evaluation of seed health assays for *P. brassicae* and *N. capsellae*. Seed health assays were evaluated to assess whether the mustard or cabbage seed lots produced were infected with *P. brassicae* or *N. capsellae* and, if so, to quantify the incidence(s) of infection. The first protocol used an NP-10 agar medium assay that was developed to detect and quantify infection of spinach seed lots by *Verticillium dahliae* and *Stemphylium botryosum* [du Toit et al. 2005, 2014; International Seed Health Initiative (ISHI) Vegetable 2015]. NP-10 agar medium, as described in the ISHI Veg (2015) protocol, was poured into acrylic boxes, each 110 mm x 110 mm x 35 mm deep (156CB, Hoffman Manufacturing, Inc., Jefferson, OR), after the boxes and lids had been sterilized with 70% ethyl alcohol in a laminar flow hood followed by exposure to UV light in a biological safety cabinet for 20 min. Sorensen's NP-10 semi-selective agar medium (Sorensen et al. 1991) was prepared as described in the ISHI-Veg (2015) protocol. The two components of the medium were prepared separately and autoclaved: suspension A included 15 g Bacto agar (Difco Laboratories, Detroit, MI), 1 g KNO_3, 1 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 ml Tergitol (NP-10; Sigma-Aldrich), and 500 ml distilled water, which were mixed using a magnetic stir bar; and suspension B contained 500 ml distilled water, 5.0 g polygalacturonic acid (Sigma-Aldrich), and 1.0 g NaOH. Once both autoclaved suspensions had cooled to <50°C, 50 mg chloramphenicol (50 ppm), 50 mg streptomycin sulfate (50 ppm), and 50 mg chlortetracycline hydrochloride (50 ppm) were added to suspension A, and the suspensions combined. The combined suspension was dispersed into the boxes (40 ml/box). Seed (100/replication/lot) were plated onto the agar medium in each of three boxes (32 to

34 seed/box), and incubated for 14 days at 20 ± 2°C with alternating 12 h periods of dark and 12 h periods of black (near-UV) plus cool white fluorescent light. The seed were then examined microscopically 4, 10, and 14 days after plating to identify fungi developing on the seed.

The NP-10 agar protocol described above was repeated with a sample of surface-sterilized seed from each of the six seed lots (three cabbage lots and one mustard lot per pathogen). The seeds were placed in a mesh tea strainer (Harold Import Co., Lakewood, NJ), immersed in 1.2% NaOCl in a glass beaker for 60 s, and then triple-rinsed in sterilized, deionized water, dried on sterilized paper towels in a laminar flow hood, stored overnight in sterilized Petri plates, and plated onto NP-10 agar medium. For both the non-sterilized and surface-sterilized seed samples, fast growing fungi (particularly *Alternaria* spp., *Cladosporium* spp., etc.) developed very rapidly on the seed, obfuscating the potential presence of *P. brassicae* and *N. capsellae* on the seed. Therefore, as a variation of the NP-10 agar protocol, seed was plated onto NP-10 agar medium as described above, and the boxes of seed were then enclosed in a cardboard box (to keep out light) at 4°C for 21 days, to use a cold incubation temperature to inhibit the development of rapidly growing fungi. Microscopic seed readings were done 21, 28, and 35 days after plating the seed. This protocol was assessed to determine if a cold incubation temperature enabled slow-growing *P. brassicae* and *N. capsellae* to develop adequately on the seed for detection microscopically (Maddock and Ingram 1981). This protocol was completed for a sample of non-sterilized seed and a sample of surface-sterilized seed of each lot, with the surface-sterilization completed as described above.

Data were analyzed by calculating the means and standard errors of the percentage of each seed lot infected with either *P. brassicae* or *N. capsellae*, as well as *Alternaria* spp. and *Cladosporium* spp., because high levels of infection of the latter two genera could obstruct the ability to detect the target fungal pathogens. The standard error was not calculated for those seed lots and protocols for

which only one replication of 100 seeds was tested, i.e., the 20°C NP-10 agar protocol with surface-sterilized and non-sterilized seed of each lot, because it became obvious that this protocol did not enable the detection of either *P. brassicae* or *N. capsellae* on seed (see Results).

2.2.3. Seed transmission of *P. brassicae.* Using the 4°C NP-10 agar seed health assay described above for both surface-sterilized and non-sterilized seed, *P. brassicae* was detected on the mustard seed lot harvested from plants of Caliente 199 that had been inoculated with this fungus, and on the seed of both the male and female lines of cabbage (see Results). However, *N. capsellae* was not detected on either of the cabbage lots or the mustard seed lot harvested from the plants inoculated with this fungus, so these three lots could not be used to assess the potential for seed transmission of *N. capsellae*.

Seed transmission assays were carried out to determine if *P. brassicae* could be transmitted from the infected mustard and cabbage seed lots to seedlings that developed from the seed. Seed transmission trials were started in West Bay 1 of the greenhouses at the WSU Mount Vernon NWREC in the spring of 2016. The *P. brassicae*-infected cabbage and mustard seed lots were each planted into RediEarth Seedling Starter Mix in five flats (representing five replications), each consisting of 200 cells (1 seed/cell, each cell 33 mm diameter x 37 mm deep). In the repeat trial for the *P. brassicae*-inoculated mustard seed lot, a revised protocol was used in which three flats of seed were planted for each of five replications, with each flat containing only 72 cells (1 seed/cell, with each cell 38 mm diameter x 57 mm deep). Using flats with the larger diameter cells provided more space between plants to minimize the spread of *P. brassicae* between plants over the duration of the assay, as the objective was to measure primary seed transmission of *P. brassicae*. Plants in the flats were irrigated with tap water until the first sign of emergence, approximately 3 to 5 days after planting. Thereafter, two microsprinklers (Modular nozzle, NaanDanJain Irrigation, Ltd, Israel) were each staked 50 cm above the surface of each bench to irrigate the seedlings while also creating ideal conditions for extended periods of leaf wetness to

promote the development of light leaf spot. Using the CycleStat II Precision Repeat Cycle Timer, the misters were set to mist for 10 s every 30 min for the first 4 days after emergence, and 10 s every 45 min for the remainder of the study, similar to the spinach seed transmission trials developed by Hernandez-Perez (2005) for the fungi that cause Stemphylium leaf spot and Cladosporium leaf spot. Depending on environmental conditions in the greenhouse, which were affected by the heaters operating in the winter months or the swamp coolers operating in the summer months, and/or the greenhouse bay used, the timing of misting was adjusted to ensure extended durations of leaf wetness as measured with a leaf wetness sensor (Spectrum Technologies, Inc., Plainfield, IL). The most common misting frequency and duration used for the seed transmission trials was misting for 30 s every 30 min.

The incidence (%) of seedlings that developed suspect symptoms of light leaf spot was recorded for each flat. Isolations were carried out from symptomatic cotyledons or first true leaves by removing each symptomatic seedling from the flat and marking the seedling location in the flat with a pin. The whole symptomatic cotyledon or the lesion on the leaf was dipped into 70% ethyl alcohol for 3 to 7 s, dried on sterilized paper towel in a laminar flow hood, plated onto ½cPDA, and incubated with cool white fluorescent light and NUV light for 8 h/day at 15°C and 16 h of dark/day at 10°C (Model 130BLL, Percival Scientific). If signs (acervuli and spores) of *P. brassicae* were observed on the tissues microscopically, the incidence (%) of seedlings from which *P. brassicae* was recovered was calculated. The rate of seed transmission was calculated as a percentage of: i) the total number of seed planted, and ii) number of *P. brassicae*-infected seed planted as using non-sterilized seed subjected to the 4°C NP-10 agar seed health assay describe above (averaged for seed health assay 1 and 2) (Table 2). The temperature was recorded hourly during each seed transmission trial using a WatchDog data logger stored inside a radiation shield (Spectrum Technologies, Inc.), and leaf wetness duration was recorded with a leaf wetness sensor placed adjacent to the data logger. Data were analyzed by calculating the

mean ± standard error of the percentage of infected seedlings detected for each seed lot based on the two calculations described above. The seed transmission assay was repeated for each of the three seed lots harvested from plants inoculated with *P. brassicae*.

2.3 Results

2.3.1 Species identity and pathogenicity of *P. brassicae* and *N. capsellae* isolates. The ITS rDNA sequences for the four putative *P. brassica* isolates, Cyc001, Cyc003, Cyc005, and Cyc006, were identical over a 626 nt length sequenced. The BLAST search in GenBank revealed the most similar ITS rDNA sequence was that of a *Cadaphora* sp. (JN859254.1) from Hungary, with only 96.00% similarity. One of the four ITS rDNA sequences of *P. brassicae* in GenBank at the time this work was completed, AJ305235.1 of an isolate collected in the UK, was 95.00% similar to the ITS rDNA sequences of the four Oregon isolates (MF143610). The other three *P. brassicae* ITS rDNA sequences in GenBank did not feature in the BLAST search results. In contrast, the β tubulin gene sequences for these four isolates were identical over a 756 nt length sequenced, and 98.00% similar to that of a single *P. brassicae* isolate in GenBank (KC342227.1) collected in France (Carter et al. 2013). In addition to the species identification based on β tubulin sequencing, the morphological characteristics of all four isolates were typical of those of *P. brassicae,* i.e., hyaline, smooth, cylindrical, mostly aseptate and eguttulate conidia (each 10 to 16 x 3 to 4 μm) produced on short (10.0 to 16.0 x 2.5 to 4.0 um), non-branching conidiophores (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977). The PCR assays attempted with primers developed by Foster et al. (1999) for the MAT-1 and MAT-2 idiomorphs were unsuccessful as DNA was not amplified for either mating type of all four isolates. However, the lack of access at that time to control isolates of *P. brassicae* of each mating type limited the capacity to assess if the results reflected the need to optimize the protocol or if the primers and PCR protocol do not work with these USA isolates.

The ITS rDNA sequences of the nine putative *N. capsellae* isolates, Psc001 to Psc009, were identical over the 533 nt amplified (MF158312). All nine sequences were 99.00% similar to the ITS rDNA sequence of an *N. capsellae* isolate in GenBank (Accession No. GU214662.1) collected in the UK. The isolates also were all confirmed morphologically as *N. capsellae* by observing hyaline, straight or slightly curved conidia that were each 1- to 5-septate, and 30 to 90 μm x 2 to 3 μm (Crossan 1954; Deighton 1973). Isolation of *N. capsellae* onto 10% clarified V8 agar resulted in the development of dematiaceous, stromatic structures that gave rise to the conidia described by Crossan (1954). Therefore, based on the ITS rDNA sequences and morphological characteristics, isolates Psc001 to Psc009 were identified as *N. capsellae.*

The two isolates of *P. brassicae* tested for pathogenicity initially both caused symptoms typical of light leaf spot on all three types of brassica plants tested (Fig. 2.1), whereas the control plants of each host remained asymptomatic. The second severity rating (20 dai) was used for data analysis because disease severity was very limited on the cauliflower and kale plants at the first rating completed 14 dai (0.67 ± 0.06% for cauliflower, 0.50 ± 0.15% for kale, and 57.08 ± 5.50% for turnip). Based on the ANOVA, significant differences in the severity of light leaf spot were detected among the three host plant species tested (P <0.001). The turnip plants were more susceptible to light leaf spot (85.42 ± 7.53% severity of symptoms) than the cauliflower plants (10.33 ± 3.50%) and kale plants (1.13 ± 0.19%) based on Fisher's protected LSD. The severity of symptoms did not differ significantly on cauliflower vs. kale plants. There was no significant difference (P = 0.564) between the two isolates of *P. brassicae* tested (30.42 ± 12.17 and 34.17 ± 11.89% when averaged across the three host species), and the interaction term between host plant type and isolate of *P. brassicae* was not significant (P = 0.914).

Turnip plants inoculated with *P. brassicae* were the first to show symptoms, with pale brown streaks on the stem and veinal browning that darkened over time (Fig. 2.1B). This was also the primary

symptom observed on the inoculated kale plants (Fig. 2.1A). Veinal browning was followed by the development of small (<5 mm diameter), chlorotic leaf spots. The chlorotic spots became diffuse and expanded in size quickly on the turnip leaves, coalescing and covering the leaf surface by 20 dai (Fig. 2.1C). Symptoms developed more slowly on the cauliflower leaves (Fig. 2.1D), although lesions on this host also coalesced, causing leaves to begin to senesce by approximately 20 dai. Hyaline, smooth, cylindrical, and mostly aseptate and eguttulate conidia were observed on short, non-branching conidiophores in slide mounts prepared from symptomatic lesions on the plants inoculated with *P. brassicae*, consistent with the typical morphology of *P. brassicae* (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977). However, the white, subcuticular conidiomata described by Rawlinson et al. (1978) and Fitt et al. (1998) were not observed on any of the plants inoculated. The repeat pathogenicity test with isolates of *P. brassicae* produced similar results (*data not shown*).

All nine isolates of *N. capsellae* caused symptoms typical of white leaf spot, whereas the control plants of all three brassica species did not develop symptoms of this disease (Fig. 2.2). The second severity rating completed 20 dai was used in the ANOVA because the severity of white leaf spot was limited at the first rating completed 14 dai (2.86 ± 0.20%). Similar to the *P. brassicae* pathogenicity test, the ANOVA revealed significant differences (P <0.001) among the three brassica hosts, with turnip plants (8.22 ± 0.67% severity of white leaf spot) more susceptible to white leaf spot than cauliflower (3.47 ± 0.37%) and kale (3.97 ± 0.54% plants), and no significant differences in severity of symptoms between the cauliflower and kale plants. Significant differences in the severity of white leaf spot were detected among the nine isolates (P <0.0001) (Fig. 2.3). The interaction term in the ANOVA for host species and fungal isolate was not significant (P = 0.065). The most virulent isolates were Psc001, Psc003, Psc004, Psc005, and Psc006, which caused a range of 5.57 to 8.15% severity of white leaf spot; while the least

virulent isolates were Psc007, Psc008, and Psc002 (listed in decreasing order of mean severity of leaf spot), which caused a range of 2.47 to 4.24% severity of symptoms (Fig. 2.3).

Symptoms of white leaf spot initially appeared as small (1- to 3-mm-diameter), pale spots, each surrounded by a chartreuse halo (Fig. 2.2A and 2.2B). Ten days after inoculation, lesions each turned white to gray with a dark, definite margin, surrounded by a pale halo (Fig. 2.2C and 2.2D). Lesions enlarged and coalesced, with infected leaves senescing by 20 dai (Fig. 2.2E and 2.2F). *N. capsellae* sporulation in the lesions was confirmed morphologically 21 dai when slide mounts were made from lesions that developed on the *Brassica* species tested, by observing hyaline, straight or slightly curved conidia that were 1- to 5-septate (Crossan 1954; Deighton 1973). Re-isolations of *N. capsellae* onto V8 agar medium from lesions on the leaves resulted in the development of dematiaceous, stromatic structures that gave rise to the conidia described by Crossan (1954).

2.3.2 Seed infection by *P. brassicae* and *N. capsellae.* Inoculating pods of the mustard and cabbage plants with *P. brassicae* resulted in the development of black flecks on the pods and stems, that expanded as infection progressed (Fig. 2.4A, 2.4B, and 2.4D). Slide mounts of sections of these lesions that were examined microscopically, and isolations from these lesions onto ½cPDA, confirmed that the symptoms were caused by *P. brassicae.* Symptoms did not develop on any of the cabbage pods of either parent line or the mustard pods inoculated with *N. capsellae* (Fig. 2.4C), though the pathogen was isolated from symptomatic leaves of the mustard plants. The Watchdog data loggers recorded an average daytime (7 am to 7 pm) RH of 78.75 ± 15.59% and temperature of 20.97 ± 2.34°C, and a nighttime (7 pm to 7 am) average RH of 80.22 ± 16.53% and temperature of 19.38 ± 0.94°C during the cabbage seed crop incubation following inoculation. During the mustard seed crop incubation after inoculation, the average daytime RH was 78.72 ± 11.87% and the temperature was 21.81 ± 2.33°C, and night time conditions had an average RH of 82.25 ± 14.97% and temperature of 19.73 ± 0.72°C.

Germination assays for the seed lots harvested from cabbage and mustard plants inoculated with *P. brassicae* revealed high rates of normal seed germination for all three lots, i.e., 97.75 ± 0.63, 98.25 ± 0.25, and 95.50 ± 1.04% for the cabbage female seed lot, cabbage male seed lot, and mustard seed lot, respectively. The incidence of seed with abnormal germination harvested from the cabbage female, cabbage male, and mustard seed lots was low, i.e., 0.75 ± 0.25, 0.25 ± 0.25, and 1.25 ± 0.75%, respectively. Likewise, the incidence of rotten seed was low (0.50 ± 0.29, 1.00 ± 0.58, and 2.75 ± 1.25%, respectively); and the incidence of non-germinated seed was very limited (1.00 ± 0.41, 0.50 ± 0.50, and 0.50 ± 0.29%, respectively). The high rates of normal germination for all three seed lots facilitated evaluation of seed transmission of *P. brassicae* for these lots that were infected with the pathogen (see below).

P. brassicae and *N. capsellae* were not detected on any of the six seed lots when tested on NP-10 agar medium with incubation at 20°C with NUV and cool white fluorescent light for 12 h/day (Table 2.1). High incidences of colonization by fast-growing *Alternaria* spp. and *Cladosporium* spp. were detected on all the seed lots (Table 2.1), obscuring the ability to observe microscopically the slow-growing target pathogens, *P. brassicae* and *N. capsellae*. *Alternaria* spp. were detected on surface-sterilized and non-sterilized seed at the following incidences when the seed were incubated at 20°C for 14 days: 21.00 and 33.00%, respectively, for the female cabbage seed lot; 29.00 and 75.00%, respectively, for the male cabbage seed lot; and 47.00 and 99.00%, respectively, for the mustard seed lot. The incidences of *Cladosporium* spp. observed on surface-sterilized and non-sterilized seed incubated on NP-10 agar medium at 20°C were 0.00 and 64.00%, respectively, for the female cabbage seed lot; 0.00 and 91.00%, respectively, for the male cabbage seed lot; and 0.00 and 96.00%, respectively, for the mustard seed lot (Table 2.1).

When the seed were incubated on NP-10 agar medium at 4°C, the incidences of *Alternaria* spp. observed on surface-sterilized and non-sterilized were 2.25 ± 0.75 and 7.00 ± 1.22%, respectively, for the female cabbage seed lot; 4.50 ± 0.87 and 6.00 ± 0.71%, respectively, for the male cabbage seed lot; and 12.75 ± 3.09 and 20.75 ± 5.11%, respectively, for the mustard seed lot by 35 days (Table 2.1). Similarly, when this 4°C version of the assay was repeated, the incidences of *Alternaria* spp. observed on surface-sterilized and non-sterilized seed were 1.50 ± 1.00 and 5.75 ± 0.75%, respectively, for the female cabbage seed lot; 4.75 ± 1.31 and 5.50 ± 0.87%, respectively, for the male cabbage seed lot; and 4.50 ± 0.87 and 14.25 ± 4.39%, respectively, for the mustard seed lot after 35 days (Table 2.1). *Cladosporium* spp. were observed on surface-sterilized and non-sterilized seed incubated at 4°C at 0.50 ± 0.29 and 84.25 ± 2.50%, respectively, for the female cabbage seed lot; 0.25 ± 0.25 and 96.75 ± 0.75%, respectively, for the male cabbage seed; and 0.25 ± 0.25 and 98.50 ± 1.19%, respectively, for the mustard seed lot (Table 2.1). Likewise, in the repeated 4°C assays, the incidences of *Cladosporium* spp., detected on surface-sterilized and non-sterilized seed were 0.25 ± 0.25 and 71.75 ± 7.23%, respectively, for the female cabbage seed lot; 1.25 ± 0.95 and 92.50 ± 1.85%, respectively, for the male cabbage seed lot; and 1.00 ± 0.41 and 94.50 ± 1.32%, respectively, for the mustard seed lot after 35 days (Table 2.1).

In contrast to the 20°C NP-10 agar seed health assay, when the seed lots harvested from plants inoculated with *P. brassicae* were incubated on NP-10 agar medium at 4°C and examined microscopically after 21, 28, and 35 days, *P. brassicae* was observed on 12.50 ± 2.06 and 12.50 ± 0.65% of the surface-sterilized and non-sterilized mustard seed, respectively, in the first assay; and 10.50 ± 1.19 and 19.75 ± 2.14% of the surface-sterilized and non-sterilized mustard seed in the repeat assay, respectively (Table 2.1). *P. brassicae* was only observed on 0.25 ± 0.25 and 0.50 ± 0.29% of the surface-sterilized and non-sterilized seed, respectively, of the female cabbage seed lot in the first assay; and on 0.50 ± 0.50 and 0.50 ± 0.29%, respectively, of the seed of that lot in the repeat assay. Likewise, *P.*

brassicae was detected on only 0.50 ± 0.29% of both the non-sterilized and surface-sterilized seed of the male cabbage seed lot in the first seed health assay when the seed was incubated on NP-10 agar medium at 4°C; but was not detected on this seed lot in the second (repeat) assay (Table 2.1). None of the seed health assay protocols evaluated revealed the presence of *N. capsellae* on the three seed lots harvested from plants inoculated with that fungus, even when the seed samples were incubated at 4°C, regardless of whether the seed were surface-sterilized or not (Table 2.1).

Signs of *P. brassicae* on infected seed incubated at 4°C on NP-10 agar medium were indistinct (Fig. 2.5). Conidiophores and conidial masses developed on the brassica seed coat, but not on the cotyledons or radicles that developed from the seed that germinated on the agar medium. Therefore, true acervuli were not observed as there was no overlying integument of host tissue that could be ruptured when the spore masses matured (Kirk et al. 2001). The initial signs of *P. brassicae* on the cabbage seed were pale pink, distinct, separate, gelatinous masses of conidia on very short conidiophores (Fig. 2.5A). As the *P. brassicae* structures matured, the gelatinous masses of conidia expanded in size and became less distinct (Fig. 2.5B). Virtually no mycelial growth was observed in association with the spore masses of *P. brassicae* on infected cabbage or mustard seed. Detailed microscopic examination of the conidia at 400 to 1,000X magnification was necessary to differentiate *P. brassicae* from spore masses of other fungi such as *Fusarium* spp. or even yeasts that can develop similar pale pink, gelatinous masses on seed (Barnett and Hunter 1998).

2.3.3 Seed transmission of *P. brassicae.* Since *N. capsellae* was not detected on any of the three seed lots harvested from plants inoculated with that pathogen, seed transmission assays could not be completed for that pathogen with those seed lots. The first set of seed transmission assays for the *P. brassicae*-infected cabbage and mustard seed lots did not result in the development of characteristic light leaf spot symptoms on the cotyledons of either host type, but did produce symptoms on the first

true leaves, so symptoms on the first true leaves were used to quantify seed transmission visually (Fig. 2.6). Subsequent seed transmission assays performed in a different greenhouse (Bay 103) resulted in more distinct light leaf spot symptoms on both the cotyledons and first true leaves of seedlings that grew from the infected mustard seed lot. Of the three *P. brassicae*-infected seed lots, seed transmission was not detected for the male cabbage seed lot, which was infected at a range of 0 to 0.50% incidence based on the seed health assays. A single seed transmission event (one infected seedling) was detected out of 1,000 seed planted in the first seed transmission trial of the female cabbage seed lot, resulting in a seed transmission rate of 0.10 ± 0.10% for the entire seed lot, or 20.00% from the 0.50% of *P. brassicae*-infected seed in that lot. Seed transmission was not detected for either cabbage seed lot in the repeat trial. For the mustard seed lot, seed transmission of *P. brassicae* occurred at a rate of 1.6 ± 0.50% for the entire seed lot, or 9.92% from the 16.13% of *P. brassicae*-infected seed in the first seed transmission assay; and at a rate of 0.50 ± 0.20% for the lot, or 3.10% for the *P. brassicae*-infected seed planted in the repeat trial; and at a rate of 0.70 ± 0.20% for the lot or 4.69% for the *P.* brassicae-infected seed in the third trial that was planted into 72-cell flats.

Symptoms of light leaf spot caused by *P. brassicae* in the seed transmission trials included chlorotic lesions on the cotyledons with light brown spots (Fig. 2.6A); and distinct, chlorotic spots, each developing a necrotic center, along with veinal browning on the lower surface of the first pair of true leaves (Fig. 2.6B). After plating and incubating the symptomatic leaves and cotyledons, gelatinous conidial masses were observed microscopically on the tissue pieces, and the conidial characteristics confirmed as those of *P. brassicae* (Fig. 2.6C). Greenhouse temperatures from 21 January to 25 April 2016, the period over which these seed transmission trials were completed, averaged 17.69 ± 1.71°C by day (7 am to 7 pm) and 16.68 ± 0.81°C by night (7 pm to 7 am); and leaf wetness readings averaged 9.77 ± 4.14 min/h by day and 11.68 ± 2.03 min/h by night.

2.4 Discussion

The potential for *P. brassicae* to be seedborne and seed transmitted was demonstrated conclusively in this study by growing infected seed crops of mustard and cabbage, developing a seed health assay to detect the pathogen on seed, and quantifying the rates of seed transmission. However, neither seed infection nor seed transmission of *N. capsellae* was detected on the seed grown from mustard and cabbage plants inoculated with the pathogen. All six brassica seed lots grown in this study had high-quality seed as demonstrated by seed germination rate of 95 to 98%. Despite inoculating the cabbage and mustard plants twice under conditions that should have been conducive for development of light and white leaf spot, symptoms were observed on the pods of plants inoculated with *P. brassicae* but not on any of the pods of plants inoculated with *N. capsellae*. Therefore, it was not surprising that *N. capsellae* was not detected on the seed harvested from the cabbage (male and female lines) and mustard plants when tested using the NP-10 agar seed health assay protocols evaluated. Very little research has been done on the seedborne aspects of *N. capsellae* (Crossan 1954; Petrie and Vanterpool 1978) and it remains unknown if different conditions are required for seed infection than the conditions used in this study. Based on Inman's dissertation (1992), the optimal temperatures for spore germination (15 to 20°C) and minimum leaf wetness requirements for infection of plants by *N. capsellae* (6 to 8 h for oilseed rape leaves) were met. This was reflected in observation of white leaf spot symptoms on the leaves of the inoculated cabbage and mustard plants, but symptoms did not develop on the pods of either species.

One factor that might have affected the development of *N. capsellae* on the inoculated mustard and cabbage pods was stomatal density. Inman (1992) found that germinating conidia of *N. capsellae* enter the host through stomata. He noted that stomatal density on abaxial leaf surfaces was two times greater than on the upper leaf surface, and observed six times more lesions on the abaxial leaf surfaces

than on the adaxial leaf surfaces of inoculated leaves of oilseed rape. Esau (1960) wrote that although stomatal frequency can vary greatly, stomata are most abundant on leaves. On a study of stomatal frequency (density) and distribution in rape, Major (1975) demonstrated that on *B. campestris* cv. Span and *B. napus* cv. Zephyr the lower leaf surface had the greatest stomatal density, pedicels and stems had the fewest stomata, and siliques (pods) and beak tissue of the pods had an intermediate density of stomata. Unlike *N. capsellae, P. brassicae* infects the plant directly through the cuticle (Rawlinson et al. 1978). Therefore, although symptoms of white leaf spot were observed on the leaves of mustard and cabbage plants inoculated with *N. capsellae* in this study, the severity of symptoms was limited on these plants, and the reduced density of stomata on the pods vs. leaves may have limited infection of the pods by *N. capsellae*. This is particularly relevant given the lower inoculum concentration of *N. capsellae* (1 x 10^4 spores/ml) vs. that of *P. brassicae* (1 x 10^5 spores/ml) used to inoculate the plants because of the more limited numbers of spores produced by *N. capsellae* vs *P. brassicae* on agar media.

In contrast to the *N. capsellae* results, *P. brassicae* was isolated from pods of the *P. brassicae*-inoculated mustard and cabbage plants, and infection of seed of all three lots by *P. brassicae* was detected using the 4°C NP-10 agar seed health assay. Compared to the 20°C NP-10 agar seed health assay, the 4°C assay slowed the growth of *Alternaria* spp. and *Cladosporium* spp. This cooler incubation temperature proved critical to prevent the obfuscation of *P. brassicae* on the seed by fast-growing fungi commonly found on brassica seed. This is consistent with the results of Maddock and Ingram (1981) who found that, although incubating *B. napus* and *B. campestris* seed in the dark at 20°C was ideal for the growth of *P. brassicae,* incubation of seed at 10°C inhibited the growth of saprophytic, fast-growing fungi and facilitated detection of *P. brassicae* on the seed microscopically. Using 4°C instead of the 10°C incubation temperature Maddock and Ingram (1981) tested, might be preferable as 4°C is a common temperature at which refrigerators are operated. This also satisfies Neergaard's (1977) recommendation that seed health assays be designed with economic considerations in mind, such as equipment.

However, a major limitation of the 4°C NP-10 agar seed health assay is the duration required for incubation of the seed, with readings completed 21, 28, and 35 days after plating the seed. A 35-day seed health assay is probably too long for many stakeholders in the brassica seed industry because of the large numbers of seed lots, for rapid test results to identify seed lots that need to be treated for the pathogen, and the short turn-around between harvesting seed crops and shipping seed lots for planting seasonally in different areas of the world (McGee 1997; Munkvold 2009; Neergaard 1977). The International Seed Health Initiative (ISHI) validated standardized seed health protocols for *Phoma lingam* on brassica seed and *V. dahliae* on spinach seed that are completed in 14 days (ISHI 2011; ISHI 2015). Agar medium seed health assays for *A. dauci* on carrot (*Daucus carota*) seed are even shorter, with 9 days of incubation (International Seed Testing Association 2017). Alternatively, research is needed to develop a molecular-based (PCR) seed health assay for *P. brassicae*, which could serve as an initial tool to assess of whether or not a seed lot is infected with *P. brassicae.* For seed lots that test positive for *P. brassicae*, a quantitative PCR assay such as a real-time PCR assay may then be necessary, or a more traditional agar or blotter plating assay, to determine the percentage of seed infected, as well as the incidence of seed on which the pathogen is viable, given that most DNA-based seed health assays cannot differentiate DNA from viable vs. non-viable propagules of the target pathogen (Munkvold 2009).

The 4°C NP-10 agar seed health assay enabled detection of *P. brassicae* on an average of 12.50 ± 2.06 and 12.50 ± 0.65% of the surface-sterilized and non-sterilized seed of the mustard seed lot, respectively. When the seed assays were repeated, 10.50 ± 1.19 and 19.75 ± 2.14% of the surface-sterilized and non-sterilized mustard seed were infected with *P. brassicae*, respectively. Producing a seed lot with this incidence of seed infection, combined with the high quality (germination) of the seed lot, meant that the mustard seed lot could be used effectively for *P. brassicae* seed transmission trials, as well as research on seed treatments for controlling seedborne inoculum of *P. brassicae* (see Chapter 4). Much lower incidences of infection (≤0.50%) were detected on surface-sterilized and non-sterilized

seed of both the male and female cabbage seed lots, which limited the value of these lots to assess seed transmission rates of *P. brassicae*. It is possible that the differences in seed infection and seed transmission rates detected in the cabbage and mustard seed lots reflect differences in the susceptibility of *B. oleracea* vs. *B. juncea* to *P. brassicae* as observed in the pathogenicity test. However, the literature on pathogenicity of *P. brassicae* on *B. juncea* cultivars currently is limited to one study by Maddock and Ingram (1981), as most published research on *Brassica* species and cultivar susceptibility to *P. brassicae* has focused on the hosts *B. oleracea, B. napus,* and *B. rapa* (Karolewski 2010).

The fact that *P. brassicae* was detected on both surface-sterilized and non-sterilized mustard and cabbage seed indicated that infection of seed by the pathogen was more than a surface contamination on the seed coat. Neergaard (1977) noted that for most seed-transmitted imperfect fungi, the most common site of infection is the seed coat. Seed coat infections of *Brassica* spp. have been reported for multiple fungal pathogens, including *A. brassicicola* and *A. brassicae* (Neergaard 1977; Rimmer et al 2007). While this also is true for *P. lingam* infection of seed, Jacobsen and Williams (1971) occasionally found *P. lingam* infection to extend into the inner epidermis of the outer integument and inner integument of brassica seed. If *P. brassicae* can reside inside the seed coat or even in the embryo of brassica seed, it may be more difficult to use seed treatments such as disinfectants and fungicides to eradicate the pathogen from infected seed lots or prevent seed transmission (Maude 1996; Neergaard 1977).

Seed transmission of *P. brassicae* was demonstrated for both the infected mustard seed lot and the infected female cabbage seed lot in this study, but not for the infected male cabbage seed lot. In the first seed transmission assay, the rate of seed transmission of *P. brassicae* for the female cabbage seed lot was 0.10 ± 0.10%, which equated to 20.00% seed transmission from the 0.50% of *P. brassicae*-infected seed in that lot. However, seed transmission was not detected for either cabbage seed lot in the repeat trial. For the mustard seed lot, seed transmission of *P. brassicae* occurred at a rate of 1.6 ±

0.50% lot, which equated to 9.92% seed transmission of *P. brassicae* from the 16.13% infected seed in that lot during the first assay. In the repeat trial, seed transmission of *P. brassicae* occurred at a lower rate, 0.50 ± 0.20%, which equated to 3.10% transmission from the 16.13% infected seed in that lot. When the seed transmission trial was completed a third time using 72-cell flats instead of 200-cell flats, *P. brassicae* was transmitted to 0.70 ± 0.20% of the seedlings that developed, which equated to 4.69% transmission from the 16.13% of *P. brassicae*-infected seed in that lot.

Differences in seed transmission rates detected between the seed lots of cabbage vs. mustard, and among trials with the same lot may have resulted from different environmental conditions among the trials based on the time of year the trials were completed, differences among the greenhouse bays in which the trials were done, and potential differences in susceptibility to seed transmission between *B. oleracea* and *B. juncea* or even among cultivars of the same species. The first seed transmission assays were conducted in West Bay 1 at the WSU Mount Vernon NWREC, from February to April of 2016, with symptoms only observed on the first true leaves, not the cotyledons. For the seed transmission assays conducted in a different greenhouse, Bay 103 (described in Chapter 4), distinct symptoms were observed on the cotyledons and first true leaves. Temperature and leaf wetness did not vary widely between the two greenhouses (West Bay 1 averaged 17.69 ± 1.71°C by day and 16.68 ± 0.81°C by night, with an average leaf wetness of 9.77 ± 4.14 min/h by day and 11.68 ± 2.03 min/h by night; whereas Bay 103 averaged 16.71 ± 1.02°C by day and 15.81 ± 1.12°C by night, with an average leaf wetness of 9.77 ± 5.95 min/h by day and 10.80 ± 5.61 min/h by night). Therefore, variations in other aspects of the trials may have impacted seed transmission, e.g., greenhouse lighting, time of the year (daylength, radiation intensity), etc. Similar differences in seed transmission rates among repeat trials were reported by Hernandez-Perez (2005) in greenhouse trials evaluating transmission of *Stemphylium botryosum* and *Cladosporium variabile* from spinach seed. Wet conditions, similar to those generated in the seed transmission trial, were a requirement for seed transmission and the development of dry rot caused by

Leptosphaeria maculans on brassicas in New Zealand (Allen and Smith 1961). In that study, seed transmission was observed from brassica seed lots with an infection rate of 0.50%, if weather conditions were wet after planting; however, if conditions were dry after planting, seed transmission was not observed, even after planting seed lots infected with *L. maculans* at up to 3.00% (Allen and Smith 1961).

This study confirmed the pathogenicity to multiple brassica hosts of the *P. brassicae* and *N. capsellae* isolates collected from a turnip seed crop in the Willamette Valley, OR. All of the *P. brassicae* and *N. capsellae* isolates were pathogenic on all three hosts tested, cauliflower, kale, and turnip, with significant differences in severity of symptoms observed among the hosts. 'Hakurei' turnip plants were more susceptible to both light leaf spot and white leaf spot than 'Candid Charm' cauliflower plants and 'Black Magic' kale plants, with no significant differences between the cauliflower and kale plants for either pathogen. However, since only one cultivar of each brassica host was tested, the susceptibility of cultivars was confounded with susceptibility of the type of brassica host. Both pathogens also caused more severe symptoms on the mustard cv. Caliente 199 than the two proprietary cabbage inbred lines inoculated during pod development. These results are consistent with the published literature on *N. capsellae,* which was shown to be more virulent on *B. napus* and *B. rapa* than *B. oleracea*, in contrast to the closely related ringspot pathogen, *N. brassicae* (teleomorph *Mycosphaerella brassicicola*) that tends to be more virulent on *B. oleracea* hosts than other *Brassica* spp. (Crossan 1954; Petrie and Vanterpool 1978). *B. juncea* was more susceptible to *N. capsellae* than *B. carinata* and *B. napus* in a study by Gunasinghe et al. (2015). In contrast to *N. capsellae*, little research has been done on the pathogenicity of *P. brassicae* on cultivars of the hosts *B. rapa* and *B. juncea*. Most of the early literature on Brassicaceae host susceptibility to *P. brassicae* focused on the pathogen effects on various subspecies of *B. oleracea* (Hickman et al. 1955). The first light leaf spot epidemic of economic importance was reported on Brussels sprouts (*Brassica oleracea* var. *gemmifera*) in Ireland in 1964 (Majer et al. 1998; Staunton 1967). Subsequent outbreaks were reported on oilseed rape (*B. napus* and *B. rapa*), starting

with the first epidemic of economic importance reported in England during the 1974-75 growing season, and followed by the disease becoming increasingly prevalent on oilseed rape crops in the UK (Majer et al. 1998; Rothamsted Research 2017).

There was no difference in virulence between the two Oregon isolates of *P. brassicae* tested in this study on three *Brassica* hosts. In contrast, there were significant differences in virulence, based on severity of white leaf spot, among the nine *N. capsellae* isolates from Oregon that were tested on the same three *Brassica* hosts. However, all of the *P. brassicae* and *N. capsellae* isolates tested were collected from one seed crop of a single species, *B. rapa,* in one location in Oregon. Pathogenicity tests with isolates collected from a diverse set of *Brassica* spp. and locations across the Pacific Northwest is needed to provide information on the genotypic and virulence diversity of populations of these pathogens that have become established very recently in this region. Both morphological characteristics and sequence analyses indicated that isolates Psc001 to Psc009, obtained from an infected turnip cover crop seed crop in the Willamette Valley, OR, were all *N. capsellae.* There was 99.00% sequence similarity for all nine isolates to the ITS rDNA sequence of an *N. capsellae* isolate in GenBank. However, the ITS rDNA sequences of all nine isolates were also 99.00% similar to that of a closely related species, *N. brassicae* (EU167607.1) from Germany. *N. brassicae,* previously named *Mycosphaerella brassicicola*, is the causal agent of ringspot of brassicas (Videira et al. 2016). Differentiating isolates of *N. brassicae* and *N. capsellae* morphologically is relatively easy as *N. brassicae* does not have a known asexual stage, while *N. capsellae* produces large, hyaline, distinct conidia (Crossan 1954; Deighton 1973). Recent molecular studies of *Ramularia* and allied genera led to the reclassification of the genus *Pseudocercosporella* (Videira et al. 2016). Videira et al. (2016) demonstrated that *Pseudocercosporella* and *Cercosporella* are polyphyletic, and recommended assignment of species in these two genera to nine new combinations and 24 species, including *Neopseudocercosporella*, of which *N. capsellae* is the type species. However, questions remain to be addressed about the genetic relatedness of species

within *Neopseudocercosporella* (P. Crous, Director of Fungal Biodiversity Centre (CBS), Utrecht Netherlands, *personal communication*).

As of April 2016, there were only 11 *P. brassicae* DNA sequences in GenBank, of which four were of the ITS rDNA (AJ305236.1, AJ305235.1, JX863406.1, and JX648200.1) and one of the β tubulin gene (KC342227.1). The 95.00% similarity between the ITS rDNA sequences of the four Oregon isolates of *P. brassicae* with only one of the four ITS rDNA sequences of *P. brassicae* in GenBank from France (AJ305235.1), was inadequate to confirm the species identity of these USA isolates (Blaalid et al. 2013; Hibbett et al. 2016). Hibbett et al. (2016) used the term "species hypotheses" to describe taxa with ITS rDNA sequences clustered above 97 to 99%. In contrast to the ITS rDNA sequences, the β tubulin gene sequences for isolates Cyc001, Cyc003, Cyc005, and Cyc006 were 98.00% similar to the only β tubulin gene sequence of *P. brassicae* in GenBank (KC342227.1). For a greater understanding of the genetic basis of this pathogen, a greater number of DNA sequences of more geographically diverse isolates are needed in GenBank so that comprehensive phylogenetic analyses can be completed. This will help to understand the genetic relatedness among isolates collected from regions where *P. brassicae* has long been established (e.g., the EU, UK, and New Zealand) and where the fungus was introduced very recently (i.e., North America).

The seed infection and seed transmission rates detected for *P. brassicae* in this study could have a significant impact on the development of light leaf spot in brassica crops (Maddock and Ingram 1981). For example, in Washington State, mustard cover crops such as those of the cv. Caliente 199 (*B. juncea*) and the cv. Nemagon White (*Sinapis alba*), are planted at approximately 11.2 kg/ha, or >2.4 million seeds/ha (D. Youngquist, Skagit Farmers Supply, *personal communication*). If 0.50% of a seed lot is infected with *P. brassicae*, and the lowest observed seed transmission rate from the study (3.10% of the *P. brassicae*-infected mustard seed lot) is used to estimate the risk of *P. brassicae* seed transmission under conducive (cool and wet) conditions, there would be approximately 3,400 plants infected with *P.*

brassicae/ha of cover crop. Environmental and seasonal conditions, brassica cropping systems, and *Brassica* spp. all are likely to play an important role in the seed transmission rate of *P. brassicae*, but the risk of seed transmission is likely to be high in the cool and wet conditions typical of the mild, maritime climate of western Oregon and Washington from fall to spring each year.

Given the potential significance of *P. brassicae*-infected brassica seed lots to this region, an important next step is to provide information to brassica growers and the seed industry to assess the risk associated with *P. brassicae*-infected seed, including assessing the prevalence of *P. brassicae* in commercially available seed lots of various types of *Brassica* spp., and the potential value of seed treatments for managing seedborne inoculum. These research objectives are addressed in Chapter 3, which describes a preliminary survey of brassica cover crops grown in the Skagit Valley, of northwestern Washington in 2016; and in Chapter 4 on the evaluation of different types of seed treatments applied to a *P. brassicae*-infected mustard seed. The limited pathogenicity tests described in this study showed differences in susceptibility to *P. brassicae* among *Brassica* species. However, given the diversity of types and species of brassica crops grown in the Pacific Northwest, more detailed information on host range and level of susceptibility is needed for various *Brassica* species as well as cultivars. Research on seed treatments to eradicate *P. brassicae* from infected seed is essential to give growers and the seed industry tools to manage seedborne infection, including an understanding of whether seed treatments commonly used to manage other seedborne fungi of brassicas are effective against *P. brassicae* seed infection (Neergaard 1977; Pesticide Information Center Online 2016). Lastly, a more detailed evaluation is needed of a larger collection of isolates of *P. brassicae* from North America in comparison to isolates from the UK, EU, and other areas of the world where this pathogen has established e.g., Oceania (Australia and New Zealand), particularly given the limited similarity of the ITS rDNA sequences of Oregon isolates evaluated in this study compared to isolates from the EU and UK, and the lack of amplification of any of the Oregon isolates with the mating type PCR assays developed by Foster et al.

(1999). To this end, results of a multi-locus sequence analysis, sexual crosses, morphological characterizations, and comparative pathogenicity test of isolates of *P. brassicae* from the USA, EU, UK, and New Zealand are presented in Chapter 3.

2.5 Literature Cited

Allen, J. D., and Smith, H. C. 1961. Dry-rot (*Leptosphaeria maculans*) of brassicas: seed transmission and treatment. N. Z. J. Agric. Res. 4:676-685.

Bakkeren, G., Kronstad, J. W., and Levesque, C. A. 2000. Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilagomycetes. Mycologia 92:510-521.

Barnett, H. L., and Hunter, B. B. 1998. Illustrated Genera of Imperfect Fungi, 4th Ed. The American Phytopathological Society, St. Paul, MN.

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., and Kauserud, H. 2013. ITS1 versus ITS2 as DNA metabarcodes for fungi. Mol. Ecol. Resour. 13:218–224.

Boyle, L. W. 1945. Cercosporella leaf spot of kale in Oregon. Plant Dis. Rep. 29:387.

Carter, H. E., Cools, H. J., West, J. S., Shaw, M. W., and Fraaije, B. A. 2013. Detection and molecular characterisation of *Pyrenopeziza brassicae* isolates resistant to methyl benzimidazole carbamates. Pest Manag. Sci. 69:1040–1048.

Cheah, L.-H., and Hartill, W. F. T. 1985. Disease cycle in New Zealand of light leaf spot of brassicas, induced by *Pyrenopeziza brassicae*. N. Z. J. Agric. Res. 28:567–573.

Chupp, C. 1935. *Macrosporium* and *Colletotrichum* rots of turnip roots. Phytopathology 25:269-274.

Commonwealth Mycological Institute Distribution Maps of Plant Diseases. 1986. Commonwealth Mycological Institute, Wallingford.

Crossan, D. F. 1954. Cercosporella leaf spot of crucifers. North Carolina Agric. Res. Stn. Tech. Bull. 109.

Deighton, F. C. 1973. Studies on *Cercospora* and allied genera. IV. *Cercosporella* SACC., *Pseudocercosporella* Gen. Nov. and *Pseudocercosporidium* Gen. Nov. Mycol. Papers 133:1–62.

du Toit, L. J., Derie, M. L., and Hernandez-Perez, P. 2005. Verticillium wilt in spinach seed production. Plant Dis. 89:4-11.

du Toit, L. J., Derie, M. L., Holmes, B.J., and Youngquist, C. P. 2014. Effect of Proline and a biosolids compost on Fusarium and Verticillium wilts in a spinach seed crop. Plant Dis. Manage. Rep. 8:V280.

Ellis, M. B., and Ellis, J. P. 1985. Microfungi on Land Plants. Macmillan Publishing, New York.

Esau, K. 1960. Anatomy of Seed Plants. John Wiley and Sons, New York.

Farr, D. F, and Rossman, A. Y. 2017. Fungal Databases, U.S. National Fungus Collections ARS, USDA. https://nt.ars-grin.gov/fungaldatabases. Accessed on 24 April 2017.

Fitt, B. D. L., Doughty, K. J., Gladders, P., Steed, J. M., and Sutherland, K. G. 1998. Diagnosis of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) in the UK. Ann. Appl. Biol. 133:155-166.

Foster, S. J., Singh, G., Fitt, B. D. L., and Ashby, A. M. 1999. Development of PCR-based diagnostic techniques for the two mating types of *Pyrenopeziza brassicae* (light leaf spot) on winter oilseed rape (*Brassica napus* spp. *oleifera*). Physiol. Molec. Plant Pathol. 55:111-119.

Greville, R. K. 1823. Scottish cryptogamic flora, or coloured figures and descriptions of cryptogamic plants, belonging chiefly to the order Fungi; and intended to serve as a continuation of English Botany. Maclachan and Stewart Publisher, Edinburgh, London.

Gunasinghe, N., You, M. P., Clode, P. L., and Barbetti, M. J. 2015. Mechanisms of resistance in *Brassica carinata, B. napus* and *B. juncea* to *Pseudocercosporella capsellae.* Plant Pathol. 65:888-900.

Harper, F. R. 1973. A key to standardize the description of growth stages in turnip rape, *Brassica campestris*. Can. Plant Dis. Surv. 53:93-95.

Hernandez-Perez, P. 2005. Management of seedborne *Stemphylium botryosum* and *Cladosporium variabile* causing leaf spot of spinach seed crops in western Washington. MS Thesis, Washington State University, Pullman, WA.

Hibbett, D., Abarenkov, K., Koljalg, U., Opik, M., Chai, B., Cole, J. R., Wang, Q., Crous, P. W., Robert, V. A. R. G., Helgason, T., Herr, J., Kirk, P., Lueschow, S., O'Donnell, K., Nilsson, H., Oona, R., Schoch, C., Smyth, C., Walker, D. M., Porras-Alfaro, A., Taylor, J. W., and Geiser, D. M. 2016. Sequence-based classification and identification of Fungi. Mycologia 108:1049–1068.

Hickman, C. J., Schofield, E. R., and Taylor, E. R. 1955. Light leaf spot of Brassica. Plant Pathol. 4:129–131.

Inman, A. J., Sivanesan, A., Fitt, B. D. L., and Evans, R. L. 1991. The biology of *Mycosphaerella capsellae* sp. nov., the teleomorph of *Pseudocercosporella capsellae*, cause of white leaf spot of oilseed rape. Mycol. Res. 95:1334-1342.

Inman, A. J. 1992. The Biology and Epidemiology of White Leaf Spot (*Pseudocercosporella capsellae*) on Oilseed Rape. Ph.D. Dissertation, University of London, London.

Inglis, D. A., du Toit, L.J., and Miller, T. W. 2013. Production of Brassica seed crops in Washington State: A case study on the complexities of coexistence. Washington State Univ. Ext. Man. EMO062E.

Ingold, C. T. 1953. Dispersal in Fungi. Oxford University Press, London, UK.

International Seed Health Initiative-Veg. 2011. Methods for the detection of *Phoma lingam* on *Brassica* spp. seed. International Seed Federation, Zurich. http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/ [Accessed on 26 April 2017].

International Seed Health Initiative-Veg. 2015. Methods for the detection of *Verticillium dahliae* on spinach seed. International Seed Federation, Zurich. http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/ [Accessed on 31 March 2017].

International Seed Testing Association (ISTA). 2017. International rules for seed testing 2017: 7-001a: Detection of *Alternaria dauci* in *Daucus carota* (carrot) seed by blotter method. ISTA, Zurich.

Jacobsen, B. J., and Williams, P. H. 1971. Histology and control of *Brassica oleracea* seed infection by *Phoma lingam.* Plant Dis. Reporter 55:10.

Jeffers, S. N. 2007. Recipes for Media Useful in the Culture and Identification of *Phytophthora* Species: V8 Agar & Broth Recipes. Clemson University, Clemson, SC.

Karolewski, Z. 2010. Development of light leaf spot (*Pyrenopeziza brassicae*) on Brassicas. Phytopathologia 55:13–20.

Kirk, P. M., Cannon, P.F., David, J. C., and Stalpers, J. A. 2001. Dictionary of the Fungi, 9th Ed. Centre for Agriculture and Biosciences International (CABI). Surrey, UK.

Kumar S., Stecher G., and Tamura K. 2015. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets (submitted) available at http://www.megasoftware.net/. [Accessed on 16 October 2016].

Maddock, S. E., and Ingram, D. S. 1981. Studies of survival and longevity of the light leaf spot pathogen of Brassicas, *Pyrenopeziza brassicae*. Trans. Br. Mycol. Soc. 77:153–159.

Majer, D., Lewis, B. G., and Mithen, R. 1998. Genetic variation among field isolates of *Pyrenopeziza brassicae*. Plant Pathol. 47:22–28.

Major, D. J. 1975. Stomatal frequency and distribution in rape. Can. J. Plant Sci. 55:1077-1078.

Maude, R. B. 1996. Seedborne Diseases and their Control: Principles and Practice. Centre for Agriculture and Biosciences International (CABI), Warwick, UK.

McGee, D. C. 1997. Plant Pathogens and the Worldwide Movement of Seeds. The American Phytopathological Society, St. Paul, MN.

McGuire, A. 2012. Mustard Types, Seed Sources, & Acreage Estimates. http://csanr.wsu.edu/mustard-green-manures/mustard-types/ [Accessed 12 August 2016].

Munkvold, G. 2009. Seed pathology progress in academia and industry. Annu. Rev. Phytopathol. 47:285-311.

Neergaard, P. 1977. Seed Pathology, Vol. 1. Halsted Press, New York.

Ocamb, C. M. 2014a. Disease Alert – Light Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/Light%20leaf%20spot%20OSU%20Disease%20Alert%20in%20crucifers%2017%20June.pdf [Accessed 12 December 2016]

Ocamb, C. M. 2014b. Disease Alert – White Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/White%20leaf%20spot%20OSU%20Disease%20Alert%20in%20Brassiceae.pdf [Accessed 12 December 2016]

Pesticide Information Center Online. 2016. Washington State University, Pullman WA. http://cru66.cahe.wsu.edu/labels/Labels.php?SrchType=C. [Accessed 12 April 2017].

Petrie, G. A., and Vanterpool, T. C. 1978. *Pseudocercosporella capsellae*, the cause of white leaf spot and grey stem of Cruciferae in western Canada. Can. Plant Dis. Surv. 58:69–72.

Rawlinson, C. J., Sutton, B. C., and Muthyalu, G. 1978. Taxonomy and biology of *Pyrenopeziza brassicae* sp. nov. (*Cylindrosporium concentricum*), a pathogen of winter oilseed rape (*Brassica napus* spp. *oleifera*). Trans. Br. Mycol. Soc. 71:425-439.

Rimmer, S. R., Shattuck, V. I., and Buchwaldt, L. 2007. Compendium of Brassica Diseases. American Phytopathological Society, St. Paul, MN.

Rothamsted Research. Historical Trends - Light Leaf Spot. http://rothamsted.ac.uk/light-leaf-spot-forecast/historical-trends-light-leaf-spot [Accessed 1 January 2017].

Sorensen, L. H., Schneider, A. T., and Davis, J. R. 1991. Influence of sodium polygalacturonate sources and improved recovery of *Verticillium* species from soil. Phytopathology 81:1347.

Staunton, W. P. 1967. Studies on light leaf spot (*Gloeosporium concentricum*) of Brassicae. Irish J. Agric. Res. 6:203–211.

Sutherland, K. G. 2001. Technical note: light leaf spot in winter oilseed rape. The Scottish Agricultural College, Edinburgh.

Sutton, D. A. 1977. *Cylindrosporium concentricum*. CMI Descr. Fungi Bact. No. 536. Commonwealth Mycological Institute, Wallingford, England.

United States Department of Agriculture National Agricultural Statistics Service. http://quickstats.nass.usda.gov/. [Accessed 19 February 2017].

Videira, S. I. R., Groenewald, J. Z., Braun, U., Shin, H. D., and Crous, P. W. 2016. All that glitters is not *Ramularia*. Stud. Mycol. 83:49–163.

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 2.1. Results of NP-10 agar seed health assays to detect the percentage of seed infected with *Pyrenopeziza brassicae* or *Neopseudocercosporella capsellae* for seed lots harvested from cabbage (*Brassica oleracea* var. *capitata*) plants and mustard (*Brassica juncea*) plants inoculated with these pathogens during pod development[a]

			Assay 1			Assay 2		
Pathogen	**Host plant seed lot[b]**	**NP-10 seed health assay[c]**	**Target Pathogen (%)**	***Alternaria* spp. (%)**	***Cladosporium* spp. (%)**	**Target Pathogen (%)**	***Alternaria* spp. (%)**	***Cladosporium* spp. (%)**
P. brassicae	Cabbage ♀	4°C	0.50 ± 0.29	7.00 ± 1.22	84.25 ± 2.50	0.50 ± 0.29	5.75 ± 0.75	71.75 ± 7.23
		4°C surface-sterilized	0.25 ± 0.25	2.25 ± 0.75	0.50 ± 0.29	0.50 ± 0.50	1.50 ± 1.00	0.25 ± 0.25
		20°C	0.00	33.00	64.00	–	–	–
		20°C surface-sterilized	0.00	21.00	0.00	–	–	–
	Cabbage ♂	4°C	0.50 ± 0.29	6.00 ± 0.71	96.75 ± 0.75	0.00 ± 0.00	5.50 ± 0.87	92.50 ± 1.85
		4°C surface- sterilized	0.50 ± 0.29	4.50 ± 0.87	0.25 ± 0.25	0.00 ± 0.00	4.75 ± 1.31	1.25 ± 0.95
		20°C	0.00	75.00	91.00	–	–	–
		20°C surface-sterilized	0.00	29.00	0.00	–	–	–
	Mustard	4°C	12.50 ± 0.65	20.75 ± 5.11	98.50 ± 1.19	19.75 ± 2.14	14.25 ± 4.39	94.50 ± 1.32
		4°C surface-sterilized	12.50 ± 2.06	12.75 ± 3.09	0.25 ± 0.25	10.50 ± 1.19	4.50 ± 0.87	1.00 ± 0.41
		20°C	0.00	99.00	96.00	–	–	–
		20°C surface-sterilized	0.00	47.00	0.00	–	–	–
N. capsellae	Cabbage ♀	4°C	0.00 ± 0.00	28.50 ± 1.19	99.00 ± 0.00	0.00 ± 0.00	27.75 ± 2.69	96.50 ± 1.19
		4°C surface-sterilized	0.00 ± 0.00	25.75 ± 1.18	3.25 ± 1.03	0.00 ± 0.00	24.00 ± 2.38	1.25 ± 0.63
		20°C	0.00	76.00	82.00	–	–	–
		20°C surface-sterilized	0.00	26.00	3.00	–	–	–
	Cabbage ♂	4°C	0.00 ± 0.00	56.00 ± 1.78	97.50 ± 0.87	0.00 ± 0.00	42.75 ± 3.20	93.00 ± 1.08
		4°C surface-sterilized	0.00 ± 0.00	37.75 ± 3.17	0.75 ± 0.25	0.00 ± 0.00	30.00 ± 2.04	0.75 ± 0.25
		20°C	0.00	82.00	80.00	–	–	–
		20°C surface-sterilized	0.00	45.00	2.00	–	–	–
	Mustard	4°C	0.00 ± 0.00	77.00 ± 2.38	92.50 ± 0.50	0.00 ± 0.00	69.00 ± 8.96	89.75 ± 1.84
		4°C surface-sterilized	0.00 ± 0.00	22.75 ± 2.21	0.25 ± 0.25	0.00 ± 0.00	27.25 ± 3.71	2.00 ± 0.00
		20°C	0.00	97.00	74.00	–	–	–
		20°C surface-sterilized	0.00	41.00	1.00	–	–	–

[a] NP-10 agar seed health assays tested to detect whether the mustard or cabbage seed lots, grown as described in the main text, were infected with *P. brassicae* or *N. capsellae.* Means ± standard errors for four replications of 100 seed/lot are shown for *P. brassicae* or *N. capsellae*, as well as fast-growing, *Alternaria* spp. and *Cladosporium* spp. Standard errors are not shown for seed health assays for which one replication of 100 seeds was tested for a particular seed lot.

[b] Seed lots of the proprietary cabbage male line (♂), cabbage female line (♀), and mustard cultivar were grown and inoculated separately with *P. brassicae* and *N. capsellae,* as described in the main text.

[c] Details of the seed health assays are described in the main text. For each assay, four replications of 100 seed/lot were plated onto NP-10 agar medium (Sorensen et al. 1991) in square acrylic boxes (32 to 34 seeds/box) and incubated at 4 or 20°C. The seed was plated with or without surface-sterilization. For surface-sterilization, seed were shaken in 1.2% NaOCl for 60 s, triple-rinsed in sterilized, deionized water, and dried before plating.

Fig. 2.1. Light leaf spot symptoms following inoculation of plants with isolates of *Pyrenopeziza brassicae*. Browning of the veins and stem on a 'Black Magic' kale (*Brassica oleracea* var. *acephala*) leaf (**A**). Symptoms observed 14 days after inoculation (dai) of 'Hakurei' turnip (*B. rapa*) (**B**). Symptoms observed 20 dai, including chlorotic spots and veinal browning, on a 'Hakurei' turnip leaf (**C**). Symptoms observed 20 dai of a 'Candid Charm' cauliflower (*B. oleracea* var. *botrytis*) plant (**D**).

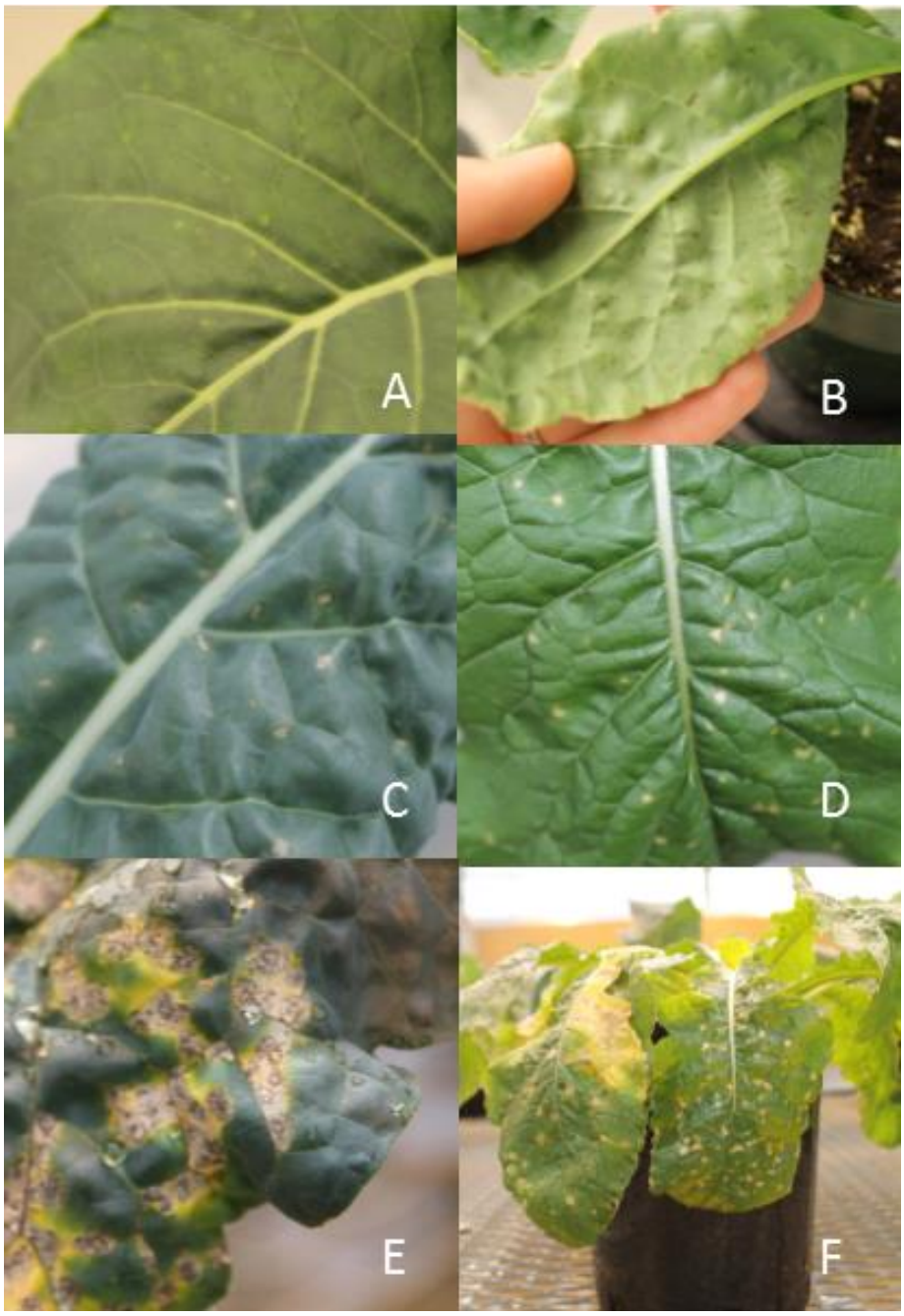

Fig. 2.2. Initial symptoms of white leaf spot caused by *Neopseudocercosporella capsellae* on 'Candid Charm' cauliflower (*Brassica oleracea* var. *botrytis*) (**A**). Symptoms on the lower surface of a leaf of 'Candid Charm' cauliflower (**B**), on a 'Black Magic' kale (*B. oleracea* var. *acephala*) leaf (**C**), and a 'Hakurei' turnip (*B. rapa*) leaf (**D**) 10 days after inoculation. Symptoms on kale (**E**) and turnip (**F**) 20 days after inoculation.

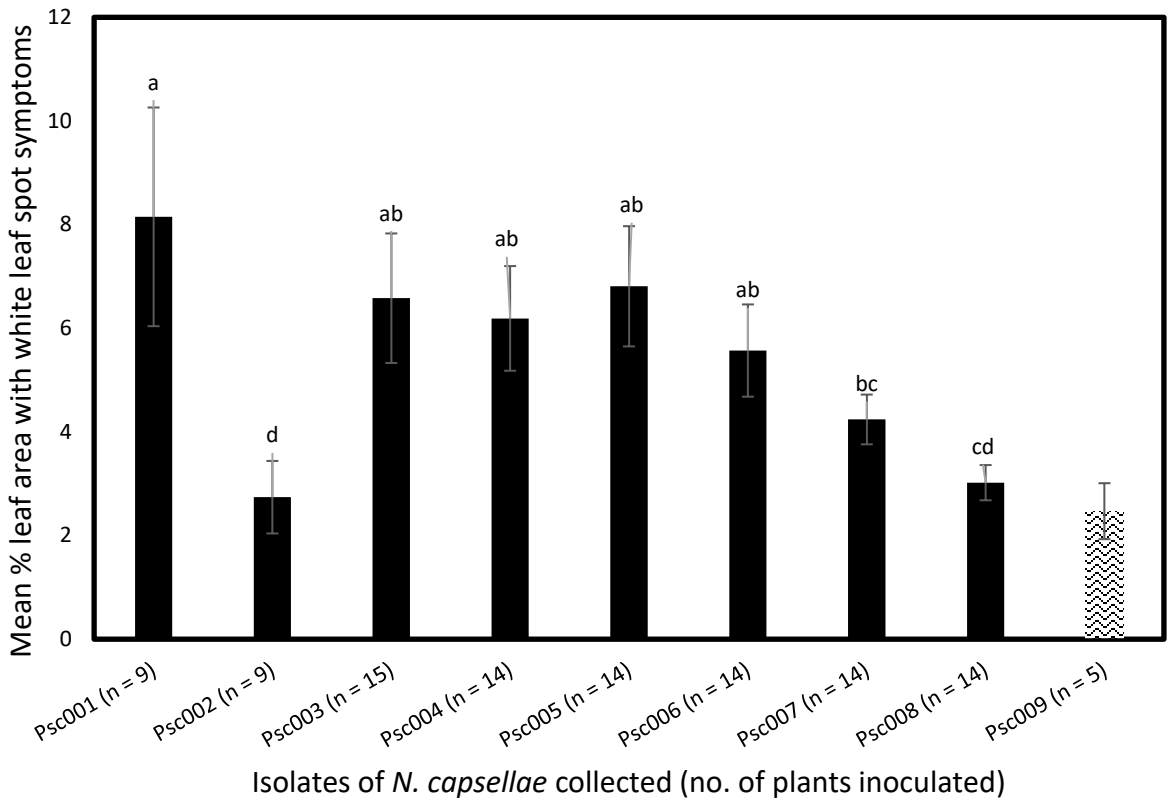

Fig. 2.3. Pathogenicity test results on cauliflower (*Brassica oleracea* var. *botrytis*), kale *(B. oleracea* var. *acephala*)*,* and turnip (*B. rapa*) for nine *Neopseudocercosporella capsellae* isolates collected from a 'Barkant' turnip seed crop in the Willamette Valley, OR, in February 2015. The means and standard errors of severity ratings for each isolate were averaged over all three host species because the interaction between plant species and isolates of the pathogen was not significant in the analysis of variance (ANOVA). Means with different letters are significantly different based on Fisher's protected least significant difference (P <0.05). Data were log-transformed to meet assumptions of normality and equal variance for parametric analysis. Plants of each host inoculated with water served as negative control treatments but data for the control plants were not included in the ANOVA as symptoms of white leaf spot were not observed on those plants. Isolates of *N. capsellae* represented by black bars were tested using a suspension of 1 x 10^4 spores/ml. Isolate Psc009 was applied at 1 x 10^3 spores/ml because of limited spore production. Similarly, limited amounts of inoculum resulted in different numbers of plants inoculated with each isolate of *N. capsellae*. Refer to the main text for details.

Fig. 2.4. Cabbage (*Brassica oleracea* var. *capitata*) pods and stems with symptoms of light leaf spot caused by *Pyrenopeziza brassicae*, including on green (**A**) and dried (**B**) pods. Cabbage pods inoculated with *Neopseudocercosporella capsellae* did not exhibit symptoms of white leaf spot (**C**). Mustard (*B. juncea*) pods inoculated with *P. brassicae* developed symptoms of light leaf spot (**D**).

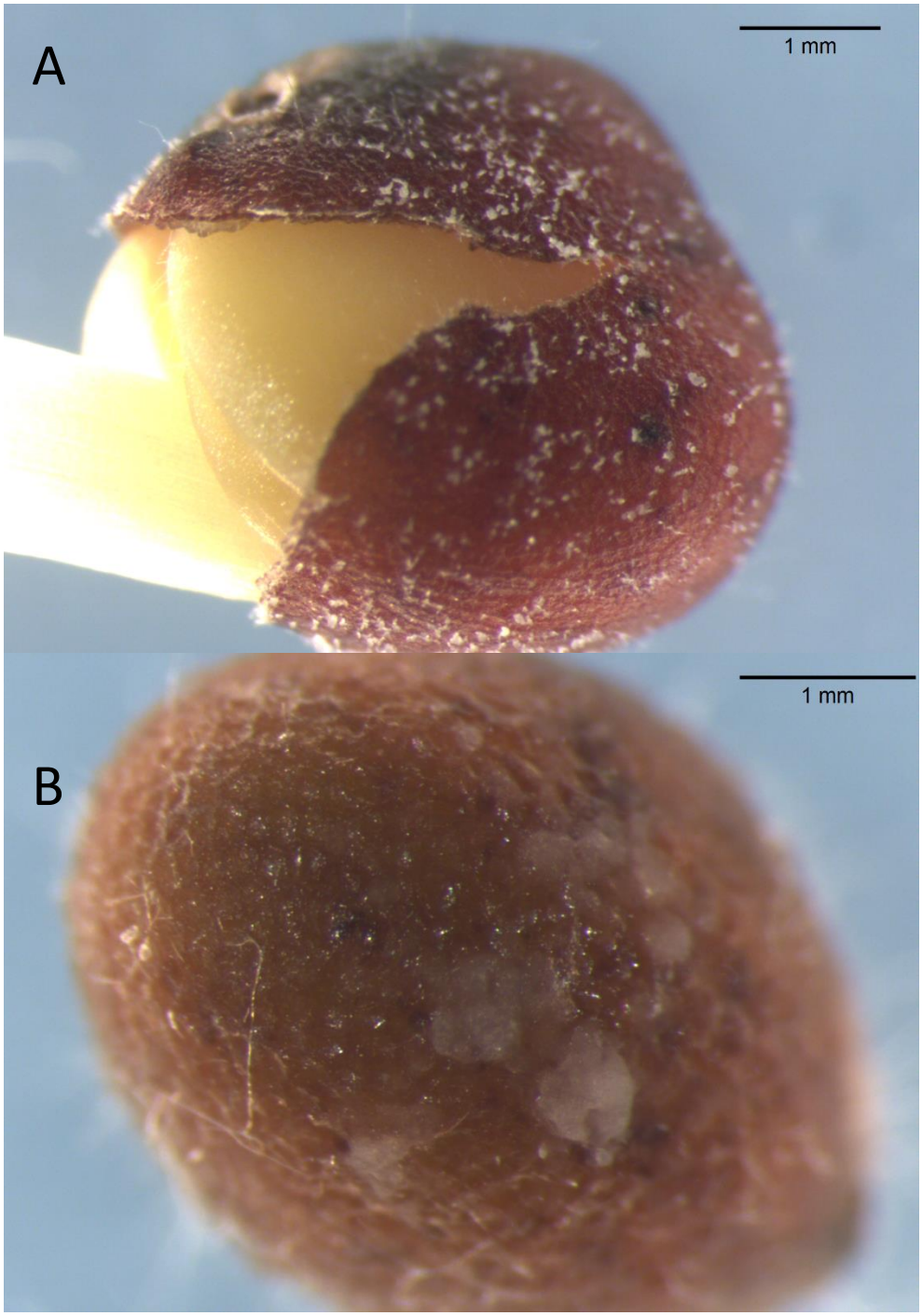

Fig. 2.5. Signs of *Pyrenopeziza brassicae* detected in a seed health assay in which brassica seed was incubated on NP-10 agar medium (Sorensen et al. 1991) at 4°C in the dark. Initially, signs of *P. brassicae* observed on cabbage (*Brassica oleracea* var. *capitata*) seed 21 days after plating (dap) included pale, pink, distinct gelatinous masses of conidia (**A**). The spore masses became larger and less distinct, as depicted on a mustard (*B. juncea*) seed 28 dap (**B**).

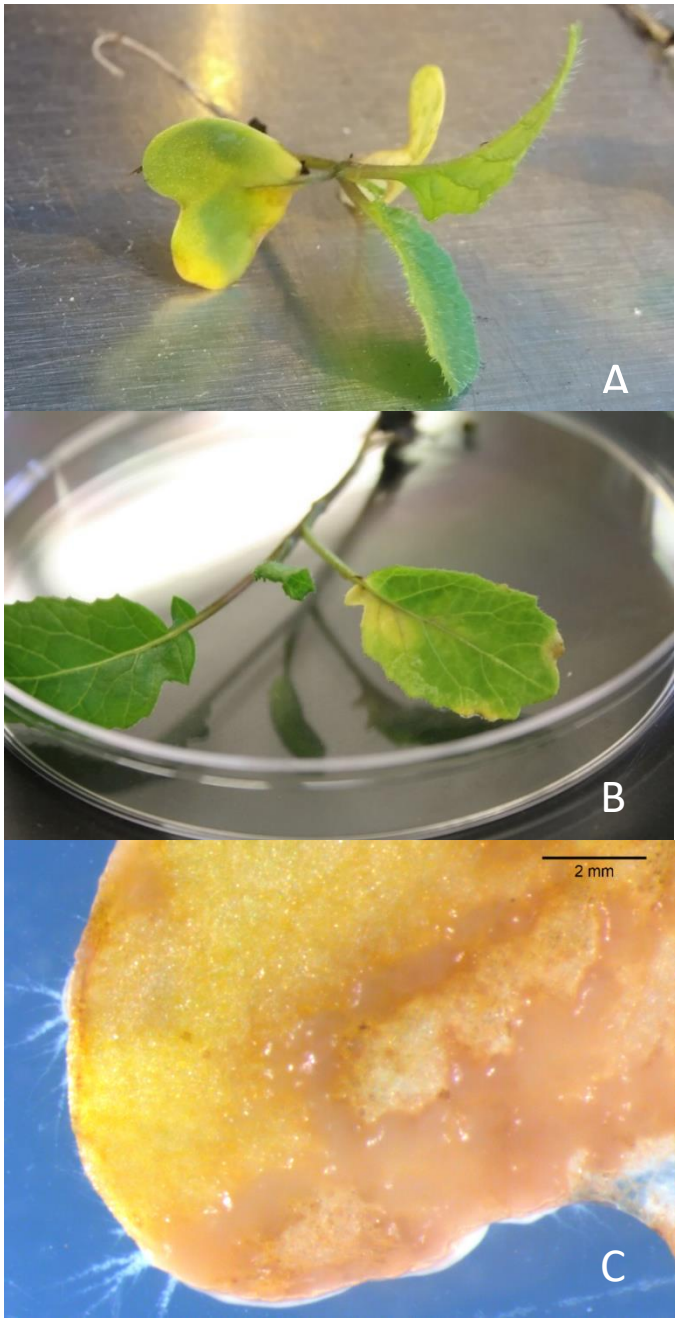

Fig. 2.6. Symptoms of light leaf spot caused by *Pyrenopeziza brassicae* on mustard (*Brassica juncea*) seedlings detected during seed transmission trials for the pathogen. Symptoms on cotyledons included chlorosis with small, pale brown spots (**A**). Symptoms on the first true leaves included more distinct, chlorotic lesions, some of which developed a brown center, and veinal browning (**B**). Microscopically observed gelatinous masses of conidia on a mustard cotyledon after the cotyledon was dipped into 70% ethyl alcohol for 3 to 7 s, dried on sterilized paper towel in a laminar flow hood, plated onto half strength potato dextrose agar, and incubated with cool white fluorescent light and near ultraviolet light for 8 h/day at 15°C followed by 16 h of dark/day at 10°C (**C**).

CHAPTER 3

DETECTION AND SPECIES IDENTIFICATION OF THE CAUSAL AGENTS OF LIGHT LEAF SPOT AND WHITE LEAF SPOT OF BRASSICAS IN WASHINGTON STATE

3.1 Introduction

In 2014, two foliar fungal diseases of brassicas, light leaf spot (caused by *Pyrenopeziza brassicae*) and white leaf spot (caused by *Neopseudocercosporella capsellae*), were detected in multiple types of brassica crops and on Brassicaceae weeds in the Willamette Valley of Oregon (Ocamb 2014a; Ocamb 2014b). Light leaf spot had never been reported before in North America (Ocamb 2014a; Farr and Rossman 2017). White leaf spot had been reported infrequently in California and Oregon, but was relatively common in the southeastern USA [Boyle 1945; Commonwealth Mycological Institute (CMI) Distribution Maps of Plant Diseases 1986; Crossan 1954]. The pathogens have since become widespread in the Willamette Valley (C. M. Ocamb, Oregon State University, *personal communication*). Because these two pathogens are considered new to the Pacific Northwest USA, the impacts of these diseases are not yet well understood in the context of the diverse brassica crop production systems in this region (Inglis et al. 2013). In the 1970s, Rawlinson et al. (1978) reported yield losses of up to 90% in winter oilseed rape crops in southern England as a result of light leaf spot. Historical trends tracked by scientists at Rothamsted Research (2017) have shown light leaf spot to be an increasing problem, with estimated costs of £30 to £160 million annually in the UK resulting from this disease. These losses are of concern to growers in the maritime Pacific Northwest where cool, rainy winter conditions are similar to those in the UK. Reports of losses associated with white leaf spot outbreaks have not been as severe as those reported for light leaf spot (Inman 1992). However, similar to light leaf spot, increases in oilseed rape production in regions like the European Union (EU) and United Kingdom (UK) have increased losses to white leaf spot, with as much as a 15% yield loss reported in France (Penaud 1987).

N. capsellae is more widespread on a global scale than *P. brassicae* (CMI Distribution Maps of Plant Diseases 1986; Rawlinson et al. 1978). White leaf spot has been found in many countries where brassicas are grown in temperate climates (CMI Distribution Maps of Plant Diseases 1986). The anamorph was first described in the UK in 1992 (Inman 1992), and the teleomorph, *Mycosphaerella capsellae*, was named in 1973 (Deighton 1973). Synonyms of *N. capsellae* include *Cercospora albo-maculans* (Ell. and Ev. Saccardo 1895), *Cylindrosporium brassicae* (Roumeguere 1891), and *Cercosporella brassicae* (Fautrey and Roumeguere Hohnel). The organism was named *Neopseudocercosporella capsellae* in 2016 by Videira et al. (2016), and is the type species for that genus. Prior to detection in the USA, the light leaf spot pathogen, *P. brassicae*, had been found in Oceania (Australia, New Zealand, and Tasmania), Europe (Denmark, France, Germany, Ireland, Latvia, Netherlands, Norway, Poland, Portugal, Romania), the UK, Japan, and the Philippines (Hickman et al. 1955; Karolewski 2010; Rawlinson et al. 1978; Sutton 1977). The anamorph, *Cylindrosporium concentricum*, was first described in the UK in the 19th century (Greville 1823); and the teleomorph, *P. brassicae*, was first described in 1978 (Rawlinson et al. 1978).

Research is needed to assess whether *P. brassicae* and *N. capsellae* occur in other regions of brassica crop production of the Pacific Northwest besides the Willamette Valley. Also, as reported in Chapter 2, the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) of isolates of *P. brassicae* from Oregon were only 95% similar to the ITS rDNA sequence of one isolate from the UK out of four ITS rDNA sequences of this species in GenBank, which is considered less similar than that of isolates of the same species of a fungus (Hibbett et al. 2016). Therefore, a more detailed assessment of a broader collection of *Pyrenopeziza* isolates associated with infected brassicas from the Pacific Northwest in comparison to isolates from other regions of the world is needed to verify whether the species of *Pyrenopeziza* associated with outbreaks of light leaf spot in the USA is *P. brassicae*. In contrast, all nine sequences of *N. capsellae* collected in Oregon in 2015 were 99.00% similar to the ITS rDNA sequence of

an *N. capsellae* isolate from the UK (GenBank Accession No. GU214662.1), supporting the species identity.

The science and art of identifying and naming fungi has changed dramatically in the past 30 years with the advent of molecular phylogenetics and evolving proposals of new species concepts (Crous et al. 2015). Species concepts emphasize species divergence in different ways: reproductive divergence [biological species concepts (BSC)], morphological divergence [morphological species concept (MSC)], and ecological or environmental divergence [ecological species concept (ESC)] (Giraud et al. 2008). Characterizing fungal species based on nucleic acid sequences has been accomplished in a number of ways: the phylogenetic species concept (PSC) (Taylor et al. 2000), sequence-based classification and identification (SBCI) (Hibbett et al. 2016), and genealogical concordance phylogenetic species recognition (GCPSR) (Crous et al. 2015). The latter is a multigene phylogenetic approach that identifies species based on genealogical concordance. Historically, the MSC has been the prevailing species concept used with fungi, but compared to the PSC or BSC, this method often groups more than one species together (Taylor et al. 2000). However, the BSC is inadequate for fungi that reproduce asexually or cannot be cultured in the lab (Taylor et al. 2000). The PSC and GCPSR provide some of the clearest species resolution, but Crous et al. (2015) wrote that the primary purpose of taxonomy and species naming is for the purpose of communication, so the connection of these species concepts to morphology remains important. Simply put, depending on the fungi, no one species concept may be entirely sufficient. Crous et al. (2015) discussed how polyphasic approaches such as the consolidated species concept (CSC) (Quaedvlieg et al. 2014), that combines morphological, ecological, biological and phylogenetic data, are commonly used to identify species, especially among economically important fungi. As *P. brassicae* is a plant pathogen of economic importance in areas of the world where this pathogen has become established, it is prudent to apply the CSC to assess differences among isolates of *Pyrenopeziza* collected from symptomatic brassicas in the USA vs. those of *P. brassicae* collected in

other regions of the world, particularly given the preliminary evidence of significant differences in ITS rDNA sequences of USA isolates.

A molecular phylogenetic approach is an essential component of the CSC to begin to explain some of the findings and hypotheses generated for *P. brassicae* in Chapter 2 of this thesis. However, as of January 2017, there were only 11 *P. brassicae* DNA sequences in GenBank, 4 of the ITS rDNA (AJ305236.1, AJ305235.1, JX863406.1, and JX648200.1) and one of the β tubulin gene (KC342227.1). The 95.00% similarity between the ITS rDNA sequences of the four Oregon isolates of *Pyrenopeziza* described in Chapter 2 (Cyc001, Cyc003, Cyc005, and Cyc006) with only one of the four ITS rDNA sequences of *P. brassicae* in GenBank from France (AJ305235.1), and no similarity level generated by GenBank for the Oregon isolates with the other three ITS rDNA sequences, precluded confirmation of the species identity of these USA isolates as *P. brassicae* (Blaalid et al. 2013; Hibbett et al. 2016). Hibbett et al. (2016) used the term "species hypotheses" to describe taxa with ITS rDNA sequences clustered at >97% similarity. In contrast to the ITS rDNA sequences for the four Oregon isolates, the β tubulin gene sequences for the same isolates were 98.00% similar to the only β tubulin gene sequence of *P. brassicae* in GenBank (KC342227.1).

The ITS rDNA region was designated as the standard barcode for fungi by the Fungal Working Group in Amsterdam in 2012 because this DNA region is amplified easily and has had wide utility in fungal taxonomic studies (Crous et al. 2015; Schoch et al. 2012). However, the ITS rDNA region has failed to identify accurately the species in some genera, such as the following ascomycetes: *Alternaria, Botryosphaeria, Cercospora,* and *Fusarium* (Schoch et al. 2014). In the process of designating the ITS rDNA as the DNA barcode for fungi, the Fungal Working Group pointed out that it is unlikely that a single marker barcode system will be capable of identifying every fungal specimen or culture to species level, and that other reliable kingdom-wide PCR amplifications for protein-coding markers, such as the β-tubulin, ribonucleic acid polymerase 1 (RPB1), RPB2, and translation elongation factor (TEF) 1-α genes,

can have more species resolving power (Schoch et al. 2012). These protein-coding genes were not chosen as the DNA barcode, however, because the genes can be more difficult to amplify by PCR assay than the ITS rDNA region (Schoch et al. 2012). For this reason, a multi-locus sequence analysis (MLSA) combining protein-coding and ribosomal markers for higher species resolution often is performed. For example, the partial large subunit (LSU) rDNA, RPB1, RPB2, and TEF 1-α gene sequences were used to resolve species limits of morels (*Morchella* spp.) in Turkey (Taskin et al. 2010).

Another component of the CSC that may be relevant to assessing brassica *Pyrenopeziza* isolates from the USA with *P. brassicae* isolates from other regions of the world is the BSC. Ilott et al. (1984) established that *P. brassicae* is heterothallic, and Gilles et al. (2001) established that mature *P. brassicae* ascospores can be produced *in vitro*. The homothallic or heterothallic nature of USA isolates of *Pyrenopeziza* from brassica plants needs to be assessed. In addition, research is needed to assess the mating types of USA isolates of this fungus, and the compatibility of the mating types of isolates within the USA with the mating types of *P. brassicae* isolates from other countries (Foster et al. 2002). The MSC is also an important consideration to assess if there might be morphological differences of *Pyrenopeziza* isolates from brassica crops in the USA with *P. brassicae* isolates in other regions of the world.

Applying the CSC to isolates of *Pyrenopeziza* collected from infected brassica plants in the USA will increase understanding of the genetic, morphological, and biological relatedness among isolates collected from regions where *P. brassicae* has long been established (e.g., the EU, UK, and New Zealand) with isolates from regions where the fungus was introduced very recently (i.e., North America). In Chapter 2, the potential for USA isolates of *P. brassicae* to be seedborne and seed transmitted was established. Understanding genetic similarities or differences of USA isolates with those from other regions of the world could provide evidence for potential sources of introduction of the pathogen into the USA, e.g., there is likely to be greater similarity among populations that originate from the spread of

infected seed lots that were produced in the same region than from infected seed lots produced in different geographic regions of the world (Barrett et al. 2008).

The recent detection of light leaf spot and white leaf spot in the Pacific Northwest, a very important vegetable, cover crop, seed, and oilseed Brassicaceae-growing region [Inglis et al. 2013; McGuire 2012; United States Department of Agriculture (USDA) National Agricultural Statistics Service (NASS)], is of concern not only to farmers planting brassica crops but also for farmers producing brassica seed crops and the brassica seed industry because of the risk of disseminating infected seed produced in this region. The objectives of this study were to: i) assess the awareness of brassica stakeholders in the Pacific Northwest of important fungal seedborne pathogens of brassicas, and the value of seed health testing and seed treatments for these pathogens; ii) survey three counties in northwestern Washington that comprise the primary region of this state for production of biennial, brassica vegetable seed crops and that have a similar climate to the Willamette Valley of Oregon, for the presence of light leaf spot and white leaf spot; and iii) determine the species identification of USA isolates of the light leaf spot pathogen compared to isolates of *P. brassicae* from the EU, UK, and New Zealand using the CSC as a framework. For the latter, pathogenicity tests; sexual compatibility tests; MLSA of the ITS rDNA, β-tubulin gene, and TEF 1-α gene; and phylogenetic analyses of the mating type genes were used. Preliminary results have been presented for the second and third objectives (Carmody et al. 2016; Carmody et al. 2017). Given the preliminary evidence that the USA isolates are potentially a different species than *P. brassicae*, the USA isolates are hereafter referred to as *P.* cf. *brassicae*.

3.2 Materials and Methods

3.2.1. Questionnaire of Pacific Northwest brassica stakeholders. In order to gain an understanding among brassica stakeholders of the awareness of seedborne brassica pathogens in the

Pacific Northwest USA, a questionnaire (Fig. 3.1) was made available to growers and other agricultural stakeholders attending three conferences in the Pacific Northwest USA: the Washington Tilth Producers' Annual Conference on 13 to 15 November 2015 in Spokane, WA (Tilth Producers 2017); the Pacific Northwest Vegetable Association (PNVA) Annual Convention and Trade Show on 17 to 18 November 2015 in Kennewick, WA (PNVA 2017); and the Organic Seed Growers' Conference on 5 to 6 February 2016 in Corvallis, OR (Organic Seed Alliance 2017). Though the conferences were not focused on brassica production, each event drew stakeholders working in the different regions of brassica crop production in the Pacific Northwest. The Washington Tilth Producers' Annual Conference focused on smaller- to medium-scale, fresh market producers and stakeholders, a significant percentage of whom grow or work with certified organic crops. The PNVA Annual Convention and Trade Show was attended by >500 medium- to large-scale vegetable growers and related stakeholders, primarily in central Washington and north-central Oregon for conventional production systems but also certified organic production systems. Attendees at the Organic Seed Growers' Conference were focused on organic seed production. At each of these events, the survey was made available to attendees following a presentation by Dr. Lindsey du Toit about black leg of brassicas, caused by the seedborne fungus *Phoma lingam,* (Paulitz et al. 2017; L. J. du Toit, Washington State University, *personal communication*), and across the Willamette Valley of western Oregon in 2014 (Ocamb et al. 2015; Ocamb 2016); as well as the quarantine status of this disease in many parts of Washington State [Washington State Department of Agriculture (WSDA) Washington Administrative Code (WAC) 16-301-490-580 and 16-301-490-495]. The WSDA implemented a quarantine ruling in 2006, WAC 16-301-490-580, at the request of brassica vegetable seed growers and seed companies, to protect six counties in northwestern Washington that are the primary region for biennial brassica vegetable seed crops in that state, from introductions of the black leg (*P. lingam*) and black rot (*Xanthomonas campestris* pv. *campestris*) pathogens on brassica seed lots (WSDA 2015). The black leg part of the regulation was extended in 2015 to include all counties east

of the Cascade Mountains (WSDA 2015), and requires that any brassica seed lot to be planted in the protected areas of Washington State must be certified as *P. lingam*-free. The completed questionnaires were collected at the end of each presentation, and the total number and percentage of responses for each question were calculated.

3.2.2. *P.* cf. *brassicae* and *N. capsellae* in northwestern Washington.

3.2.2.1. Survey for *P.* cf. *brassicae* and *N. capsellae* on brassica plants in northwestern Washington.

In early April 2016, plants of birds rape mustard (*B. rapa*), a weedy brassica, with classic symptoms of light leaf spot, i.e., large chlorotic spots that coalesced, sometimes covering the leaf surface, and often accompanied by veinal browning of the leaves (Rawlinson et al. 1978), were found along roadsides in Skagit Co., WA. As a result, a preliminary survey of mustard cover crops (*B. juncea*), weedy brassicas (*B. rapa* and an unknown *Brassica* sp.), vegetable brassica crops (various *B. oleracea* subspecies), and cabbage seed crops (*B. oleracea* var. *capitata*) was initiated in late spring (March) to early summer (June) of 2016 in northwestern Washington. Leaves and stems with suspect symptoms of light leaf spot were collected from a total of 20 locations in Skagit, Snohomish, and Whatcom Counties (Table 3.1). Isolations were made by plating small pieces (up to 5 mm^2) of surface-sterilized, symptomatic leaf and stem tissue onto chloramphenicol-amended (100 mg/liter), clarified V8 (cV8) agar [(100 ml of clarified V8 juice (with the clarified V8 juice made by mixing 1.0 g of $CaCO_3$/100 ml V8 juice, centrifuging the suspension at 7,000 rpm for 10 minutes, and collecting the supernatant); 15 g agar, and 900 ml deionized water/liter] (Jeffers 2007). Each leaf or stem section was surface-sterilized in 70% ethyl alcohol for 3 to 7 s, then placed on sterilized paper towel in a laminar flow hood to dry, and plated onto clarified cV8 agar medium. The agar plates with the tissue pieces were placed in an incubator (Model 130BLL, Percival Scientific, Perry, IA) at 15°C with cool white fluorescent light and near-ultraviolet (NUV) light for 8 h/day, and at 10°C in the dark for 16 h/day.

Fungi that developed from the tissue pieces were examined microscopically for characteristics typical of *P. brassicae*, i.e., pale pink, gelatinous, conidial masses with very slow-growing hyphae; and of *N. capsellae*, i.e., hyaline, upright, 1- to 5-septate conidia and slow-growing, dematiaceous hyphal growth (as described in Chapter 2) (Crossan 1954; Deighton 1973). Single-spore isolates were obtained by streaking conidia onto half-strength-potato dextrose agar amended with chloramphenicol at 100 mg/liter (½cPDA) for *P. brassicae* or onto clarified cV8 for *N. capsellae*. Individual colonies that developed from the streaks were each transferred to a plate of ½cPDA and/or clarified cV8 on which five sterilized filter disks (each 1.5 cm diameter; VWR, Visalia, CA) had been placed. Once colonized, the filter disks were transferred into sterilized No. 1 coin envelopes (Model #478-259, Office Depot, Boca Raton, FL), dried overnight in a laminar flow hood, and placed in an air-tight container with desiccant at -20°C for long-term storage. The genus and putative species identities of a subset of the isolates collected (Table 3.1) were confirmed morphologically and by sequencing the ITS rDNA, as described in Chapter 2. In addition, the β-tubulin gene was sequenced for *P.* cf. *brassicae* isolates because of evidence from Chapter 2 that the ITS rDNA region of Oregon isolates of this pathogen was too dissimilar from that of isolates in GenBank for verification as *P. brassicae*. Once the filter disks had been removed, the agar medium in each Petri plate was flooded with 1 ml of sterilized, deionized water and the spore suspension used to inoculate sterilized potato dextrose broth (PDB) in 125 ml, screw top, Erlenmeyer flasks (50 ml/flask). Cultures in the inoculated flasks were incubated on a gyratory shaker at 100 rpm at room temperature (24 ± 2°C) for three weeks. DNA was then extracted from the mycelial mat and spores in each flask using the standard protocol for the DNeasy Plant Mini Kit (Qiagen, Germantown, MD), as described in Chapter 2.

3.2.2.2. Pathogenicity of Washington isolates of *P.* cf. *brassicae* and *N. capsellae*. Four pathogenicity tests were carried out for a total of 16 isolates of *P.* cf. *brassicae* and 6 isolates of *N. capsellae* collected in northwestern Washington in 2016, with each test lasting six weeks (Table 3.1). The

tests included two additional *P.* cf. *brassicae* isolates (Cyc023a and Cyc024a) and two additional *N. capsellae* isolates (Psc012 and Psc015) obtained from *B. rapa* samples collected from the Willamette Valley, OR in April 2016 by Dr. Lindsey du Toit (Table 3.1). Isolates Cyc001 of *P.* cf. *brassicae* and Psc007 of *N. capsellae* obtained from a turnip cover crop seed crop in Benton, Co., OR in 2015 were used as positive control isolates in each of the four tests, which started on 12 May, 17 May, 10 June, and 2 September 2016 for pathogenicity tests 1, 2, 3 and 4, respectively. The control isolates were verified as pathogenic on multiple *Brassica* species, as detailed in Chapter 2. Seeds of 'Hakurei' turnip (*B. rapa*) (Osborne International Seed Co., Mount Vernon, WA) and 'Caliente 199' mustard (High Performance Seeds, Inc., Moses Lake, WA) were sown in RediEarth Seedling Starter Mix (SunGro, Agawam, MA) in 72-cell flats (2 seed/cell, with each cell 3.8 cm diameter x 5.7 cm deep) in a greenhouse set at 20 ± 3°C by day and 15 ± 3°C at night with supplemental lights on for 12 h/day, at the Washington State University (WSU) Mount Vernon Northwestern Washington Research and Extension Center (NWREC). Approximately three weeks later, seedlings were transplanted into Sunshine Mix #1 (SunGro) in 15-cm diameter plastic pots. Six weeks after transplanting, the plants were put into plastic containers (each 41.0 cm x 28.0 cm x 16.5 cm deep) for a total of 4 replicate plants/plant species/isolate, and each container with plants was enclosed in a large (38.1 cm wide x 22.9 cm deep x 81.3 cm tall) plastic bag to create a humidity chamber. A 45-cm-long bamboo stake was put into one of the pots in each bin to prevent the bag from collapsing onto the plants. The plants were incubated overnight under a greenhouse bench that was covered with two layers of Remay cloth for shading.

The next day, a spore suspension was made of each of the test isolates of *P.* cf. *brassicae* or *N. capsellae* and the positive control isolate (Cyc001 or Psc007), to inoculate the plants using an atomizer (Rescende Model 175, Badger Air-Brush Co., Franklin Park, IL) powered by a compressor (0523-V4B-G180DX, Gast Manufacturing, Benton Harbor, MI). The conidial suspension of each isolate was made by flooding each plate (five plates/isolate for *N. capsellae*, and two plates/isolate for *P.* cf. *brassicae*) with

20 ml sterilized, deionized water, and gently rubbing the surface of the plate with a sterilized, bent glass rod. The spore suspension was strained through two layers of cheesecloth and quantified using a hemocytometer. Spore suspensions were each diluted to 1 x 10^6 spores/ml for *P.* cf. *brassicae* and 1 x 10^4 spores/ml for *N. capsellae*, and the surfactant Tween 20 was added (0.01%). A lower spore concentration was used for *N. capsellae* because of the limited amount of sporulation of these isolates on agar media (Chapter 2). Each spore suspension was atomized onto both sides of each leaf of all four replicate plants/plant species/isolate. Four plants/plant species were included as a negative control treatment by atomizing the foliage similarly with water and Tween 20. After inoculation, plants were placed back into the plastic bags for two days under greenhouse benches covered with Remay. The plants were then maintained at the WSU Mount Vernon NWREC greenhouse Bay 102 or West Bay 1, set at 20 ± 3°C by day and 15 ± 3°C by night, with supplemental lighting for 12 h/day.

The tests were set up as 2 x 6, 2 x 4, 2 x 13, and 2 x 5 factorial treatment designs for the first, second, third, and fourth pathogenicity tests, respectively. Each pathogenicity test consisted of four replications of each treatment combination of two plant species (turnip and mustard) and the number of inoculation treatments [the number of fungal isolates inoculated, a negative water control treatment, and positive control isolate(s)]. Plants were laid out in a randomized complete block (RCB) experimental design when removed from the plastic bags and placed on greenhouse benches. Each plant was evaluated for the percentage of leaf area with light leaf spot symptoms by rating three leaves/plant 14 and 21 days after inoculation (dai). For plants on which limited veinal browning was the primary symptom, disease severity was rated as 1%. Glass slide mounts made from leaf lesions that had developed by 21 dai were examined microscopically, and isolations completed from the lesions onto clarified cV8 agar medium to verify the causal agent of symptoms. Isolations from symptomatic leaves were completed 21 dai for three of the four replicate plants by dipping symptomatic leaf tissue into 70% ethyl alcohol for 3 to 7 s, drying the tissue on sterilized paper towel in a laminar flow hood, plating the

leaf sections onto ½cPDA, and incubating the leaf pieces with cool white fluorescent light and NUV light for 8 h/day at 15°C, and 16 h of dark/day at 10°C. Of the three re-isolates per original isolate of *P.* cf. *brassicae* and *N. capsellae*, one re-isolate was single-spored, stored, and DNA extracted for ITS rDNA or β-tubulin sequencing as described above and in Chapter 2. DNA sequences of the re-isolates were compared to those of the original isolates by alignment using Mega 7 (Kumar et al. 2015). The pathogenicity tests for the isolates were conducted shortly after the various isolates were obtained from samples of brassica plants collected in northwestern Washington in 2016, necessitating four tests. Pathogenicity tests for isolates of *N. capsellae* were included in the last two tests because those isolates were obtained later in the survey and took longer to sporulate adequately in culture to produce enough inoculum for the tests.

The mean severity ratings of three leaves/plant for each replication of each treatment combination were subjected to analyses of variance (ANOVA), with replication a random effect, and plant species and inoculation treatment fixed effects in the model. The non-inoculated control plants of each species were excluded from the ANOVA because symptoms were not observed on those plants (see Results), so the data were analyzed as 2 x 5, 2 x 3, 2 x 12, and 2 x 4 factorial designs for the first, second, third, and fourth pathogenicity tests, respectively. Assumptions of normality and equal variance were tested for parametric analysis. Treatment means were compared using Fisher's protected least significant difference (LSD) at $P < 0.05$.

3.2.2.3. Survey for *P.* cf. *brassicae* on brassica cover crop seed lots planted in northwestern Washington.

In the summer of 2016, a request was sent to members of the Puget Sound Seed Growers' Association (PSSGA) and the Western Washington Small Seed Association (WWSSA) for samples of brassica cover crop seed lots that were planted in northwestern Washington that season or that would be planted in fall 2016, to assay for *P.* cf. *brassicae.* Based on the results described in Chapter 2, *P.* cf. *brassicae* can infect brassica seed, be transmitted from infected seed to seedlings, and be

detected on brassica seed using the 4°C NP-10 agar seed health assay. Samples of five seed lots were received from Skagit Valley growers and agricultural supply companies: 1) a yellow mustard (*B. juncea*) seed lot of the cv. Caliente 199, produced in central Washington in 2014; 2) a radish (*Raphanus raphanistrum*) seed lot of cv. CCS779 (unknown region of production); 3) a white mustard (*Sinapis alba*) seed lot of the cv. Nemagon White, grown in western Oregon (Willamette Valley) in 2016; 4) a second mustard seed lot of Caliente 199, grown in central Washington in 2015; and 5) a daikon radish seed lot of the cv. VNS Daikon, grown in central Washington in 2014.

The 4°C NP-10 agar seed health assay described in Chapter 2 was used to assay the five seed lots for *P.* cf. *brassicae*. Each seed health assay was set up as a randomized complete block design (RCBD) with four replications of 100 seed tested. The seed for each replication were plated onto NP-10 agar medium (ISHI-Veg 2015; Sorenson et al. 1991) in three acrylic boxes, each 110 mm x 110 mm x 35 mm deep (model 156CB, Hoffman Manufacturing, Inc., Jefferson, OR), after the boxes and lids had been sterilized with 70% ethyl alcohol in a laminar flow hood followed by exposure to UV light in a biological safety cabinet for 20 min. Once seed were plated, the boxes were enclosed in a cardboard box at 4°C for 21 days. Microscopic examinations of the seed were done 21, 28, and 35 days after plating. A second set of samples of four replications of 100 seed/lot was surface-sterilized by placing the seeds in a mesh tea strainer (Harold Import Co., Lakewood, NJ), immersing the strainer with the seeds into 1.2% NaOCl in a beaker for 60 s, and then triple-rinsing the seed in sterilized, deionized water, drying the seed on sterilized paper towels in a laminar flow hood, and storing the seed overnight in sterilized Petri plates before plating the seed. Data were analyzed by calculating the means and standard errors of the percentage of each seed lot infected with *P.* cf. *brassicae.*

3.2.3. Species identity of USA isolates of *P.* cf. *brassicae.* Isolates of *P.* cf. *brassicae* collected in northwestern Washington (Table 3.1) were confirmed to be similar to the isolates Cyc001 to Cyc004 collected from Benton Co., OR in 2015, as described in Chapter 2. Because there were only four ITS rDNA

sequences of *P. brassicae* isolates in GenBank in 2016, and because of the limited maximum sequence similarity (95.00%) of the ITS rDNA of Oregon isolates of *P. brassicae* with the ITS rDNA sequences in GenBank (see Chapter 2), as well as the inability to amplify ITS rDNA of the USA isolates of *P.* cf. *brassicae* with the primers and PCR assay published by Foster et al. (1999), collaboration was initiated with Drs. Jonathon West and Kevin King at Rothamsted Research to determine the genetic relationship of USA isolates of *P.* cf. *brassicae* with isolates of *P. brassicae* from various regions of the world. Genomic DNA extracts of 18 *P. brassicae* isolates from the EU, UK, and New Zealand (NZ) were provided by West and King (Table 3.2) to compare with genomic DNA extracted from 12 isolates of *P.* cf. *brassicae* from northwestern Washington and western Oregon (also extracted by West and King) using molecular phylogenetics and MLSA of three DNA regions, ITS rDNA, β-tubulin, and TEF 1-α, as detailed below (Bakkeren et al. 2000, Carter et al. 2013; Taşkin et al. 2010). In addition, sequences for two of the mating type genes of *P. brassicae*, *MAT1-3* and *MAT1-2* (representing mating type idiomorphs MAT-1 and MAT-2, respectively), were produced by West and King using the protocol described by Foster et al. (2002), and subjected to phylogenetic analysis. In addition to the MLSA and mating type study, a sexual crossing study was completed with 10 USA isolates and 10 isolates from the EU and UK provided by West and King (Table 3.2), and a pathogenicity test was performed on the turnip cv. Hakurei with the same 10 isolates from the EU and UK to compare with the USA isolate Cyc001 to assess biological differences between the EU/UK and USA isolates. This combination of studies was used to assess the species identity of the USA isolates using the CSC (Crous et al. 2015; Quaedvlieg et al. 2014). Relevant permits were obtained to receive and handle the isolates - Defra Plant Health License No. PHL 174B/4630 (10/2004) for the Rothamsted Research lab in the UK to receive isolates from the USA, and the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Plant Protection and Quarantine (PPQ) Permit No. P526-16-04179 for Dr. Lindsey du Toit's lab in the USA to receive isolates from the EU, UK, and NZ. Isolates were grown in the UK on 3% malt extract agar (MEA)

medium (30 g malt extract, 5 g mycological peptone, and 15 g agar per liter, with a pH of 5.4 ± 0.2) (CM0059, ThermoScientific, Waltham, MA) in 9-cm-diameter Petri plates, from single-spore isolates prepared from frozen glycerol stocks. One set of plates of each isolate was sent to Dr. du Toit's lab on 27 February 2017. Plates were incubated at 18˚C in the dark until used for the sexual crosses and pathogenicity test, as detailed below.

3.2.3.1. Pathogenicity test of USA isolates of *P.* cf. *brassicae* vs. EU and UK isolates of *P. brassicae*. Pathogenicity tests were completed on 'Hakurei' turnip plants for 10 isolates from the EU/UK (Table 3.2) compared to the USA isolate Cyc001 (positive control isolate). Plants treated with water served as a negative control treatment. Four replicate plants were used for each of the 12 treatments. The 'Hakurei' turnip plants were sown in a greenhouse at the WSU Mount Vernon NWREC on 27 March 2017, as described above for the pathogenicity test of USA isolates, and the seedlings transplanted after two weeks into Sunshine Mix No. 1 in 10-cm-diameter pots. On 5 April 2017, the plants were put into plastic containers (4 plants/container, representing 4 replicate plants/isolate), and each container with plants was enclosed in a large plastic bag to create a humidity chamber, as detailed for the pathogenicity test of the USA isolates. The plants were incubated overnight in a growth chamber at 18°C with cool white fluorescent light for 12 h/day and without light for 12 h/day. The next day, a spore suspension was made from each of the 10 *P. brassicae* isolates and the *P.* cf. *brassicae* isolate to inoculate the turnip plants, as described above. A conidial suspension of each isolate was produced by flooding each 9-cm-diameter plate with 1 ml sterilized, deionized water, and gently rubbing the surface of the plate with a sterilized, bent glass rod. The resulting conidial suspension was filtered through two layers of cheesecloth, quantified using a hemocytometer, and diluted to 1 x 10^6 spores/ml. After inoculation, the plants were placed back into the plastic bags for 2 days in the growth chamber.

The turnip plants were then laid out in an RCB design when removed from the plastic bags and placed back into the growth chamber. Each plant was evaluated for the percentage of leaf area with

light leaf spot symptoms by rating three leaves/plant 14 dai. However, this rating system only sufficed for the positive control isolate, Cyc001, as very different signs and symptoms were observed on the turnip plants inoculated with the EU and UK isolates. By 14 dai, none of the plants inoculated with isolates from the EU or UK had symptoms of light leaf spot. Instead, these plants had developed white conidiomata of *P. brassicae*, very similar to the white, subcuticular conidiomata described by Rawlinson et al. (1978) and Fitt et al. (1998). Therefore, the presence or absence of circular patches of white conidiomata on three leaves/plant was rated 14 dai. The ratings were modified 21 and 28 dai to record the following: the total number of leaves present at the time of inoculation, the number of inoculated leaves that were chlorotic, the number of inoculated leaves that were necrotic (senesced), and the number of inoculated leaves with white conidiomata. Isolations were completed from a lesion on each plant, from an asymptomatic leaf with white conidiomata, or an asymptomatic leaf section of each negative control plant onto MEA medium. Leaf sections were dipped into 70% ethyl alcohol for 3 to 7 s, dried on sterilized paper towel in a laminar flow hood, plated onto MEA, and incubated with cool white fluorescent light and NUV for 8 h/day at 15°C, followed by 16 h of dark/day at 10°C. The leaf sections were examined microscopically for gelatinous masses of conidia typical of *P. brassicae* (Rawlinson et al. 1978), to verify the causal agent of symptoms and/or signs.

Leaf rating data were subjected to ANOVAs for the number of inoculated leaves with white conidiomata/plant, the number of inoculated necrotic leaves/plant, and the number of inoculated chlorotic leaves/plant 28 dai for each replication of each treatment. Replication was treated as a random effect and inoculation treatment as a fixed effect in the model. The control plants treated with water were excluded from the ANOVAs for rating of leaves with white conidiomata and chlorosis because symptoms were not observed on those plants (see Results). The positive control isolate of *P.* cf. *brassicae*, Cyc001, was excluded from the ANOVA for the number of inoculated leaves with white conidiomata, as white conidiomata were not observed on plants inoculated with that isolate.

Assumptions of normality and equal variance were tested for parametric analysis. Treatment means were compared using Fisher's protected LSD at $P < 0.05$.

3.2.3.2. *In vitro* sexual compatibility test of USA isolates of *P.* cf. *brassicae* with EU and UK isolates of *P. brassicae.* Sexual compatibility of *P.* cf. *brassicae* isolates from the USA with isolates from the EU and UK (Table 3.2) of both mating type idiomorphs, MAT-1 and MAT-2 (Foster et al. 1999; Foster et al. 2002; Ilott et al. 1984), was tested to assess whether or not the isolates might belong to the same species. Ten EU or UK isolates, 5 of each mating type (K. King, *unpublished data*), were crossed with 10 USA isolates, 5 of each mating type, in pairs of opposite mating types. This was started on 9 March 2017 in the lab of Dr. Lindsey du Toit at the WSU Mount Vernon NWREC. The collaborators at Rothamsted Research had tested all 20 isolates to determine the mating types using the multiplex PCR assay of Foster et al. (2002) (Table 3.2).

To make the sexual crosses, a conidial suspension of each isolate was produced by flooding each colonized, 9-cm-diameter MEA plate with 1 ml sterilized, deionized water, and gently rubbing the surface of the plate with a sterilized, bent glass rod. The resulting conidial suspension was filtered through sterilized, 25 ml syringe filters (Pall Life Science, New Port Richey, FL), quantified using a hemocytometer, and diluted to 1 x 10^6 spores/ml. Three replicate crosses were made for each pair of isolates by adding 40 µl of conidial suspension of each of the appropriate isolates to the center of a 9-cm-diameter Petri dish containing 3% MEA medium, and distributing the 80 µl across each plate using a sterilized, bent glass rod. Isolates of *P.* cf. *brassicae* from the USA, 5 each of the MAT-1 and MAT-2 idiomorphs, and isolates from the EU and UK, 5 of each of the two idiomorphs, were used to pair all possible combinations of MAT-1 x MAT-2 crosses for the USA and EU/UK isolates, for 100 total crosses. Crosses also were made for all of the USA MAT-1 x USA MAT-1 isolates (15 total crosses) and all of the USA MAT-2 x USA MAT-2 isolates (15 crosses), to test whether or not the USA isolates might be homothallic (Ilott et al. 1984). This was not done for the EU/UK isolates because these isolates

originated from known heterothallic populations of *P. brassicae* (Ilot and Ingram 1984). Each Petri plate was sealed with Parafilm, and the plates were kept in plastic containers (each 41.0 cm x 28.0 cm x 16.5 cm deep and closed with a lid) in the dark at 18˚C in an incubator (Model 130BLL).

Using a dissecting microscope (up to 100x magnification), the Petri plates were checked visually each week for the development of apothecia, starting two weeks after plating (Gilles et al. 2001). Once apothecial initials were observed, the maturity of any apothecia that developed was assessed by making slide mounts of thin sections of the apothecia, using a scalpel, to examine microscopically for the presence of asci and ascospores. Plates were examined weekly in this manner for three months. Each replicate plate of each cross was scored as positive or negative for apothecial initials, apothecia, and asci with ascospores (Table 3.3). Photographs of these structures were taken with a Leica camera (DFC295, Wetzlar, Germany) and Leica Application Software Version 3.8 (Leica Microsystems, Heerbrugg, Switzerland).

3.2.3.3. Phylogenetic analyses and MLSA of USA isolates of *P.* cf. *brassicae* vs EU, UK, and New Zealand isolates of *P. brassicae*. Drs. West and King sent genomic DNA extracts of 18 *P. brassicae* isolates from the UK, EU, and NZ to compare with that of 12 isolates of *P.* cf. *brassicae* from northwestern Washington and western Oregon (Table 3.2). DNA of four additional fungal isolates, *Oculimacula acuformis* isolate 22-443 collected from wheat (*Triticum aestivum*), *Rhynchosporium orthosporum* isolate 27-DG collected from cat grass (*Dactylis glomerata*), and isolates AJ549759 and AJ537511 of *R. commune*, also were provided by West and King to serve as outgroups to root the phylogenetic trees. Two nuclear genes, β–tubulin and TEF 1-α, and the ITS rDNA region were sequenced by PCR assays with β tubulin primers F-Btub3 (TGG GCY AAG GGT YAY TAY AC) and F-Btub2r (GGR ATC CAY TCR ACR AA) (Carter et al. 2013), TEF 1-α primers EF5AR (CCA GCA ACR TTA CCA CGA CG) and EF2F (AAC ATG ATS ACT GGT ACY TCC) (Taşkin et al. 2010), and the ITS rDNA primers UNUP18S42 and

UNLO28S576B (Bakkeren et al. 2000). PCR amplification of the ITS rDNA and the β-tubulin gene were performed as described in Chapter 2. PCR amplification of the TEF1-α DNA was done in a total reaction volume of 20 μl, which included 2 μl 10x buffer, 0.48 μl (25 mM) $MgCl_2$, 0.3 μl (2mM) of each dNTP, 0.3 μl (10 um) of each primer, 0.2 μl (5 units/μl) of *Taq* DNA polymerase, and 2 μl of genomic DNA. The TEF1-α PCR assays were performed in a Thermohybaid PCR Express thermocycler (ThermoFisher Scientific, Waltham, MA) using the following cycling parameters: 95°C for 2 min; 35 cycles of 95°C for 15 s, 58°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. Amplified DNA was run on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, MO) in 1x TBE buffer with a 100 bp DNA ladder (Invitrogen Life Technologies), and visualized using UV light. ExoSAP-IT (ThermoFisher Scientific) was used to clean each PCR product for sequencing, using 2 μl of ExoSAP-IT for every 5 μl of PCR product. For each amplified product of each isolate, 10 to 15 ng of cleaned PCR product and 8 pmole of each primer were sent to Elim Biopharmaceuticals, Inc. (Hayward, CA) for both forward and reverse sequencing of the amplified DNA.

The generated sequences for each gene or DNA region were aligned, edited, and concatenated using MEGA 7 (Kumar et al. 2015). The phylogenetic analyses used in this study included a Maximum Likelihood analysis performed with PAUP v 4.0 (Swofford 2002) and a Bayesian analysis performed with MrBayes Version. 3.2 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2011). All analyses were run using the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway Version 3.3, which provides public access to the National Science Foundation (NSF) Extreme Science and Engineering Discovery Environment (XSEDE) large computational resources through an online interface (CIPRES 2017). Evolutionary models were determined using jModelTest (Darriba et al. 2012) for each gene and for the concatenated data. For the Maximum Likelihood analyses, the TPM2+I model was used for the concatenated data and the ITS rDNA region, the Trn model was used for the β tubulin gene, and the TrNEF model was used for the TEF 1-α gene. Each analysis included 1,000 bootstrap replicates.

jModelTest was used to determine the best nucleotide substitution model for each gene or gene region sequenced as well as the concatenated data in order to perform a model-optimized Bayesian phylogeny. For each Bayesian analysis, the Markov Chain Monte Carlo (MCMC) analysis of four chains was started in parallel from a random tree topology, the temperature parameter was set to 0.2, trees were saved every 1,000 generations for a total of 1,000,000 generations, and the burn-in was set to 25% (CIPRES 2017).

In addition to the MLSA, phylogenetic analyses of the *MAT1-3* and *MAT1-2* gene sequences were performed. Sequences were generated by Dr. King (Table 3.2) using the Foster et al. (2002) multiplex PCR assay with the *MAT1-3* specific forward primer PbM-1-3 (GATCAAGAGACGCAAGACCAAG), the *MAT1-2* specific forward primer PbM-2 (CCCGAAATCATTGAGCATTACAAG), and the common reverse primer Mt3 (CCAAATCAGGCCCCAAAATATG). The phylogenetic methods described above were used for these two gene regions. The outgroup isolate used for the *MAT1-3* phylogenetic analysis was *R. commune* sequence AJ549759, and the outgroup isolate for the *MAT1-2* phylogenetic analysis was *R. commune* sequence AJ537511. For the Maximum Likelihood analysis, the model TPM1 was used for *MAT1-3* and K80 was used for *MAT1-2*, while the jModelTest was used to determine the best nucleotide substitution model for *MAT1-3* and *MAT1-2* to perform a model-optimized Bayesian phylogeny. All resulting phylogenetic trees were printed in FigTree Version 1.4.2 (FigTree 2017). The DNA sequences generated in this study were deposited in GenBank, the National Center for Biotechnology Information (NCBI) DNA database.

3.3 Results

3.3.1. Questionnaire of Pacific Northwest brassica stakeholders. A total of 64 questionnaires were completed: 15 by respondents from the Washington Tilth Producers' Annual Conference, 35 by

respondents from the PNVA Annual Convention and Trade Show, and 14 by respondents from the Organic Seed Growers' Conference. Of the respondents, 22 (38.6%) were farmers, 15 (26.3%) worked in the seed industry, 14 (24.6%) were crop consultants, 5 (8.8%) were researchers, 3 (5.3%) worked in extension or education, and 8 (14.0%) categorized themselves as "other." Most respondents grew or worked with brassicas for fresh markets (34), followed by brassicas grown for seed crops (24), cover crops (19), processing (11), oilseed (3), and "other" (2) crops. Of the *Brassica* species listed, kale was the crop grown or worked with most commonly (32 respondents), followed by cabbage and kale (31), mustard (28), arugula (24), Brussels sprouts and cauliflower (22), and canola (7). Ten respondents indicated that they grew or worked with a type of Brassica crop not listed, and 14 did not grow or work with any brassica crops.

When asked about brassica diseases in general, 21 respondents (47.7%) said they had observed diseases in brassica crops, while 14 (31.8%) had not, and 10 (22.7%) were unsure. When asked if they were aware of black leg, caused by *P. lingam*, 31 respondents (50.8%) were aware. In contrast, only 4 respondents (6.6%) were aware of light leaf spot and/or white leaf spot, 15 (24.6%) were aware of all three brassica diseases, and 11 (18%) were not aware of any of these three brassica diseases. For the questions about testing for seedborne pathogens, 23 respondents (35.9%) indicated they did not know if the brassica seed with which they work had been tested for seedborne pathogens, 9 respondents (14.1%) indicated they only purchase brassica seed that has been tested for seedborne pathogens (although the specific pathogens were not listed), 2 respondents (3.1%) grew and saved their own seed, 1 respondent (1.6%) did not purchase seed that had been tested for seedborne pathogens, and 29 respondents (45.3%) did not feel this question was applicable to them.

When queried about awareness of state regulations aimed at preventing the dissemination of brassica seed infected with *P. lingam* and/or *X. campestris* pv. *campestris*, 27 respondents (42.9%) were not aware of the Oregon Department of Agriculture (ODA) Crucifer Rule OAR 603-052-0870 about black

leg that applies to all Brassicaceae crops planted in Oregon, while 14 respondents (22.2%) were aware, 7 (11.1%) were unsure, and 15 (23.8%) felt that is was not applicable to their work. Participants' responses when asked about the WSDA Crucifer Quarantine Rule WAC 16-301-495 to 16-301-580 in western Washington, and the proposed amendment to the rule to include all counties east of the Cascade Mountains, revealed that 26 (41.3%) and 33 (54.1%) were not aware of either, 18 (28.6%) and 11 (18.0%) were aware of both, and 7 (11.1%) and 6 (9.8%) were unsure of the original quarantine rule or the amendment, respectively. Twelve (19.0%) and 11 (18.0%) respondents felt the WSDA Crucifer Quarantine Rule and amendment, respectively, were not applicable to their work.

3.3.2. Survey for *P.* cf. *brassicae* and *N. capsellae* in northwestern Washington.

3.3.2.1. Survey, species identity, and pathogenicity of *P.* cf. *brassicae* and *N. capsellae* isolates from brassica plants in northwestern Washington.

In the survey of brassica crops and weeds in three counties in northwestern Washington in 2016, 20 sites were visited, from which 19 single-spore isolates of *P.* cf. *brassicae* and 4 isolates of *N. capsellae* were obtained (Table 3.1). *P.* cf. *brassicae* was isolated from birds' rape mustard weeds collected from each of three sites in Snohomish Co. Both *P.* cf. *brassicae* and *N. capsellae* were isolated from birds' rape mustard plants from one site in Whatcom Co. In Skagit Co., 16 sites were surveyed, with both *P.* cf. *brassicae* and *N. capsellae* detected and isolated from mustard (*B. juncea*) cover crops in each of 2 sites, and *P.* cf. *brassicae* alone was isolated from birds' rape mustard weeds in each of 7 sites. Neither *P.* cf. *brassicae* nor *N. capsellae* was detected in cabbage seed crops surveyed at 7 sites. A subset of 16 of the isolates of *P.* cf. *brassicae* and 4 of the isolates of *N. capsellae* collected was selected for sequencing and pathogenicity testing to represent all three counties and both hosts, for a total of 15 of the 20 sites surveyed. Two additional *P.* cf. *brassicae* isolates (Cyc023a and Cyc024a) and two additional *N. capsellae* (Psc012 and Psc015) isolates from the Willamette Valley, OR were included in the pathogenicity tests, as detailed above (Table 3.1).

The ITS rDNA sequences for 16 of the 17 *P.* cf. *brassica* isolates (Cyc007, Cyc008, Cyc009, Cyc010, Cyc011, Cyc012, Cyc013a, Cyc014, Cyc015, Cyc023a, Cyc024a, Cyc025, Cyc026, Cyc027, Cyc028, and Cyc029a) were identical over a 604 nt sequence (GenBank accession numbers listed in Table 3.1). These ITS rDNA sequences were also identical to those of the Oregon isolates described in Chapter 2, i.e., Cyc001, Cyc003, Cyc005, and Cyc006. Therefore, the BLAST search in GenBank again revealed the most similar ITS rDNA sequence was that of a *Cadaphora* sp. (JN859254.1) from Hungary, with 96.00% similarity; and one of the four ITS rDNA sequences of *P. brassicae* in GenBank, AJ305235.1 of an isolate collected in the UK, was only 95.00% similar to the ITS rDNA sequences of the 16 isolates from Washington State. The other three *P. brassicae* ITS rDNA sequences in GenBank did not show a match with the sequences of the Washington isolates in the BLAST search. The ITS rDNA sequence of isolate Cyc030b differed from that of the other 16 isolates by 1 nt, and did not match the *P. brassicae* ITS rDNA sequence AJ305235.1 in GenBank. However, the closest match was still that of the *Cadaphora* sp. (JN859254.1). The morphological characteristics of all 17 of the putative *P.* cf. *brassicae* isolates from Washington were typical of those of *P. brassicae,* i.e., hyaline, smooth, cylindrical, mostly aseptate and eguttulate conidia of similar dimensions produced on short, non-branching conidiophores (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977). The β tubulin sequences for a subset of 10 of the isolates of *P.* cf. *brassicae* collected in northwestern Washington (Cyc007, Cyc009, Cyc011, Cyc013a, Cyc015, Cyc017, Cyc024, Cyc025, Cyc029, Cyc031a) and 1 isolate (Cyc023a) collected in Oregon in 2016, were identical to the 602 nt sequence of isolate Cyc001 collected in 2015 from a 'Barkant' turnip seed crop, as detailed in Chapter 2. These β tubulin gene sequences were 98.00% similar to that of a single *P. brassicae* isolate in GenBank (KC342227.1) collected in France (Carter et al. 2013). Therefore, based on the morphological characteristics and the ITS rDNA and β tubulin gene sequences, the Washington isolates were identified as *P.* cf. *brassicae*.

The ITS rDNA sequences of the four putative *N. capsellae* isolates from Washington State, Psc010, Psc011, Psc013, and Psc014, and two from Oregon State, Psc012 and Psc015, were identical over a 531 nt sequence amplified for all isolates. The six sequences were identical to those of the nine isolates collected in Oregon in 2015 (Psc001 to Psc009, see Chapter 2) and were 99.00% similar to the ITS rDNA sequence of an *N. capsellae* isolate in GenBank (GU214662.1) collected in the UK. The isolates also were confirmed morphologically as *N. capsellae* by observing hyaline, straight or slightly curved conidia that were each 1- to 5-septate, and of similar dimensions to those described for this species (Crossan 1954; Deighton 1973). Isolation of *N. capsellae* onto 10% clarified cV8 agar resulted in the development of dematiaceous, stromatic structures that gave rise to the conidia similar to those described by Crossan (1954). Therefore, based on the ITS rDNA sequences and morphological characteristics, isolates Psc010 to Psc015 were identified as *N. capsellae.* The ITS rDNA sequences from one isolate for each host and sampling location were submitted to GenBank (Table 3.1).

The 17 isolates of *P.* cf. *brassicae* from Washington, and isolate Cyc001 (positive control isolate) from Oregon, which were evaluated in pathogenicity tests 1, 2, and 3 (Table 3.1), all caused symptoms typical of light leaf spot on both mustard and turnip, whereas the negative control plants of each host remained asymptomatic. The second severity rating (21 dai) was used for data analysis because disease severity was very limited on the mustard plants at the first rating completed 14 dai (average of 14.51 ± 7.45%, 19.22 ± 4.99%, and 2.40 ± 1.03% severity on the mustard plants in pathogenicity tests 1, 2, and 3, respectively). Data met assumptions for parametric analysis in pathogenicity tests 1 and 2, but the data for pathogenicity test 3 had to be square root-transformed to meet the assumptions of equal variance. Based on the ANOVAs, significant differences in severity of light leaf spot 21 dai were detected among the two plant species tested ($P = 0.0004$, $P < 0.001$, and $P < 0.001$ for pathogenicity tests 1, 2, and 3, respectively). The turnip plants of cv. Hakurei were more susceptible to light leaf spot (100.00 ± 0.00, 99.72 ± 0.28, and 84.09 ± 3.80% severity of symptoms in pathogenicity tests 1, 2, and 3, respectively)

than the mustard plants of cv. Caliente 199 (84.79 ± 3.72%, 77.03 ± 4.00%, and 21.45 ± 2.85% severity in pathogenicity tests 1, 2, and 3, respectively). There were no significant differences (P = 0.7384 and P = 0.1910 for pathogenicity tests 1 and 2, respectively) among the isolates of *P.* cf. *brassicae* tested (average 93.38 ± 4.67% light leaf spot severity for Cyc001, Cyc007, Cyc008, Cyc009, and Cyc010 in test 1; and average 88.37 ± 5.23% severity of light leaf spot for Cyc001, Cyc011, and Cyc012 in test 2 across the two host species). In pathogenicity test 3, there was a significant difference (P <0.0001) among the 11 Washington and 1 Oregon isolates tested. The interaction term between plant species and isolate of *P.* cf. *brassicae* was not significant for any of the pathogenicity tests (P = 0.738, 0.258, and 0.106, for pathogenicity tests 1, 2, and 3, respectively). The most virulent isolates in pathogenicity test 3 were Cyc024a (69.50 ± 10.27% severity of symptoms), Cyc026 (68.17 ± 12.93%), Cyc023a (66.42 ± 13.80%), Cyc025 (61.50 ± 14.77%), Cyc001 (60.00 ± 13.43%), Cyc015 (56.17 ± 16.22%), Cyc028 (55.50 ± 13.89%), and Cyc029a (54.79 ± 12.42%) with no significant differences among these eight isolates. Isolates Cyc027 (48.29 ± 14.53%) and Cyc013a (42.67 ± 14.39%) had intermediate virulence, while Cyc014 (29.75 ± 13.82%) and Cyc030 (9.78 ± 6.74%) were the least virulent. For isolate Cyc030 (9.78 $\pm$ 6.74%), only 3 plants could be inoculated per host species due to an inoculum shortage.

As reported in Chapter 2, turnip plants inoculated with *P.* cf. *brassicae* developed symptoms before the mustard plants, with pale brown streaks on the stems and veinal browning on the leaves that darkened over time. Veinal browning was followed by the development of small (<5 mm diameter), chlorotic leaf spots. The chlorotic spots became diffuse and expanded in size quickly on the turnip leaves, coalescing and covering the leaf surface by 21 dai. Symptoms were similar but developed more slowly on the mustard leaves. Hyaline, smooth, cylindrical, and mostly aseptate and eguttulate conidia were observed on short, non-branching conidiophores in slide mounts prepared from symptomatic lesions on the plants inoculated with *P.* cf. *brassicae*, confirming the typical morphology of *P. brassicae* (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977). Koch's postulates were completed by re-

isolating the pathogen from symptomatic leaves of three of the four replicate plants for each combination of treatment and host, and sequencing the ITS rDNA and β tubulin DNA of one isolate from each of the three re-isolates for genus and species identification as similar to *P. brassicae*. However, the white, subcuticular conidiomata described by Rawlinson et al. (1978) and Fitt et al. (1998) as being produced on the leaves of plants infected with *P. brassicae* were not observed on any of the plants inoculated.

The *N. capsellae* isolates, which were tested in pathogenicity tests 3 and 4 (Table 3.1), included five of the six isolates of *N. capsellae* collected from *B. juncea* and *B. rapa* plants in Washington (4 isolates) and Oregon (2 isolates), and the positive control isolate (Psc007 from Oregon). All of these isolates caused symptoms typical of white leaf spot on the mustard and turnip plants, while the negative control plants and plants inoculated with isolate Psc014 did not develop symptoms of this disease on either host (Fig. 3.2). The second severity rating completed 21 dai was used in the ANOVA because the severity of white leaf spot was limited at the first rating 14 dai (4.15 ± 0.94 and 3.00 ± 0.55% averaged across isolates in pathogenicity tests 3 and 4, respectively). Because assumptions for parametric analysis could not be met, data were subjected to Friedman's non-parametric rank test, with significant differences ($P = 0.0003$ and $P < 0.0001$ for pathogenicity tests 3 and 4, respectively) among the two brassica hosts, and significant differences among the isolates ($P < 0.0001$ for both pathogenicity tests). However, unlike the *P.* cf. *brassicae* pathogenicity tests, a significant host-by-treatment interaction was detected ($P = 0.0046$ and 0.0105 in pathogenicity tests 3 and 4, respectively). In pathogenicity test 3, the control isolate, Psc007, was the most virulent on both hosts with 33.75 ± 11.17 and 32.67 ± 18.46% severity of white leaf spot on mustard and turnip plants, respectively (Fig. 3.2A). The severity of symptoms was 4.50 ± 2.28 and 4.59 ± 1.13% for isolate Psc010 on mustard and turnip, respectively; 0.34 ± 0.39 and 4.33 ± 2.72% for isolate Psc011 on mustard and turnip, respectively; and 0.92 ± 0.99 and 9.42 ± 5.49% for Psc013 on mustard and turnip, respectively. In pathogenicity test 4, isolate Psc012 was the

most virulent on turnip with 13.75 ± 6.72% severity of symptoms compared to just 4.25 ± 1.97% severity on mustard plants (Fig. 3.2B). The severity of symptoms was 2.17 ± 0.64 and 4.00 ± 1.16% for Psc007 on mustard and turnip, respectively; and 3.67 ± 0.77 and 5.34 ± 2.52% for isolate Psc015, respectively. Symptoms were not detected on plants of mustard or turnip inoculated with isolate Psc014.

Symptoms of white leaf spot initially appeared approximately 10 dai as small (1- to 3-mm-diameter), light spots, each surrounded by a chartreuse halo, as described in Chapter 2 for Oregon isolates of *N. capsellae*. Lesions enlarged and coalesced, with infected leaves senescing by 21 dai. *N. capsellae* sporulation in the lesions was confirmed morphologically 21 dai when slide mounts were made from lesions that developed on the plants, by observing hyaline, straight or slightly curved conidia that were 1- to 5-septate (Crossan 1954; Deighton 1973). Koch's postulates were completed by re-isolating the pathogen from three of the four replicates for each combination of treatment and host, and sequencing the ITS rDNA of one isolate from each of the three re-isolates for genus and species verification, as described above.

3.3.2.2. Survey of *P.* cf. *brassicae* in brassica cover crop seed lots planted in northwestern Washington.

P. cf. *brassicae* was detected in only one of the five cover crop seed lots tested with the 4°C NP-10 agar seed health assay, and in very limited amounts on that lot, i.e., 0.33 ± 0.58% of the surface-sterilized seed of the white mustard cover crop cv. Nemagon, but not on any of the non-surface-sterilized seed of this lot. The signs of *P.* cf. *brassicae* on the 'Nemagon' seed were indistinct, pale pink, gelatinous masses of conidia, as described in Chapter 2. This infected seed lot was produced in the Willamette Valley, OR.

3.3.3. Species identification of USA isolates of *P.* cf. *brassicae.*

3.3.3.1. Pathogenicity test of USA isolates of *P.* cf. *brassicae* vs. EU and UK isolates of *P brassicae*.

The 10 EU and UK isolates of *P. brassicae* tested on the turnip cv. Hakurei each produced

signs (white conidiomata) on the leaves, that were first observed 11 dai (Fig. 3.3A and 3.3B). These resembled the white, subcuticular conidiomata on leaves of inoculated rapeseed that were described by Rawlinson et al. (1978) and Fitt et al. (1998). However, white conidiomata were not observed on the leaves of any of the plants inoculated with the USA isolate of *P.* cf. *brassicae* (Fig. 3.3C and Chapter 2). By 21 dai, leaves with white conidiomata had senesced more quickly than those on the negative control plants. Some chlorosis of the inoculated leaves was observed, but not the bright, yellow chlorotic spots observed on plants inoculated with the USA isolates (Fig. 3.3C). The positive control isolate, Cyc001, caused symptoms typical of those seen in previous pathogenicity tests with the USA isolates of *P.* cf. *brassicae*, but plants inoculated with this isolate never produced the patches of white conidiomata that were observed on leaves of plants inoculated with the EU/UK isolates (Fig. 3.3C).

The severity ratings 28 dai were used for the ANOVAs because the number of necrotic leaves was much greater than at 21 dai. For the ANOVAs of the number of inoculated leaves with white conidiomata and the number of necrotic inoculated leaves, assumptions for parametric analysis were met, while the data for the number of chlorotic leaves had to be analyzed using Friedman's non-parametric rank test. There were significant differences among isolates of *P.* cf. *brassicae* for all three variables. For the number of necrotic inoculated leaves, the ANOVA (coefficient of determination, R^2 = 0.647), revealed a significant main effect of isolates (P <0.0001) (Fig. 3.4A). However, there were no significant differences in the mean number of necrotic inoculated leaves caused by 10 of the 11 *P. brassicae* isolates (4.50 to 5.75 necrotic leaves/plant for isolates Fr2, Cyc001, 2016-34, 2016-26, UK73, 8CAB, 2016-50, E3a, 5a, and 2016-9). Only isolate 2016-5 caused significantly fewer necrotic leaves (4.50 ± 0.29 leaves/plant) than that caused by isolates Fr2 and Cyc001. The negative control plants averaged 2.50 ± 0.29 necrotic leaves/plant, which was significantly less than that of any of the plants inoculated with an isolate of *P. brassicae.* In summary, the USA isolate Cyc001 did not differ from 9 of the 10 EU/UK isolates in terms of the number of necrotic leaves that developed on inoculated plants (Fig. 3.4A).

The ANOVA results for the mean number of chlorotic leaves (R^2 = 0.489), also revealed a significant main effect of isolates of *P. brassicae* (*P* = 0.012). The water control treatment was excluded from this ANOVA as chlorotic leaves were not detected on those plants. Isolate Cyc001 caused the greatest mean number of leaves to turn chlorotic (1.75 ± 0.25 leaves/plant); however, this isolate did not differ significantly from 4 of the 10 EU/UK isolates in terms of number of chlorotic, inoculated leaves/plant, i.e., 2016-50 (1.00 ± 0.00 chlorotic leaves/plant); and isolates 2016-26, 2016-9, and UK73 (0.75 ± 0.25 chlorotic leaves/plant) (Fig. 3.4B). All other isolates caused significantly fewer chlorotic leaves/plant than Cyc001, i.e., isolates E3a, 2016-5, and 5a caused 0.50 ± 0.29 chlorotic leaves/plant; isolates 2016-34 and Fr2 caused 0.25 ± 0.25 chlorotic leaves/plant; and isolate 8CAB did not cause any leaves to turn chlorotic (Fig. 3.4B). For the ANOVA for the mean number of leaves with patches of white conidiomata, neither the positive control treatment (Cyc001) nor the negative control treatment were included in the analysis as plants for neither treatment developed these conidiomata (Fig. 3.4A and 3.4B). The ANOVA (R^2 = 0.634), revealed a significant main effect of isolates (*P* = 0.0005). Isolate 2016-26 from the UK caused the greatest mean number of leaves to produce white conidiomata (4.25 ± 0.63 leaves/plant), while isolate UK73 resulted in significantly fewer leaves with white conidiomata (0.50 ± 0.29 leaves/plant) than any other isolate (Fig. 3.4C). The other isolates did not differ significantly, with a range of 2.75 ± 0.25 to 3.75 ± 0.25 leaves with white conidiomata/plant. Koch's postulates were completed by re-isolating the pathogen from all three of the replicate plants used for re-isolations for each isolate, but not from the negative control plants. In summary, all 10 of the EU/UK isolates of *P. brassicae* caused patches of white conidiomata to form on the leaves, but not the symptoms of light leaf spot caused by the USA isolate, revealing a significant difference in pathogenicity effects of the USA isolates than the EU/UK isolates.

3.3.3.2. *In vitro* test for sexual compatibility of USA isolates of *P.* cf. *brassicae* with EU and UK isolates of *P. brassicae.* After nine weeks of incubating paired EU/UK and USA isolates of

opposite mating types on MEA, with weekly observations starting two weeks after plating, apothecia, asci, and ascospores had not been observed for any of the EU/UK isolates crossed with the USA isolates, or for any of the USA isolates crossed with other USA isolates, whether the crosses were a homothallic test (same mating type) or a heterothallic test (different mating types) of USA isolates. In contrast, mature apothecia, asci, and ascospores of *P. brassicae* were observed on plates for 16 of the 25 possible EU/UK x EU/UK sexual crosses of opposite mating type (Fig. 3.5A and 3.5B, Table 3.3). By nine weeks after plating, all three replicate plates for the following crosses had produced ascospores: 2016-5 with each of 2016-9, 2016-26, 2016-34, and 8CAB; 5a with 2016-34; and E3A with 2016-9 and 2016-26. Crosses of the following isolates had produced apothecia with asci and ascospores in one or two replicate plates by 9 weeks: 2016-5 x Fr2; 2016-50 with each of 2016-9, 2016-34, and 8CAB; 5a with each of 2016-26, 8CAB, and Fr2; E3a with each of 2016-34, 8CAB, and Fr2; and UK73 with 2016-26 and 8CAB. The following five EU/UK x EU/UK crosses had not produced mature apothecia with ascospores by nine weeks after plating: 2016-50 with each of 2016-9 (only immature apothecia observed), 2016-26, and Fr2; and UK73 with 2016-34 (immature apothecia) and Fr2 (apothecium initials only). What appeared to be apothecial initials were observed in several EU/UK x USA crosses, but did not mature to form apothecia with asci with ascospores, i.e., 2016-5 with each of Cyc015, Cyc017, and Cyc023a; 5a x 14CC2 and Cyc017; E3a x Cyc015; Cyc001 with 2016-26 and 8CAB; and Cyc009a x 2016-9, 2016-34, and 8CAB (Fig. 3.5C). Apothecial initials did not develop in any of the heterothallic or homothallic USA x USA crosses (*data not shown*). Variable colony color was observed on the MEA medium with the different isolates plated individually and in pairs. Colors ranged from cream, grey, and black, to pink, yellow, orange, and red, and all of the colonies that formed mature apothecia in the sexual crosses were grey and black (not lightly or brightly-colored).

3.3.3.3. Phylogenetic analyses and MLSA of USA isolates of *P.* cf. *brassicae* vs. EU, UK, and New Zealand isolates of *P. brassicae*. The PCR amplification and sequencing of the ITS rDNA, β

tubulin gene, and TEF 1-α gene was successful for the 32 isolates of *P. brassicae* evaluated in this study. The individual gene trees showed that the ITS rDNA, β tubulin, and TEF 1-α gene trees each resulted in discrimination of two distinct clades, with the 12 USA isolates of *P.* cf. *brassicae* in one clade and the 18 EU, UK, and New Zealand isolates of *P. brassicae* in the other clade, that were strongly supported by both the Maximum Likelihood and Bayesian analyses (Fig. 3.6, 3.7, and 3.8). The final alignment for the ITS rDNA included 30 isolates of *P. brassicae* or *P.* cf. *brassicae*, covering 690 nt (including alignment gaps). The Bayesian analysis supported a posterior probability of 1 for the 12 USA isolates grouping together and the EU, UK, and New Zealand isolates grouping separately (Fig. 3.6). The Maximum Likelihood analysis showed bootstrap support values of 100% for the EU/UK/NZ isolates as a group or clade, and 99.855% for the USA isolates. Therefore, the ITS rDNA trees showed strong support for separation of the USA isolates from the EU, UK, and New Zealand isolates (Fig. 3.6). The final alignment of the β tubulin gene sequences included the same 30 isolates, covering 602 nt (including alignment gaps). For this gene, all 12 of the USA isolates were discriminated clearly from the 18 EU, UK, and New Zealand isolates (Fig. 3.7). The Bayesian analysis supported the posterior probability for USA isolates grouping together at a probability of 1, and the EU, UK, and New Zealand isolates grouping together at 0.996. The Maximum Likelihood analysis showed bootstrap support of 99.400% for the clade of USA isolates and 95.913% for the clade of EU, UK, and New Zealand isolates. For the β tubulin tree, three subclades within the EU, UK, and New Zealand isolates were identified. Isolates PC28, PC20, PC50, and PC18 formed a subclade comprising isolates collected in Scotland (0.947/60.400 probabilities for the BPP/MLBS analyses, respectively). Two isolates from England, 4e and 5a, comprised a second subclade (0.986/62.812 probabilities for BPP/MLBS analyses, respectively). The remaining 12 EU, UK, and New Zealand isolates formed a third subclade (Fig. 3.7). The final alignment of the TEF 1-α gene sequences included 30 isolates, covering 597 nt (including alignment gaps). The Bayesian analysis supported the posterior probability for USA isolates grouping together at a probability of 0.748, and the EU, UK, and

New Zealand isolates grouping together at 1.000. The Maximum Likelihood analysis showed bootstrap support of 78.367% for the clade of USA isolates and 99.592% for the clade of EU, UK, and New Zealand isolates. As expected based on comparing these sequences with sequences of isolates in GenBank, the ITS rDNA strongly supported separate clades of *P.* cf. *brassicae* isolates collected from the USA vs. those from the EU, UK, and New Zealand, as did the β tubulin and TEF 1-α gene sequence gene trees.

The concatenated alignment of the three loci (ITS rDNA, β tubulin, and TEF 1-α) generated a phylogenetic tree that further supported the differentiation of the USA isolates of *P.* cf. *brassicae* from the EU, UK, and New Zealand isolates of *P. brassicae* (Fig 3.9). The final alignment included 30 isolates and 1,579 nt (including alignment gaps). The Bayesian analysis supported differentiation of the USA isolates from the EU, UK, and New Zealand isolates with a posterior probability of 1, while the Maximum Likelihood analysis showed bootstrap support values of 100% for this grouping of isolates.

The phylogenetic analysis of the mating type idiomorphs showed similar results to the individual and MLSA phylogenetic trees described above for the ITS rDNA, β tubulin, and TEF 1-α gene. The final alignment for sequences of the *MAT1-3* gene included 22 isolates of *P. brassicae* and *P.* cf. *brassicae* covering 571 nt (including alignment gaps) (Fig. 3.10). The Bayesian analysis supported the posterior probability of all eight USA isolates with *MAT1-3* grouping together at 0.9986, and a posterior probability of 0.9211 for all 14 EU and UK isolates of this mating type grouping together (Fig. 3.10). The Maximum Likelihood analysis showed bootstrap support values of 100% for both groups. The alignment for *MAT1-2* sequences included 26 isolates and 606 nt (including alignment gaps) (Fig. 3.11). The Bayesian analysis supported the posterior probability of the 8 USA of *MAT1-2* isolates grouping together at 0.9534 (Fig. 3.11), and the 18 EU, UK, and New Zealand isolates grouping together at a posterior probability of 1.000. The Maximum Likelihood analysis showed bootstrap support values of 100% for

both groups. Therefore, both of the mating type trees strongly supported separate clades for P. *brassicae* isolates collected from the USA vs. those collected from the EU, UK, and New Zealand.

3.4 Discussion

In the spring of 2016, *N. capsellae* and *P.* cf. *brassicae* were isolated from plants of the weed bird's rape mustard as well as mustard cover crops with symptoms of white leaf spot and light leaf spot, respectively, in three counties in northwestern Washington: Skagit, Snohomish, and Whatcom. To our knowledge, this the first report of white leaf spot, caused by *N. capsellae*, and light leaf spot, caused by *P.* cf. *brassicae,* in Washington State. *P.* cf. *brassicae* was detected on plants in Washington, Skagit, Snohomish, and Whatcom Counties; while *N. capsellae* was detected on plants in Skagit and Whatcom Counties. This is a significant concern to brassica seed growers and the brassica seed industry as these counties comprise three of the six counties that form the main region of biennial brassica vegetable seed production in the state and, together with the Willamette Valley, the primary region of production of seed of these crops for the USA (du Toit 2007; Inglis et al. 2013).

Sequencing of the ITS rDNA region of 4 isolates of *N. capsellae* and 16 isolates of *P.* cf. *brassicae* from Washington, as well as the β tubulin gene for the *P.* cf. *brassicae* isolates, revealed that the isolates were genetically identical to those collected in the Willamette Valley in a 'Barkant' turnip cover crop seed crop in 2015. The *N. capsellae* isolates matched morphologically those described previously by Crossan (1954) and Deighton (1973), and matched (99% similarity) the ITS rDNA sequence of an *N. capsellae* isolate in GenBank from the UK. In contrast, the ITS rDNA sequences of the Washington and Oregon isolates of *P.* cf. *brassicae* were only 95% similar to one ITS rDNA sequence of this species in GenBank, which was inadequate to confirm the species identity of these USA isolates. However, the β tubulin gene sequences for the Washington isolates indicated the isolates were most closely related to *P. brassicae* based on 98% similarity to the single β tubulin sequence of a *P. brassicae* isolate in GenBank

(Carter et al. 2013). The latter, combined with the conidial and conidiophore characteristics of the USA isolates of *P.* cf. *brassicae* (Ellis and Ellis 1985; Rawlinson et al. 1978; Sutton 1977), indicated the species of the USA isolates was most closely related to *P. brassicae*.

In addition to the ITS rDNA sequence discrepancy for the *P.* cf. *brassicae* isolates from the USA with sequences in GenBank, results of pathogenicity tests with USA isolates differed from what has been published about *P. brassicae* in other countries and with the results of pathogenicity tests with 10 EU and UK isolates of *P. brassicae* on turnip in this study. Although the turnip plants inoculated with *P.* cf. *brassicae* that originated from Oregon and Washington States developed typical light leaf spot symptoms, the plants failed to develop the patches of white, subcuticular conidiomata of *P. brassicae* described by Rawlinson et al. (1978) and Fitt et al. (1998) on various types of *Brassica* species in the UK, whereas all 10 of the EU and UK isolates of *P. brassicae* tested on turnip in this study produced these white conidiomata on the turnip leaves. Furthermore, none of the 10 EU and UK isolates produced the distinct, chlorotic lesions that were observed on plants inoculated with the USA isolates. Although the β tubulin sequences of these isolates were 98% similar to that of an isolate of *P. brassicae* in GenBank, phylogenetic analyses of the ITS rDNA, β tubulin gene, and TEF 1-α gene sequences; MLSA of these three sequences of each isolate; and phylogenetic analyses of sequences of each mating type idiomorph, *MAT1-3* and *MAT1-2*, all grouped the USA isolates separately from the EU, NZ, and UK isolates evaluated, as well as in the sexual crossing study. Therefore, the morphological, pathological, and phylogenetic criteria of the CSC described by Crous et al. (2015) provide strong evidence that the isolates causing light leaf spot of brassicas in the Pacific Northwest USA are a different species of *Pyrenopeziza* than *P. brassicae*, and possibly a new species. The limited number of DNA sequences of this species (and even the genus) in GenBank preclude a conclusion at this point.

Interestingly, although light leaf spot and white leaf spot were detected on mustard cover crops as well as birds' rape mustard weeds in Washington State, neither pathogen was detected on *B.*

oleracea crops surveyed in the same area of Washington State in 2016, including cabbage seed crops and brassica vegetable crops, even on those crops surveyed immediately adjacent to sites where these pathogens were found. The lack of evidence of infection of cabbage seed crops in this region by either pathogen might reflect the fact that these crops are sprayed regularly with fungicides to help growers produce pathogen-free seed lots for distribution nationally and internationally (du Toit 2007), and/or greater susceptibility of *B. juncea* and *B. rapa* to isolates of these pathogens that have been introduced into the region. However, in spring 2017, a Willamette Valley organic brassica seed grower reported nearly 100% incidence of infection by *P.* cf. *brassicae* in each of a *B. juncea* seed crop and a *B. rapa* seed crop, estimating >50% yield losses, whereas adjacent *B. napus* and *B. oleracea* seed crops on that farm had very little infection (L.J. du Toit, Washington State University, *personal communication*). These organic crops had not been treated with fungicides, suggesting that host species susceptibility to the isolates of *P.* cf. *brassicae* present in Oregon and Washington, not fungicide use, might account for the lack of infection of *B. oleracea* crops in Washington by the light leaf spot pathogen. In contrast to observations in Washington State, Claassen et al. (2016) and Ocamb et al. (2015) documented *P.* cf. *brassicae* on a diverse range of Brassicaceae hosts in Oregon State, including numerous weedy species. Furthermore, *P. brassicae* has been reported most commonly in the EU, UK, and New Zealand on *B. napus* and *B. oleracea* plants (Hickman et al. 1955; Rawlinson et al. 1978). A more extensive survey of a diversity of brassica crops and weeds in Washington State, accompanied by host range tests, is needed to clarify potential differences in the host range of Washington and Oregon isolates compared to isolates of *P. brassicae* from other regions of the world.

Following documentation of *P.* cf. *brassicae* and *N. capsellae* in Washington State on mustard cover crops and *B. rapa* weeds, seed lots of *Brassica* spp. planted as cover crops in northwestern Washington in 2016 were tested to assess the possibility that *P.* cf. *brassicae* was introduced into Washington on cover crop seed lots. Only five cover crop seed lots were tested, but one of these five

lots tested using the 4°C NP-10 agar seed health assay was found to be infected with *P.* cf. *brassicae*, i.e., seed of a white mustard cover crop of the cv. Nemagon that was harvested from a seed crop grown in the Willamette Valley of Oregon in 2015-16. The Willamette Valley is where *P.* cf. *brassicae* was first found in the USA in 2014, and where the pathogen has since become well established (Ocamb et al. 2015). The seed lots that tested negative for *P.* cf. *brassicae* were grown in central Washington or central Oregon, semi-arid regions where this pathogen has not been documented and where the climate is very different than the cool, moist, maritime region of western Oregon and Washington that is highly favorable for light leaf spot and more similar to the UK climate than central Washington and central Oregon. Brassica cover crops were a suspected source of introduction of *P.* cf. *brassicae* into the Pacific Northwest USA because *P.* cf. *brassicae* was detected in numerous brassica cover crops in Oregon and Washington; cover cropping with *Brassica* spp. has become increasingly popular in the USA, including in the Pacific Northwest (Watts 2016); and seeding rates for cover crops typically are much greater than for brassica vegetable and seed crops. In addition, the seed lots used to plant conventional *B. oleracea* vegetable and seed crops in Washington State usually are treated with fungicides, some of which are likely to be at least partially efficacious against seedborne inoculum of this pathogen based on the seed treatment trials described in Chapter 4. In contrast, most *Brassica* cover crop seed lots are not treated with fungicides because of the expense for growers to invest in seed treatments for non-cash crops, and brassica cover crops are used commonly on certified organic farms (D. Gies, High Performance Seeds, Inc., *personal communication*). For example, an estimated 11,800 ha in Washington State were planted with mustard green manure crops in 2012 (McGuire 2012). The significant growth in brassica cover cropping in this state and many other regions of the USA (Watts 2016) includes growers who may not have planted brassica crops previously and, therefore, may not be aware of the risk of introducing seedborne pathogens on cover crop seed lots. For example, in northwestern Washington, brassica cover

crops are being planted increasingly in rotation with potato crops because the cover crops improve soil tilth (C. Benedict, Washington State University Whatcom Co., Extension Chair, *personal communication*).

The responses to a questionnaire about awareness of seedborne pathogens of brassicas in the Pacific Northwest showed very limited awareness of white leaf spot and light leaf spot among respondents in this region in 2015 to 2016. This was not surprising as, prior to 2014, white leaf spot had rarely been detected in the Pacific Northwest USA, and light leaf spot had never been documented in the USA. The potential effects of these diseases on the diverse brassica cropping systems in the Pacific Northwest remain to be determined, although there has been empirical evidence of losses to light leaf spot in individual fields in Oregon. Almost 50% of the respondents indicated they had observed diseases in their brassica crops, and yet only 14.1% of respondents indicated they purchase brassica seed lots that have been tested or treated for potential seedborne pathogens. Almost half of the respondents were not aware of state regulations aimed at preventing the dissemination of brassica seed infected with *P. lingam* and/or *X. campestris* pv. *campestris*, i.e., 42.9% were not aware of the ODA Crucifer Rule OAR 603-052-0870, and 41.3% were not aware of the WSDA Crucifer Quarantine Rule WAC 16-301-495 to 16-301-580 in western Washington despite the fact that this rule was implemented at the request of brassica seed growers and seed companies, and had been in place for approximately 10 years. In addition, 54.1% of respondents were not aware of the proposed amendment to the WSDA crucifer rule to include all counties east of the Cascade Mountains (WSDA 2015). These results reveal the very limited awareness of seedborne pathogens of brassicas, including the value of only purchasing seed lots that have been tested for key seedborne pathogens that pose a major risk of outbreaks of diseases (e.g., *P. lingam* and *X. campestris* pv. *campestris*), even among growers/stakeholders who work with a variety of *Brassica* spp.

The pathogenicity test of EU and UK isolates of *P. brassicae* on turnip, the sexual crossing study with isolates of this fungus of opposite mating types, and the MLSA and mating type phylogenetic

analyses in this study all showed distinct differences between the USA vs. EU, UK, and even New Zealand isolates of *P. brassicae.* The pathogenicity test on 'Hakurei' turnip plants demonstrated significantly different symptoms and signs caused by the EU and UK isolates than what was observed in pathogenicity tests with the USA isolates. Although the USA and EU/UK isolates caused foliar necrosis, the isolates differed in that symptoms on leaves inoculated with isolates from the USA developed characteristic chlorotic leaf spots and showed significantly more extensive foliar chlorosis but never developed patches of white conidiomata of the pathogen, in contrast to the abundant white conidiomata that developed on otherwise asymptomatic leaves, as described by Fitt et al. (1998), on turnip plants inoculated with all 10 EU/UK isolates of *P. brassicae*. Fitt et al. (1998) observed that, although these conidiomata are "diagnostic" when incubating infected leaves at high relative humidity, the signs can be difficult to detect in fields, particularly following rains. In the UK, symptoms described as developing on plants infected with *P. brassicae* are similar to those observed on plants inoculated with isolates of *P.* cf. *brassicae* from the USA in this study, i.e., indistinct, pale green, roughly circular lesions with a mottled appearance that turn pale green, coalesce and can kill the leaf (Fitt et al. 1998). In this study, symptom expression on brassica plants inoculated with isolates of *P.* cf. *brassicae* from the USA included bright yellow lesions, veinal browning, and very rapid expansion and coalescence of lesions.

In the sexual crossing study, mature apothecia with ascospores were not observed for any of the pairings of USA with EU/UK isolates of opposite mating types, suggesting that the USA isolates originate from distinct populations that are incompatible sexually with isolates from the EU and UK. In contrast, 19 of 25 crosses of EU/UK x EU/UK isolates of different mating types produced apothecia that matured to develop ascospores in at least one of the three replicate plates per cross. Another three crosses produced apothecia that did not mature to form ascospores, one cross resulted in development of apothecial initials, and only two crosses did not result any evidence of apothecia. Ilott et al. (1984)

explained that it can be difficult in experiments with heterothallic ascomycetes to achieve ideal conditions for sexual crossing that enables the development of mature apothecia. Of the USA x EU/UK crosses attempted with isolates in this study, 11 resulted in apothecial initials forming that had not matured into apothecia with ascospores by 11 weeks (almost 3 months) after setting up the crosses. Similarly, fruiting structures were not observed in either the homothallic or heterothallic crosses among the USA isolates. Ilott et al. (1984) observed that when cultured alone, isolates of both mating types were capable of developing a range of apothecial stages of development. This might explain the USA x EU/UK crosses that resulted in development of immature apothecia. Although Hickman et al. (1955) and Maddock and Ingram (1981) reported homothallic isolates of *P. brassicae*, Ilott et al. (1984) found no evidence of homothallism for isolates from the UK. Likewise, results of this study indicated no evidence of homothallism for the USA isolates. Although small (2- to 3-mm in diameter), short-stipitate apothecia were observed on weedy *B. rapa* stem residues collected in late fall of 2016 in Skagit Co., WA were morphologically similar to the apothecia of *P. brassicae* (Rawlinson et al. 1978), the ITS rDNA sequences of DNA extracted from these fruiting bodies revealed the structures were not of *P. brassicae*, and the colony characteristics of these isolates differed from those of *P. brassicae* (too rapid a growth rate for the latter, *data not shown*). Ilott et al. (1984) noted that *P. brassicae* apothecia can be difficult to detect in the field. In addition, Inman et al. (1992) observed apothecia of a species of *Unguicularia* on oilseed rape crop residues that had similar morphology to apothecia of *P. brassicae*, and suggested the abundance of apothecia of this genus may have been responsible for overestimating counts of *P. brassicae* ascospores in studies monitoring airborne spores of the light leaf spot pathogen in the UK. To date, there is no evidence of apothecia of *P.* cf. *brassicae* having been found in the USA. The MLSA as well as the phylogenetic analyses of the ITS rDNA region, β tubulin gene, TEF 1-α gene, and mating type genes trees all strongly supported differentiation of the USA isolates from the EU, UK, and New Zealand isolates of *P. brassicae*.

Given the potential significance of *P.* cf. *brassicae* in the Pacific Northwest USA, an important brassica growing region for the USA, it is important to understand how *P.* cf. *brassicae* and *N. capsellae* were introduced into the Pacific Northwest USA. Additional *Brassica* cover crop seed lots should be tested to understand the potential risk of disseminating *P.* cf. *brassicae* infected seed lots to regions where *P.* cf. *brassicae* has not yet been detected, or introducing new isolates of the fungus. Other potential sources of introduction of the pathogen should also be investigated, e.g., brassica transplants imported into Washington State from nurseries. Pathogenicity tests should also be completed using more diverse *Brassica* spp. and cultivars to understand how *P. brassicae* isolates from the EU/UK and *P.* cf. *brassicae* isolates from the USA may affect the many different types of brassica crops grown in this region.

To fulfil the requirements of the CSC for naming a new species of fungus, which the USA isolates of *Pyrenopeziza* on brassicas appear to be, it will be important also to compare the morphology of *P.* cf. *brassicae* isolates from the USA with isolates from the EU/UK (Hawksworth et al. 1995). This includes detailed measurements of conidia and other relevant microscopic characteristics such as conidiophores (Siefert and Rossman 2010), and comparing colony morphology and growth rates on media (Siefert and Rossman 2010). Whether or not detailed morphological analyses add to the evidence of the USA isolates being a different species of *Pyrenopeziza* than *P. brassicae*, naming of the new species will need to follow the criteria of the International Code of Botanical Nomenclature (ICBN), i.e., publishing the comparative study in a peer-reviewed journal with a unique binomial and a detailed Latin or English description of the fungus, and submitting a permanently preserved specimen to a public herbarium to serve as the type specimen (Seifert and Rossman 2010). Seifert and Rossman (2010) outlined criteria that should be addressed in a publication proposing a new species, i.e., to confirm that the new species has been assigned to the correct genus and is monophyletic, and that a sufficient number of isolates has been assessed (Seifert and Rossman 2010). Accession numbers from MycoBank should be included in

the description (Crous et al. 2004). We propose naming the USA isolates *Pyrenopeziza cascadia*, after the geographic region west of the Cascade Mountains where this pathogen has been detected in the USA since 2014.

Determining the origin of the USA isolates of *P.* cf. *brassicae* will be important. Isolates of this fungus from many countries where *P. brassicae* has been reported were not included in the MLSA in this study, e.g., the pathogen has been reported in Japan, Latvia, the Netherlands, Norway, the Philippines, Poland, Portugal, and Romania (Hickman et al. 1955; Karolewski 2010; Rawlinson et al. 1978). *P. brassicae* isolates from these countries should be incorporated into phylogenetic analyses as well as morphological and pathogenicity comparisons. Development of a more robust set of DNA sequences of *P. brassicae* isolates in GenBank from diverse geographic regions and host plant species will facilitate an understanding of the global distribution of *P. brassicae*, how this might be related to the global movement of brassica seed that can potentially serve as a source of inoculum of this pathogen, the importance of testing seed lots for this pathogen, and the need for effective seed treatments for this pathogen that can be used in the diversity of organic and conventional agricultural systems of brassica crop production.

3.5 Literature Cited

Agostini, A., Johnson, D. A., Hulbert, S., Demoz, B., Fernando, W. G. D., and Paulitz, T. 2013. First report of blackleg caused by *Leptosphaeria maculans* on canola in Idaho. Plant Dis. 97:842.

Bakkeren, G., Kronstad, J. W., and Levesque, C. A. 2000. Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilagomycetes. Mycologia 92:510-521.

Barrett, L. G., Thrall, P. H., Burdon, J. J., and Linde, C. C. 2008. Life history determines genetic structure and evolutionary potential of host – parasite interactions. Trends Ecol. Evol. 23:678-685.

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., and Kauserud, H. 2013. ITS1 versus ITS2 as DNA metabarcodes for fungi. Mol. Ecol. Resour. 13:218–224.

Boyle, L. W. 1945. Cercosporella leaf spot of kale in Oregon. Plant Dis. Rep. 29:387.

Carmody, S. M., King, K. M., Claassen, B. J., Fraaije, B. A., West, J. S., Ocamb, C. M., and du Toit, L. J. 2017. Genetic analysis of *Pyrenopeziza brassicae*, cause of light leaf spot of brassicas, in the European Union, Oceania, and North America. Phytopathology 107:*in press* (Abstract).

Carmody, S. M., Ocamb, C. M, and du Toit, L. J. 2016. The potential for seed transmission of *Pyrenopeziza brassicae* and *Mycosphaerella capsellae* on brassicas in the Pacific Northwest. APS Pacific Division Annual Meeting, 28-30 June 2016, La Conner, WA. Phytopathology 106:S4.196 (Abstract).

Carter, H. E., Cools, H. J., West, J. S., Shaw, M. W., and Fraaije, B. A. 2013. Detection and molecular characterisation of *Pyrenopeziza brassicae* isolates resistant to methyl benzimidazole carbamates. Pest Manag. Sci. 69:1040–1048.

Claassen, B. J., Serdani, M., Thomas, W. J., Putnam, M. L., and Ocamb, C. M. 2014. Pathogenicity of *Cylindrosporium concentricum* on cultivated and weedy Brassica hosts. Phytopathology 105:S4.30 (Abstract).

Commonwealth Mycological Institute Distribution Maps of Plant Diseases. 1986. Commonwealth Mycological Institute, Wallingford.

Crossan, D. F. 1954. Cercosporella leaf spot of crucifers. North Carolina Agric. Res. Stn. Tech. Bull. 109.

Crous, P. W., Gams, W., Stalpers, J. A., Robert V., and Stegejuis, G. 2004. MycoBank: an online initiative to launch mycology into the 21st century. Stud. Mycol. 50:19-22.

Crous, P. W., Hawksworth, D. L., and Wingfield, M. J. 2015. Identifying and naming plant-pathogenic fungi: past, present, and future. Annu. Rev. Phytopathol. 53:247-267.

Cyberinfrasturcutre for Phylogenetic Research (CIPRES). 2017. www.phylo.org. [Accessed on 10 May 2017].

Darriba D., Taboada G. L., Doallo, R., and Posada, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9:772.

Deighton, F. C. 1973. Studies on *Cercospora* and allied genera. IV. *Cercosporella* SACC., *Pseudocercosporella* Gen. Nov. and *Pseudocercosporidium* Gen. Nov. Mycol. Papers 133:1–62.

du Toit, L. J. 2007. Crop Profile for Cabbage Seed in Washington. USDA Pest Management Centers. http://www.ipmcenters.org/CropProfiles/docs/WAcabbageseed.pdf.

Ellis, M. B., and Ellis, J. P. 1985. Microfungi on Land Plants. Macmillan Publishing, New York.

Farr, D. F, and Rossman, A. Y. 2017. Fungal Databases, U.S. National Fungus Collections ARS, USDA. https://nt.ars-grin.gov/fungaldatabases. [Accessed on 24 April 2017].

FigTree. 2017. http://tree.bio.ed.ac.uk/software/figtree/. [Accessed on 10 May 2017].

Fitt, B. D. L., Doughty, K. J., Gladders, P., Steed, J. M., and Sutherland, K. G. 1998. Diagnosis of light leaf spot (*Pyrenopeziza brassicae*) on winter oilseed rape (*Brassica napus*) in the UK. Ann. Appl. Biol. 133:155-166.

Foster, S. J., Singh, G., Fitt, B. D. L., and Ashby, A. M. 1999. Development of PCR-based diagnostic techniques for the two mating types of *Pyrenopeziza brassicae* (light leaf spot) on winter oilseed rape (*Brassica napus* spp. *oleifera*). Physiol. Molec. Plant Pathol. 55:111-119.

Foster, S. J., Ashby, A. M., and Fitt, B. D. L. 2002. Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape. Eur. J. Plant Pathol. 108:379-383.

Greville, R. K. 1823. Scottish cryptogamic flora, or coloured figures and descriptions of cryptogamic plants, belonging chiefly to the order Fungi; and intended to serve as a continuation of English Botany. Maclachan and Stewart Publisher, Edinburgh, London.

Gilles T., Ashby, A. M., Fitt, B. D. L., and Cole, T. 2001. Development of *Pyrenopeziza brassicae* apothecia on agar and oilseed rape debris. Mycol. Res. 105:705-714.

Giraud, T., Refre, G., Vienne, D. M. De, and Hood, M. E. 2008. Speciation in fungi. Fung. Gen. Biol. 45:791–802.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. N. 1995. Ainsworth and Bisby's Dictionary of the Fungi, Eighth Ed. CAB International Mycological Institute, Surrey, UK.

Hibbett, D., Abarenkov, K., Koljalg, U., Opik, M., Chai, B., Cole, J. R., Wang, Q., Crous, P. W., Robert, V. A. R. G., Helgason, T., Herr, J., Kirk, P., Lueschow, S., O'Donnell, K., Nilsson, H., Oono, R., Schoch, C.,

Smyth, C., Walker, D. M., Porras-Alfaro, A., Taylor, J. W., and Geiser, D. M. 2016. Sequence-based classification and identification of Fungi. Mycologia 5514:16–130.

Hickman, C. J., Schofield, E. R., and Taylor, E. R. 1955. Light leaf spot of Brassica. Plant Pathol. 4:129–131.

Historical Trends - Light Leaf Spot. Rothamsted Research. http://rothamsted.ac.uk/light-leaf-spot-forecast/historical-trends-light-leaf-spot [Accessed 1 January 2017].

Huelsenbeck, J. P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754-755.

Ilott, T. W., Ingram, D. S., and Rawlinson, C. J. 1984. Heterothallism in *Pyrenopeziza brassicae*, cause of light leaf spot of brassicas. Brit. Mycol. Soc. 82:477-483.

Inglis, D. A., du Toit, L.J., and Miller, T. W. 2013. Production of Brassica seed crops in Washington State: A case study on the complexities of coexistence. Washington State Univ. Ext. Man. EMO062E.

Inman, A. J. 1992. The Biology and Epidemiology of White Leaf Spot (*Pseudocercosporella capsellae*) on Oilseed Rape. Ph.D. Dissertation, University of London, London.

Inman, A. J., Fitt, B. D. L., Evans, R. L., and End, M. 1992. A species of *Unguicularia* on oilseed rape, and its importance in studies of the epidemiology of light leaf spot (*Pyrenopeziza brassicae*). Plant Pathol. 41:646–652.

International Seed Health Initiative-Veg. 2015. Methods for the detection of *Verticillium dahliae* on spinach seed. International Seed Federation, Zurich. http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/ [Accessed on 31 March 2017].

Jeffers, S.N. 2007. Recipes for Media Useful in the Culture and Identification of *Phytophthora* species: V8 Agar & Broth Recipes. Clemson University.

https://fhm.fs.fed.us/sp/sod/misc/culturing_species_phytophthora.pdf [Accessed on 3 March 2017].

Karolewski, Z. 2010. Development of light leaf spot (*Pyrenopeziza brassicae*) on Brassicas. Phytopathologia 55:13–20.

Kumar, S., Stecher, G., and Tamura, K. 2015. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets (submitted) available at http://www.megasoftware.net/. [Accessed on 16 October 2016].

McGuire, A. 2012. Mustard Types, Seed Sources, & Acreage Estimates. http://csanr.wsu.edu/mustard-green-manures/mustard-types/ [Accessed 12 August 2016].

Ocamb, C. M. 2014a. Disease Alert – Light Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/Light%20leaf%20spot%20OSU%20Disease%20Alert%20in%20crucifers%2017%20June.pdf [Accessed 12 December 2016].

Ocamb, C. M. 2014b. Disease Alert – White Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/White%20leaf%20spot%20OSU%20Disease%20Alert%20in%20Brassiceae.pdf [Accessed 12 December 2016].

Ocamb, C. M. 2016. A Clinic Close-Up: Black leg, light leaf spot, and white leaf spot in western Oregon. Oregon State University, Corvallis, OR. https://pnwhandbooks.org/sites/pnwhandbooks/files/insect/document/turnip-rutabaga-brassica-sp-light-leaf-spot/black-leg-light-leaf-spot-white-leaf-spot-crucifers16.pdf [Accessed on 12 December 2016].

Ocamb, C. M., Mallory-Smith, C., Thomas, W. J., Serdani, M., Putman, M. 2015. New and re-emerging fungal pathogens affecting Brassicaceae plants in western Oregon: black leg, light leaf spot, and white leaf spot. Phytopathology 105:S4.103. (Abstract).

Organic Seed Alliance. 2017. https://seedalliance.org/conference/. [Accessed on 19 May 2017].

Pacific Northwest Vegetable Association. 2017. www.pnva.org. [Accessed on 19 May 2017].

Paulitz, T. C., Knerr, A. J., Carmody, S. M., Schlatter, D., Sowers, K., Derie, M., and du Toit, L. J. 2017. First report of *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, causal agents of black leg, on canola in Washington State. Plant Dis. 101:504.

Quaedvlieg, W., Binder, M., Groenewald, J. Z., Summerell, B. A., Carnegie, A. J., Burgess, T. I., and Crous, P. W. 2014. Introducing the consolidated species concept to resolve species in the *Teratosphaeriaceae.* Mol. Phylogeny Evol. Fungi. 33:1–40.

Rawlinson, C. J., Sutton, B. C., and Muthyalu, G. 1978. Taxonomy and biology of *Pyrenopeziza brassicae* sp. nov. (*Cylindrosporium concentricum*), a pathogen of winter oilseed rape (*Brassica napus* spp. *oleifera*). Trans. Br. Mycol. Soc. 71:425-439.

Ronquist F., Teslenko, M., and van der Mark, P. 2011. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. System. Biol. 61:539-542.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., and Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. PNAS 109:6241-6246.

Schoch, C. L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Irinyi, L., Meyer, W., Nilsson, R. H., Hughes, K., Miller, A. N., Kirk, P. M., Abarenkov, K., Aime, M. C., Ariyawansa, H. A., Bidartondo, M., Boekhout, T., Buyck, B., Cai, Q., Chen, J., Crespo, A., Crous, P., Damm, U., De Beer, Z. W., Dentinger, B. T. M.,

Divakar, P. K., Duenas, M., Feau, N., Fliegerova, K., Garcia, M. A., Ge, Z., Griffith, G. W., Groenewald, J. Z., Groenewald, M., Grube, M., Gryzenhout, M., Gueidan, C., Guo, L., Hambleton, S., Hamelin, R., Hansen, K., Hofstetter, V., Hong, S. B., Houbraken, J., Hyde, K. D., Inderbitzin, P., Johnston, P. R., Karunarathna, S. C., Koljalg, U., Kovacs, G. M., Kraichak, E., Krizsan, K., Kurtzman, C. P., Larsson, K. H., Leavitt, S., Letcher, P.M., Liimatainen, K., Liu, J. K., Lodge, D. J., Luangsaard, J. J., Lumbsch, H. T., Maharachchikumbura, S. S. N., Manamgoda, D., Martin, M. P., Minnis, A. M., Moncalvo, J. M., Mule, G., Nakasone, K. K., Niskanen, T., Olariaga, I., Papp, T., Petkovits, T., Pino-Bodas, R., Powell, M. J., Raja, H. A., Redecker, D., Sarmiento-Ramirez, J. M., Seifert, K. A., Shrestha, B., Stenroos, S., Stielow, B., Suh, S. O., Tanaka, K., Tedersoo, L., Telleria, M. T., Udayanga, D., Untereiner, W. A., Uribeondo, J. D., Subbarao, K. V., Vagvolgyi, C., Visagie, C., Voigt, K., Walker, D. M., Weir, B. S., Weiß, M., Wijayawardene, N. N., Wingfield, M. J., Xu, J. P., Yang, Z. L., Zhang, N., Zhuang, W. Y., and Federhen, S. 2014. Finding needles in haystacks: Linking scientific names, reference specimens and molecular data for fungi. Database 2014:1–21.

Seifert, K. A., and Rossman, A. Y. How to describe a new fungal species. IMA Fung. 2:109-116.

Sorensen, L. H., Schneider, A. T., and Davis, J. R. 1991. Influence of sodium polygalacturonate sources and improved recovery of *Verticillium* species from soil. Phytopathology 81:1347.

Sutton, D. A. 1977. *Cylindrosporium concentricum*. CMI Descr. Fungi Bact. No. 536. Commonwealth Mycological Institute, Wallingford, England.

Swofford, D. L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.

Taylor, J. W., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S., and Fisher, M. C. 2000. Phylogenetic species recognition and species concepts in fungi. Fung. Genet. Biol. 31:21–32.

Taşkin, H., Büyükalaca, S., Doğan, H. H., Rehner, S. A., and O'Donnell, K. 2010. A multigene molecular phylogenetic assessment of true morels (*Morchella*) in Turkey. Fung. Genet. Biol. 47:672-682.

Tilth Producers. 2017. http://tilthproducers.org/ [Accessed 19 May 2017].

United States Department of Agriculture National Agricultural Statistics Service. 2017. http://quickstats.nass.usda.gov/. [Accessed 19 February 2017].

Videira, S. I. R., Groenewald, J. Z., Braun, U., Shin, H. D., and Crous, P. W. 2016. All that glitters is not *Ramularia*. Stud. Mycol. 83:49–163.

Washington State Department of Agriculture. 2015. Crucifer Seed Quarantine. http://agr.wa.gov/Inspection/SeedInspection/docs/478-CruciferSeedQuarantineFactSheet-Web.pdf. [Accessed on 1 January 2017].

Watts, C. 2016. Annual Report. 2015-2016 Cover Crop Survey. Sustainable Agriculture Research and Education/Conservation Technology Information Center, College Park, MD.

Table 3.1. Isolates of *Pyrenopeziza* cf. *brassicae* and *Neopseudocercosporella capsellae* collected from *Brassica* species in northwestern Washington and western Oregon in 2016[a]

Fungus	Isolate	County, State, GPS coordinates	Original *Brassica* species	Patho-genicity test[b]	GenBank Accession #
P. cf. *brassicae*	Cyc007	Skagit, WA, 48.417335 N, -122.444508	*B. rapa*	1	MF143611
P. cf. *brassicae*	Cyc008	Skagit, WA, 48.417335 N, -122.444508	*B. rapa*	1	MF143612
P. cf. *brassicae*	Cyc009	Skagit, WA, 48.417335 N, -122.444508	*B. rapa*	1	MF143613
P. cf. *brassicae*	Cyc010	Skagit, WA, 48.548727 N, -122.472012	*B. rapa*	1	MF143614
P. cf. *brassicae*	Cyc011	Skagit, WA, 48.548727 N, -122.472012	*B. rapa*	2	MF143615
P. cf. *brassicae*	Cyc012	Skagit, WA, 48.548727 N, -122.472012	*B. rapa*	2	MF143616
P. cf. *brassicae*	Cyc013a	Skagit, WA, 48.355423 N, -122.458390	*B. rapa*	3	MF143617
P. cf. *brassicae*	Cyc014	Skagit, WA, 48.417335 N, -122.444508	*B. rapa*	3	MF143618
P. cf. *brassicae*	Cyc015	Skagit, WA, 48.448770 N, -122.465548	*B. juncea*	3	MF143619
P. cf. *brassicae*	Cyc016	Skagit, WA, 48.483144 N, -122.377808	*B. rapa*	-	-
P. cf. *brassicae*	Cyc017	Skagit, WA, 48.442493 N, -122.389717	*B. rapa*	-	MF143620
P. cf. *brassicae*	Cyc023a	Benton, OR, -	*B. rapa*	3	MF143621
P. cf. *brassicae*	Cyc024a	Whatcom, WA, 48.891872 N, -122.285325	*B. rapa*	3	MF143622
P. cf. *brassicae*	Cyc025	Snohomish, WA, 48.198019 N, -122.138091	*B. rapa*	3	MF143623
P. cf. *brassicae*	Cyc026	Snohomish, WA, 48.198019 N, -122.138091	*B. rapa*	3	MF143624
P. cf. *brassicae*	Cyc027	Snohomish, WA, 48.231512 N, -122.325615	*B. rapa*	3	MF143625
P. cf. *brassicae*	Cyc028	Snohomish, WA, 48.289039 N, -122.361304	*B. rapa*	3	MF143626
P. cf. *brassicae*	Cyc029a	Snohomish, WA, 48.289039 N, -122.361304	*B. rapa*	3	MF143627
P. cf. *brassicae*	Cyc030	Skagit, WA, 48.554366 N, -122.459415	*B. rapa*	3	MF143628
P. cf. *brassicae*	Cyc031	Skagit, WA, 48.371385 N, -122.368416	*B. rapa*	-	-
P. cf. *brassicae*	Cyc001	Benton, OR[c]	*B. rapa*	1, 2, 3[c]	MF143610
N. capsellae	Psc007	Benton, OR[c]	*B. rapa*	3, 4[c]	MF158312
N. capsellae	Psc010	Skagit, WA, 48.451571 N, -122.444705	*B. juncea*	3	MF158311
N. capsellae	Psc011	Whatcom, WA, 48.891872 N, -122.285325	*B. rapa*	3	-
N. capsellae	Psc012	Benton, OR, -	*B. rapa*	4	-
N. capsellae	Psc013	Whatcom, WA, 48.891872 N, -122.285325	*B. rapa*	3	-
N. capsellae	Psc014	Whatcom, WA, 48.451571 N, -122.444705	*B. rapa*	4	MF158313
N. capsellae	Psc015	Linn, OR, -	*B. rapa*	4	-

[a] Isolates were collected from a survey of brassica cover crops (*B. juncea*), weedy brassicas (*B. rapa* and an unknown *Brassica* sp.), vegetable brassicas (*B. oleracea* with various subspecies), and cabbage seed (*B. oleracea* var. *capitata*) in late spring until early summer of 2016. Leaves and stems with symptoms of light leaf spot were collected from 20 locations in 3 counties in northwestern Washington.

[b] Each pathogenicity test was a set up as a randomized complete block design with four replications of each isolate and two brassica species: the turnip (*B. rapa*) 'Hakurei' and the mustard (*B. juncea*) 'Caliente 199,' as detailed in the main text. Each pathogenicity test was run for six weeks, with a total of 16 isolates of *P.* cf. *brassicae* and 6 isolates of *N. capsellae* assessed over the four tests. Pathogenicity tests 1, 2, 3, and 4 were initiated on 12 May, 17 May, 10 June, and 2 September 2016, respectively, as detailed in the main text.

[c] The same control isolate of each pathogen (Cyc001 for *P.* cf. *brassicae* and Psc007 for *N. capsellae*) and a non-inoculated control treatment of each host species were included in each pathogenicity test. The isolates were collected from a turnip cover crop seed crop grown in the Willamette Valley, OR, from 2014 to 2015, and verified as pathogenic on *Brassica* species in 2015 (Chapter 2).

Table 3.2. Isolates of *Pyrenopeziza brassicae* from the EU, UK, and New Zealand, and isolates of *P.* cf. *brassicae* from the USA used for phylogenetic analyses and multi-locus sequence analysis (MLSA) to assess the genetic diversity of these isolates[a]

						GenBank accession number[f]			
Isolate[b]	**Geographic origin**	**Original plant**	**Mating type[c]**	**Source[d]**	**Assays[e]**	**ITS rDNA**	**β tubulin**	**TEF 1-α**	**Mating type**
17Kale02	Lincolnshire, England	*B. oleracea*	MAT1-3	K. M. King 2017	*MAT1-3*	-	-	-	-
2016-9	Northumberland, England	*B. napus*	MAT1-3	N. J. Hawkins 2016	P, *MAT1-3*, SX	-	-	-	MF314442
2016-26	Northumberland, England	*B. napus*	MAT1-3	N. J. Hawkins 2016	P, *MAT1-3*, SX	-	-	-	MF314441
2016-34	Northumberland, England	*B. napus*	MAT1-3	N. J. Hawkins 2016	P, *MAT1-3*, SX	-	-	-	-
4E	England	*B. napus*	MAT1-3	N. J. Hawkins 2013	P, MLSA, *MAT1-3*	MF187532	MF314350	MF314394	MF314431
8CAB	East Lothian, Scotland	*Brassica* sp.	MAT1-3	Rothamsted Research collection	P, SX	-	-	-	-
CBS157.35	Victoria, Australia	*B. oleracea*	MAT1-3	E. McLennan 1935	*MAT1-3*	-	-	-	MF314438
Fr2	Le Rheu, France		MAT1-3	M. Mayer 1997	P, SX	-	-	-	-
PC13	Rostock, Germany	*B. napus*	MAT1-3	M. Mayer 1997	MLSA, *MAT1-3*	MF187545	MF314352	MF314381	-
PC19	Rostock, Germany	*B. napus*	MAT1-3	M. Mayer 1997	MLSA, *MAT1-3*	MF187546	MF314355	MF314378	MF314436
PC23	Rostock, Germany	*B. napus*	MAT1-3	M. Mayer 1997	MLSA, *MAT1-3*	MF187543	MF314358	MF314375	MF314432
PC28	Edinburgh, Scotland	*B. napus*	MAT1-3	M. Mayer 1997	MLSA, *MAT1-3*	MF187538	MF314359	MF314374	MF314437
PC35	Le Rheu, France	*B. napus*	MAT1-3	M. Mayer 1997	MLSA, *MAT1-3*	MF187534	MF314362	MF314371	MF314430
PC39	Aberdeen, Scotland	*B. napus*	MAT1-3	M. Mayer 1997	MLSA, *MAT1-3*	MF187541	MF314364	MF314369	MF314433
PC50	Aberdeen, Scotland	*B. napus*	MAT1-3	D. Majer 1998	MLSA, *MAT1-3*	MF187540	MF314366	MF314367	MF314434

14CC2B	Polk, Co., OR, USA	*B. napus*	MAT1-3	B. Claassen 2014	*MAT1-3*, SX	-	-	-	MF314426
14CC4A	Polk, Co., OR, USA	*B. napus*	MAT1-3	B. Claassen 2014	*MAT1-3*	-	-	-	MF314427
14CC8A	Polk, Co., OR, USA	*Raphanus* sp.	MAT1-3	B. Claassen 2014	*MAT1-3*	-	-	-	MF314428
15LS13B	Benton, Co., OR, USA	*B. juncea*	MAT1-3	B. Claassen 2015	*MAT1-3*	-	-	-	MF314429
Cyc011	Skagit Co., WA, USA	*B. rapa*	MAT1-3	S. M. Carmody 2016	MLSA, *MAT1-3*, SX	MF143615	MF314340	MF314389	MF314425
Cyc015	Skagit Co., WA, USA	*B. juncea*	MAT1-3	S. M. Carmody 2016	MLSA, *MAT1-3*, SX	MF143619	MF314342	MF314387	MF314422
Cyc017	Skagit Co., WA, USA	*B. rapa*	MAT1-3	S. M. Carmody 2016	MLSA, *MAT1-3*, SX	MF143620	MF314343	MF314386	MF314423
Cyc023a	Benton Co., OR, USA	*B. rapa*	MAT1-3	L. J. du Toit 2016	MLSA, *MAT1-3*, SX	MF143621	MF314344	-	MF314424
2016-5	Northumberland, England	*B. napus*	MAT1-2	N. J. Hawkins 2016	P, *MAT1-2*, SX	-	-	-	MF314404
2016-50	Northumberland, England	*B. napus*	MAT1-2	N. J. Hawkins 2016	P, *MAT1-2*, SX	-	-	-	MF314405
204290	Oxfordshire, England	*B. napus*	MAT1-2	C.J. Rawlinson 1975	*MAT1-2*	-	-	-	MF314408
233715	New Zealand	*B. oleracea*	MAT1-2	W.F. Harthill, C.J. Rawlinson 1978	*MAT1-2*	-	-	-	MF314409
233716	New Zealand	*B. oleracea*	MAT1-2	W.F. Harthill, C.J. Rawlinson 1978	MLSA, *MAT1-2*	MF187548	-	-	MF314410
233717	New Zealand	*B. oleracea*	MAT1-2	W.F. Harthill, C.J. Rawlinson 1978	*MAT1-2*	-	-	-	MF314411
5A	England	*B. napus*	MAT1-2	N. J. Hawkins 2013	P, MLSA, *MAT1-2*, SX	MF187533	MF314349	MF314393	MF314406
E3a	Hertfordshire, England	*B. napus*	MAT1-2	E. Boys 2007	P, *MAT1-2*, SX	-	-	-	MF314407
JT2A	Hertfordshire, England	*B. rapa*	MAT1-2	J. S. West 2009	*MAT1-2*	-	-	-	MF314412

PC17	Cambridge, England	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187536	MF314353	MF314380	MF314413
PC18	Aberdeen, Scotland	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187547	MF314354	MF314379	MF314414
PC20	Edinburgh, Scotland	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187539	MF314356	MF314377	MF314415
PC22	Cambridge, England	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187535	MF314357	MF314376	MF314416
PC30	Cambridge, England	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187531	MF314360	MF314373	MF314417
PC32	Cambridge, England	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187537	MF314361	MF314372	MF314418
PC38	Cambridge, England	*B. napus*	MAT1-2	M. Mayer 1997	MLSA, *MAT1-2*	MF187544	MF314363	MF314370	MF314419
PC45	Yorkshire, England	*B. oleracea*	MAT1-2	P. Gladders 1996	MLSA, *MAT1-2*	MF187542	MF314365	MF314368	MF314420
UK73	Angus, England	*B. napus*	MAT1-2	Unknown 2005	P, *MAT1-2*, SX	-	-	-	MF314421
223	Douglas, Co., OR, WA	*B. rapa*	MAT1-2	B. Claassen 2016	*MAT1-2*	-	-	-	MF314403
Cyc001	Benton, Co., OR, USA	*B. rapa*	MAT1-2	S. M. Carmody 2015	P, MLSA, *MAT1-2*, SX	MF143610	MF314337	MF314392	MF314396
Cyc007	Skagit Co., WA, USA	*B. rapa*	MAT1-2	S. M. Carmody 2016	MLSA, *MAT1-2*	MF143611	MF314338	MF314391	MF314397
Cyc009	Skagit Co., WA, USA	*B. rapa*	MAT1-2	S. M. Carmody 2016	MLSA, *MAT1-2*, SX	MF143613	MF314339	MF314390	MF314398
Cyc013a	Skagit Co., WA, USA	*B. rapa*	MAT1-2	S. M. Carmody 2016	MLSA, *MAT1-2*, SX	MF143617	MF314341	MF314388	MF314399
Cyc024a	Whatcom Co., WA, USA	*B. rapa*	MAT1-2	S. M. Carmody 2016	MLSA, *MAT1-2*	MF143622	MF314345	MF314385	MF314400
Cyc025	Snohomish Co., WA, USA	*B. rapa*	MAT1-2	S. M. Carmody 2016	MLSA, *MAT1-2*, SX	MF143623	MF314346	MF314384	MF314401
Cyc029a	Snohomish Co., WA, USA	*B. rapa*	MAT1-2	S. M. Carmody 2016	MLSA, *MAT1-2*, SX	MF143627	MF314347	MF314383	MF314402
Cyc031a	Skagit Co., WA, USA	*B. rapa*	Unknown	S. M. Carmody 2016	MLSA	-	MF314349	MF314382	-

[a] MLSA completed using the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), β-tubulin, and translation elongation factor (TEF) 1-α sequences of 32 isolates from the EU, New Zealand, the UK, and the USA. Isolates noted with *MAT1-3* and *MAT1-2* were used in a phylogenetic analysis of the mating type genes (Foster et al. 2002; Ilott et al. 1984).

[b] Non *P. brassicae* isolates were sequenced and used as outgroups to root the phylogenetic analyses, including: isolate 22-443 of *Oculimacula acuformis* from Somme, France; two isolates of *Rhynchosporium commune* for rooting the outgroups for the *MAT1-2* (AJ537511) and *MAT1-3* (AJ549759); and isolate 27DG09 of *R. orthosporum* from Aberystwyth, England.

[c] Mating type sequences of isolates from the EU, New Zealand, UK, and the USA were prepared by Drs. Kevin King and Jonathon West at Rothamsted Research, UK, using the multiplex PCR assay of Foster et al. (2002) with three primers: PbM-1-3, PbM-2, and Mt3. Mating types was not determined for Cyc031a.

[d] Source = person who collected the isolate, and the year the isolate was collected.

[e] Assays = assays used to assess the consolidated species concept (CSC) of isolates as defined by Crous et al. (2015). Ten EU and UK isolates were tested for pathogenicity on the turnip (*Brassica rapa*) cv. Hakurei (P). MLSA = isolates used in the MLSA study. *MAT1-3* and *MAT1-2* = isolates used in one of the two mating type phylogenetic analyses. SX = isolates (n = 20) used in the sexual crossing study.

[f] GenBank accession numbers for sequences of the ITS rDNA, β tubulin gene, TEF 1-α gene, and *MAT1-3* and *MAT1-2* genes.

Table 3.3. *In vitro* tests for sexual compatibility of isolates of *Pyrenopeziza brassicae* collected from *Brassica* spp. from the European Union (EU) and the United Kingdom (UK)[a]

Sexual cross[b]		Weeks after plating paired conidial suspension[c]					
MAT1-3	**MAT1-2**	**4**	**5**	**6**	**7**	**8**	**9**
2016-5	2016-9	Ai^1 As^2	As^3	As^3	As^3	As^3	As^3
2016-5	2016-26	Ai^1	Ap^2	Ap^1 As^1	As^3	As^3	As^3
2016-5	2016-34	-	As^1	As^1	As^3	As^3	As^3
2016-5	8CAB	Ai^1	Ai^1Ap^1 As^1	As^3	As^3	As^3	As^3
2016-5	Fr2	Ai^1	As^1	As^1	As^1	As^1	As^1
2016-50	2016-9	Ap^1	Ap^1	Ap^1	Ap^1	Ap^1	Ap^1
2016-50	2016-26	-	-	-	-	-	-
2016-50	2016-34	-	-	-	As^1	As^1	As^1
2016-50	8CAB	-	-	As^2	As^2	As^2	As^2
2016-50	Fr2	-	-	-	-	-	-
5a	2016-9	Ap^1	As^2	As^2	As^3	As^3	As^3
5a	2016-26	Ai^1 Ap^1	Ap^2	As^2	As^2	As^2	As^2
5a	2016-34	As^1	As^1	As^1	As^1	As^3	As^3
5a	8CAB	-	-	-	As^1	As^2	As^2
5a	Fr2	Ai^1	Ai^1 Ap^1	As^2	As^2	As^2	As^2
E3A	2016-9	-	-	-	As^2	As^3	As^3
E3A	2016-26	-	-	-	As^2	As^3	As^3
E3A	2016-34	Ap^1	As^1	As^1	As^1 Ai^2	Ai^1 As^2	Ai^1 As^2
E3A	8CAB	Ai^1 Ap^1	As^2	As^2	As^2	As^2	As^2
E3A	Fr2	-	-	-	As^2	As^2	As^2
UK73	2016-9	-	-	Ai^2	Ai^2	Ai^1 Ap^1	Ai^1 Ap^1
UK73	2016-26	Ai^1	Ap^1	Ap^1	Ap^2	Ap^1 As^1	As^2
UK73	2016-34	-	-	Ai^1	Ai^1	Ap^1	Ap^1
UK73	8CAB	Ai^1	Ai^1 As^1	Ai^1 As^1	Ai^1 As^1	Ap^1 As^2	Ap^1 As^2
UK73	Fr2	-	-	Ai^1	Ai^1	Ai^1	Ai^1

[a] Sexual compatibility of *P. brassicae* isolates from the EU and UK (Table 3.2) of different mating type genes, *MAT1-3* or *MAT1-2*, were tested. In addition, 10 USA isolates, 5 of each of *MAT1-3* and *MAT1-2* genes, and 10 EU/UK isolates, 5 each of *MAT1-3* and *MAT1-2*, were paired in all possible combinations of MAT-1 x MAT-2 idiomorph crosses. Only results for the EU/UK *MAT1-3* x EU/UK *MAT1-2* crosses are shown because mature apothecia and/or ascospores were not observed for any other crosses (USA x USA of opposite mating types, USA x USA of the same mating type, and USA x EU/UK of opposite mating types).

[b] Three replications/pairing were made for isolates with MAT-1 and MAT-2 idiomorphs on 3% malt extract agar, as described in the main text.

[c] Starting two weeks after plating, plates were examined microscopically on a weekly basis for apothecium initials (Ai), apothecia (Ap), and asci/ascospores (As). The superscript number represents the number of three replicate plates on which Ai, Ap, and/or As developed. " –" = no fruiting structures observed. Data are shown for observations of the plates four to nine weeks after setting up the crosses with the pairs of isolates.

Seedborne Pathogens of Brassicas in the Pacific Northwest

Shannon Carmody, MS student, and Dr. Lindsey du Toit, Professor in Plant Pathology,
Washington State University

Objective: The overarching goal of this Washington State University research project is to provide farmers and seed companies that produce brassica crops in this region with tools and awareness of methods to detect and manage seedborne pathogens of brassica/crucifer species in brassica fields and seed lots. Your responses will remain anonymous. This project is funded by the Western Sustainable Agriculture Research and Education program, and the Clif Bar/Seed Matters Foundation. If you would like more information on this survey or topic, please contact **Dr. Lindsey du Toit at dutoit@wsu.edu or 360-848-6140**.

What best describes your work in agriculture?

☐ Farmer ☐ Seed industry ☐ Extension/Educator
☐ Researcher ☐ Consultant ☐ Other:

Which types of brassicas do you currently work with?

☐ Arugula ☐ Broccoli ☐ Brussel sprouts
☐ Cabbage ☐ Canola ☐ Cauliflower
☐ Kale ☐ Mustard ☐ I don't work with brassicas
☐ Other(s):

Which types of brassicas do you currently work with?

☐ Fresh Market ☐ Seed Crops ☐ Processing Crops
☐ Cover Crops ☐ Oilseed Crops ☐ Other(s)

If you work with brassica crops, please indicate if you:

☐ Do not know if the seed you use has been tested for any seedborne pathogens
☐ Only purchase seed that has been tested for potential seedborne pathogens
☐ Do not purchase seed that has been tested for potential seedborne pathogens
☐ Grow and save your own seed
☐ Not applicable to my work

Have you observed any diseases in your brassica crops?

☐ Yes ☐ No ☐ Unsure

Have you heard of brassica black leg *(Phoma lingam)*, light leaf spot (*Cylindrosporium concentricum*), &/or white leaf spot *(Pseudocercosporella capsellae)?*

☐ Black Leg ☐ Light Leaf Spot ☐ White leaf spot
☐ All of the above ☐ None of the above

Are you aware of the **Oregon Department of Agriculture** Crucifer Rule OAR 603-052-0870 about black leg (*Phoma lingam*) that applies to all Brassicaceae crops planted in Oregon?

☐ Yes ☐ No ☐ Unsure ☐ Not applicable to me

Fig. 3.1. Questionnaire for Pacific Northwest brassica stakeholders to gain an understanding of awareness of seedborne brassica pathogens in the Pacific Northwest USA. The questionnaire was distributed to growers at three conferences: the Washington Tilth Producers' Annual Conference on 13 to 15 November 2015 in Spokane, WA; the Organic Seed Growers' Conference on 5 to 6 February 2016 in Corvallis, OR; and the Pacific Northwest Vegetable Association Annual Convention and Trade Show on 17 to 18 November 2015 in Kennewick, WA.

Are you aware of the **Washington State Department of Agriculture** Crucifer Quarantine Rule WAC 16-301-495 to 16-301-580 that currently applies to 6 counties in **western Washington** to protect against seedborne black leg (*Phoma lingam*) and black rot (*Xanthomonas campestris* pv. *campestris*)?

☐ Yes ☐ No ☐ Unsure ☐ Not applicable to me

Are you aware of the **proposed amendment** to the **WSDA** Crucifer Quarantine Rule WAC 16-301 to provide protection against black leg in brassica/crucifer crops in all counties **east of the Cascade Mountains** in Washington?

☐ Yes ☐ No ☐ Unsure ☐ Not applicable to me

Thank you!

Fig. 3.1. Continued.

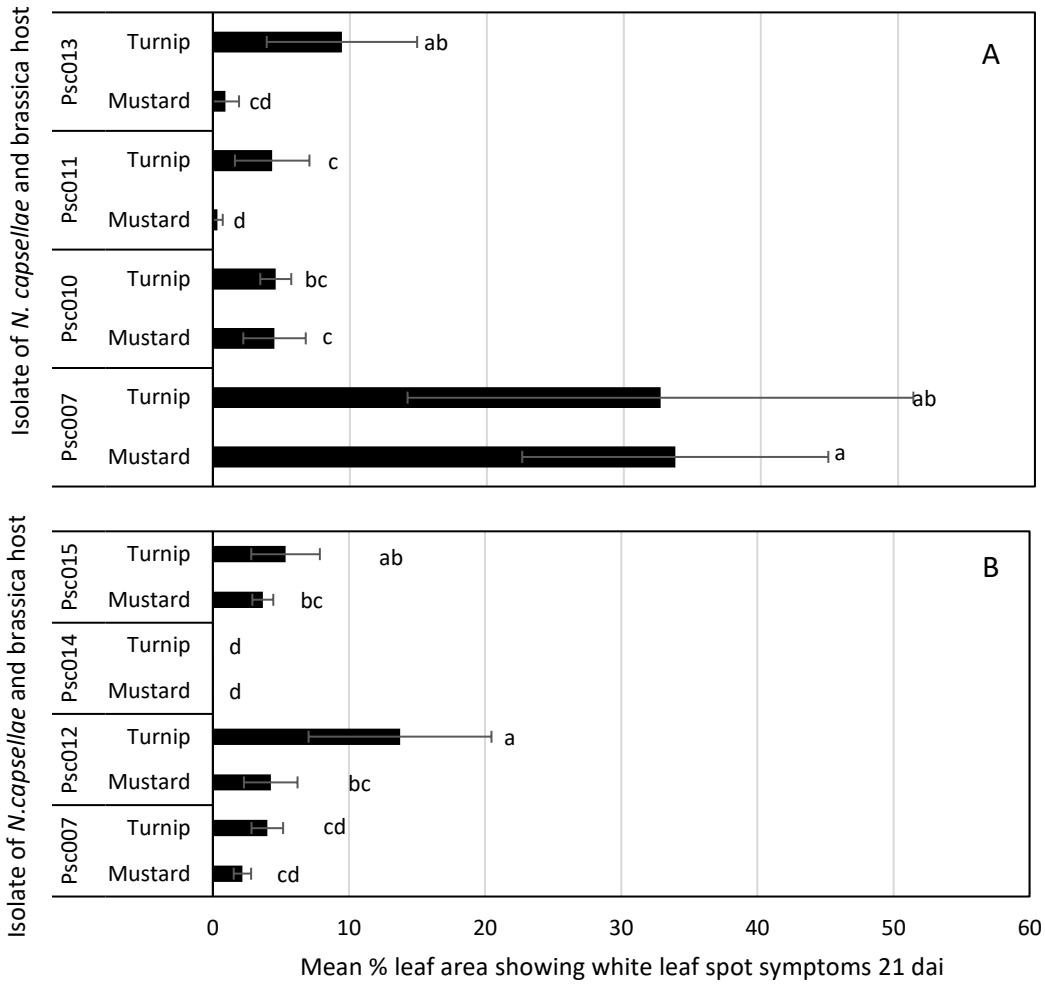

Fig. 3.2. Severity of white leaf spot on 'Hakurei' turnip (*Brassica rapa*) and 'Caliente 199' mustard (*B. juncea*) caused by *Neopseudocercosporella capsellae* isolates collected in northwestern Washington in 2016. The first (**A**) and second (**B**) pathogenicity tests with isolates of this pathogen were started on 10 June and 2 September 2017, respectively, corresponding to tests 3 and 4, respectively, in Table 3.1, to assess a total of 6 isolates. Details about the isolates are in Table 1. Isolate Psc007, the positive control isolate, was collected in the Willamette Valley, OR, in 2015, and demonstrated previously to be pathogenic on multiple *Brassica* spp. (see Chapter 2). Original means are presented, but data did not meet assumptions for parametric analyses so were analyzed using Friedman's non-parametric rank test. Significant interactions between host and isolate were detected in both pathogenicity tests. Means separations were calculated using Fisher's protected least significant difference (LSD) at $P < 0.05$ of rank-transformed data. Each bar represents the mean ± standard error of four replicate plants of that isolate-host species combination. Plants treated with water served as the negative control treatment on which symptoms of white leaf spot did not develop. Refer to Table 1 and the main text for details of the isolates, inoculation procedure, and rating.

Fig. 3.3. Light leaf spot signs (patches of white conidiomata) produced by *Pyrenopeziza brassicae* isolates from the EU and UK 14 days after inoculation of 'Hakurei' turnip (*Brassica rapa*) plants (**A** and **B**). Close-up image of white conidiomata on a turnip leaf surface (**B**). Symptoms of light leaf spot caused by isolate Cyc001 from the Willamette Valley, OR, were typical of those observed for other isolates of *P.* cf. *brassicae* collected in Washington and Oregon States, i.e., coalescing chlorotic spots and veinal browning without white conidiomata (**C**).

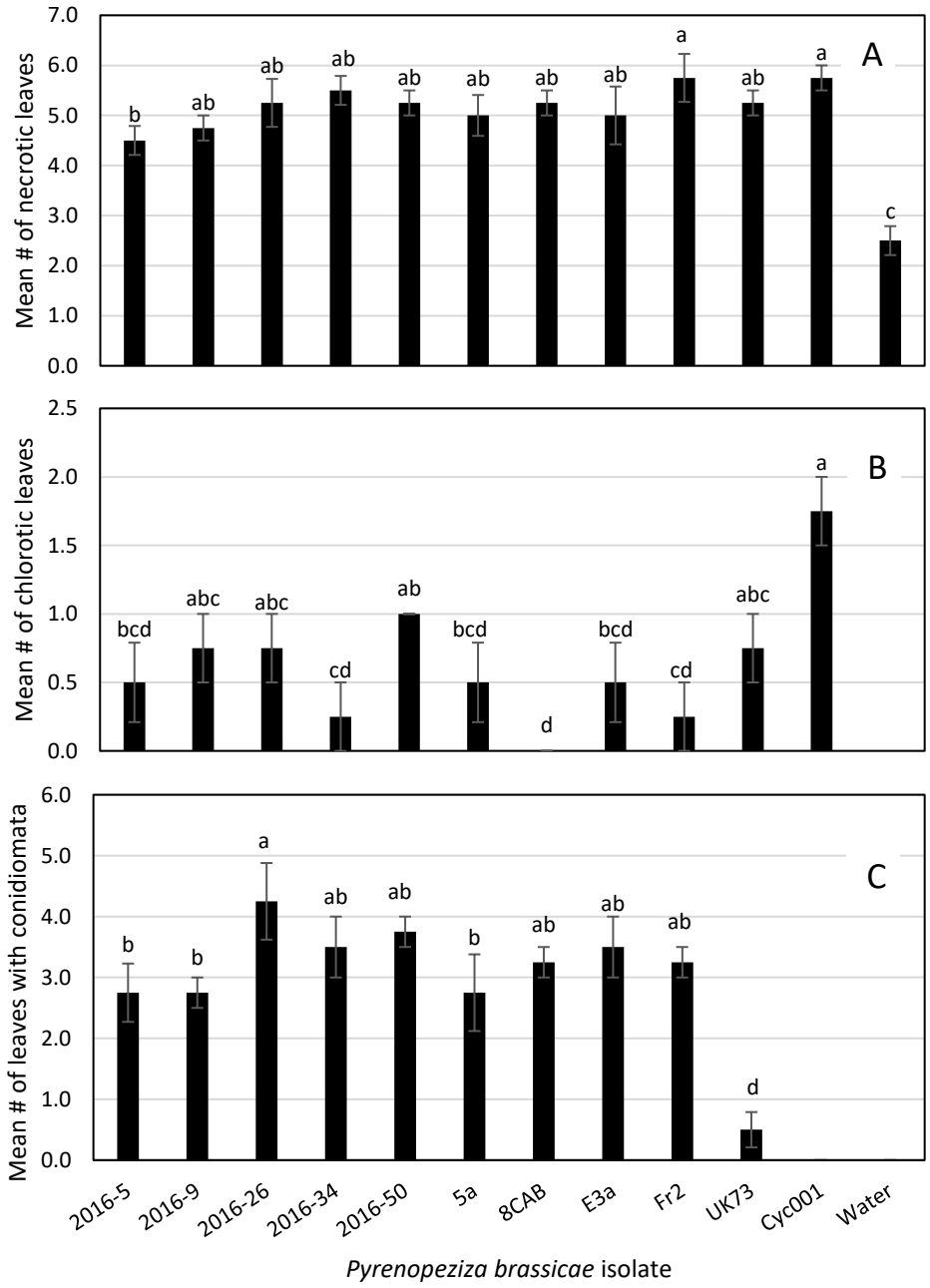

Fig. 3.4. Results of a pathogenicity test of EU and UK isolates of *Pyrenopeziza brassicae* on 'Hakurei' turnip (*Brassica rapa*) compared to USA isolate Cyc001 of *P.* cf. *brassicae*. Plants treated with water served as a control treatment. Ratings were done 28 days after inoculation, including the number of necrotic leaves (**A**), number of chlorotic leaves (**B**), and number of leaves that developed white conidiomata of *P. brassicae* (**C**). Each bar represents the mean ± standard error of four replicate plants. Means with different letters are significantly different based on Fisher's protected least significant difference at $P < 0.05$. Data were rank-transformed although original means are shown. The control treatment was removed from the analysis for **B** and **C** as none of the leaves was chlorotic or developed white conidiomata.

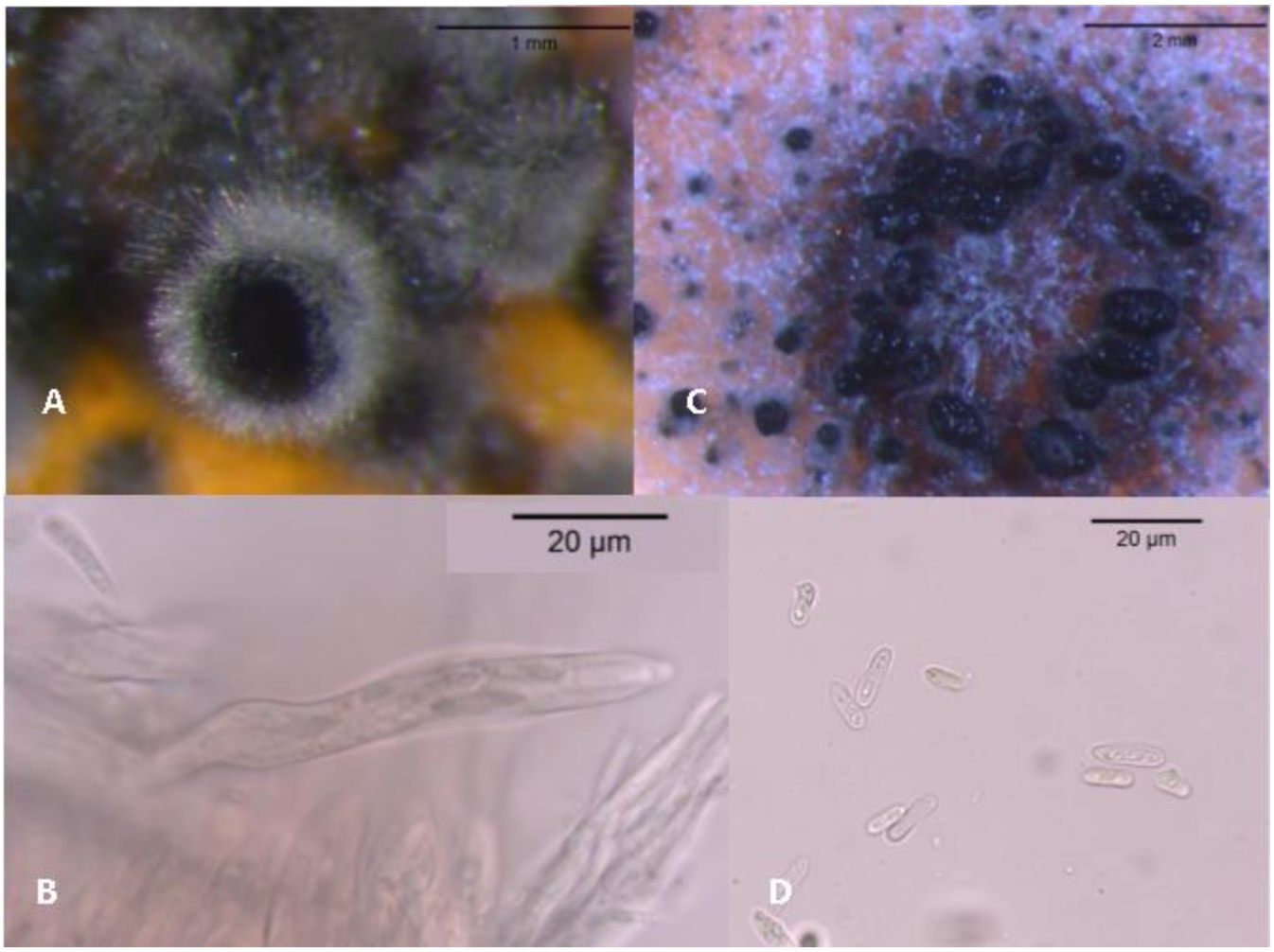

Fig. 3.5. Mature apothecia of *Pyrenopeziza brassicae* formed from UK isolate 5a (mating type idiomorph MAT-2) crossed with UK isolate 2016-9 (MAT-1 idiomorph) (**A**). *P. brassicae* ascospores in an ascus from the sexual crosses of UK isolate UK73 (MAT-2) with UK isolate 8CAB (MAT-1) (**B**). *P. brassicae* ascospores (**D**) closely resemble conidia of *P. brassicae* (= *Cylindrosporium concentricum*), so confirmation of the presence of ascospores in asci is important. Apothecial initials formed from the UK isolate 2016-5 (MAT-1) plated with the USA isolate Cyc017 (MAT-2) of *P.* cf. *brassicae* (**C**). Mating type idiomorphs were identified by West and King using the protocol described by Foster et al. (2002). Refer to Tables 3.2 and 3.3 as well as the main text for details of the isolates and sexual crosses.

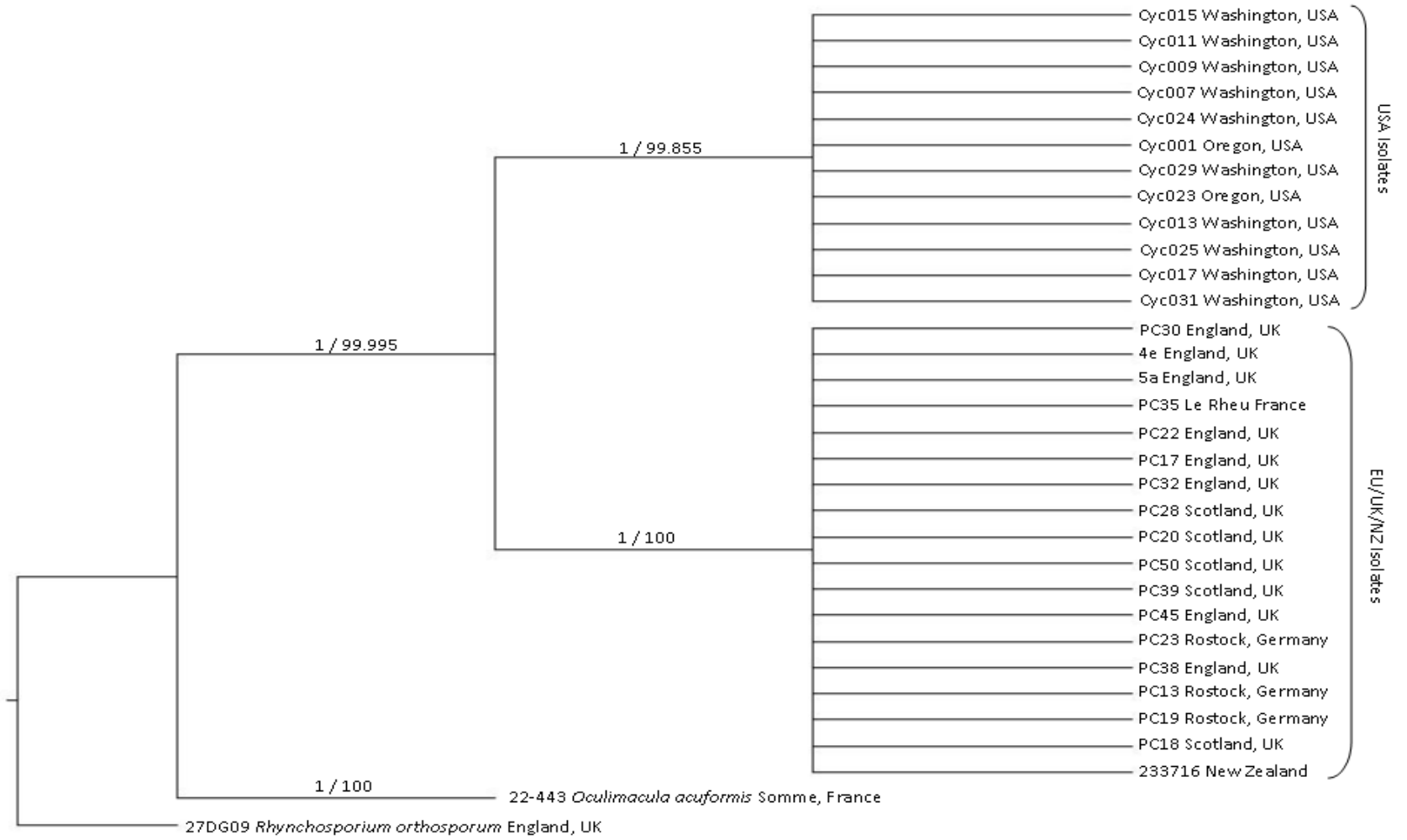

Fig. 3.6. Maximum likelihood phylogenetic tree with the Bayesian posterior probability (BPP) support indicated on the branches for the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) of isolates of *Pyrenopeziza brassicae* from the EU, New Zealand (NZ), and the UK, and *P.* cf. *brassicae* isolates from the USA. BPP and maximum likelihood bootstrap support (MLBS) values are indicated at the nodes (BPP/MLBS). Putative species clades are delimited as shown. The tree was rooted to *Rhynchosporium orthosporum* isolate 27DG09. Refer to Table 3.2 for details of the isolates.

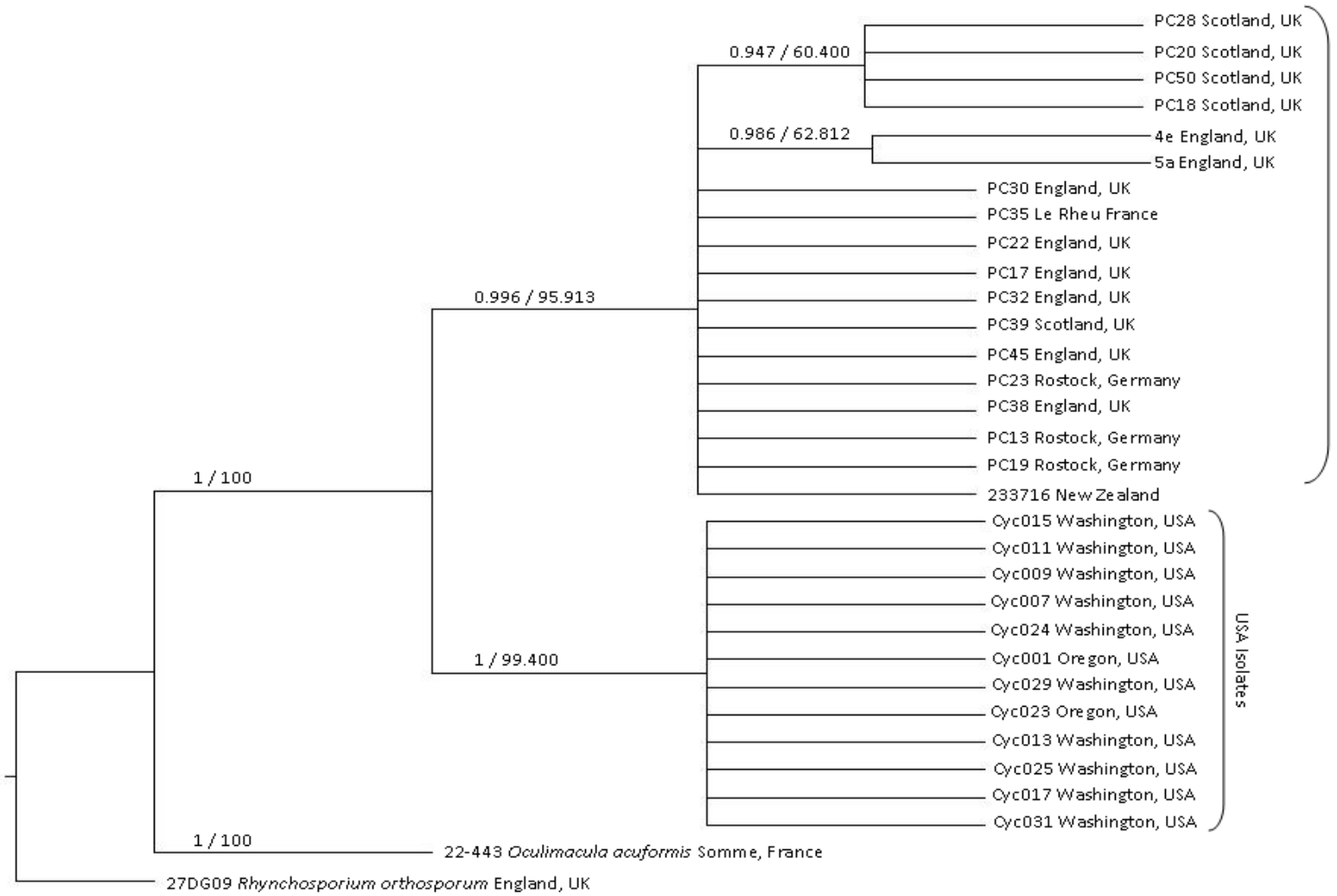

Fig. 3.7. Maximum likelihood phylogenetic tree with the Bayesian posterior probability (BPP) support indicated on the branches for the β tubulin gene of isolates of *Pyrenopeziza brassicae* from the EU, New Zealand (NZ), and the UK, and *P.* cf. *brassicae* isolates from the USA. BPP and maximum likelihood bootstrap support (MLBS) values are indicated at the nodes (BPP/MLBS). Putative species clades are delimited as shown. The tree was rooted to *Rhynchosporium orthosporum* isolate 27DG09. Refer to Table 3.2 for details of the isolates.

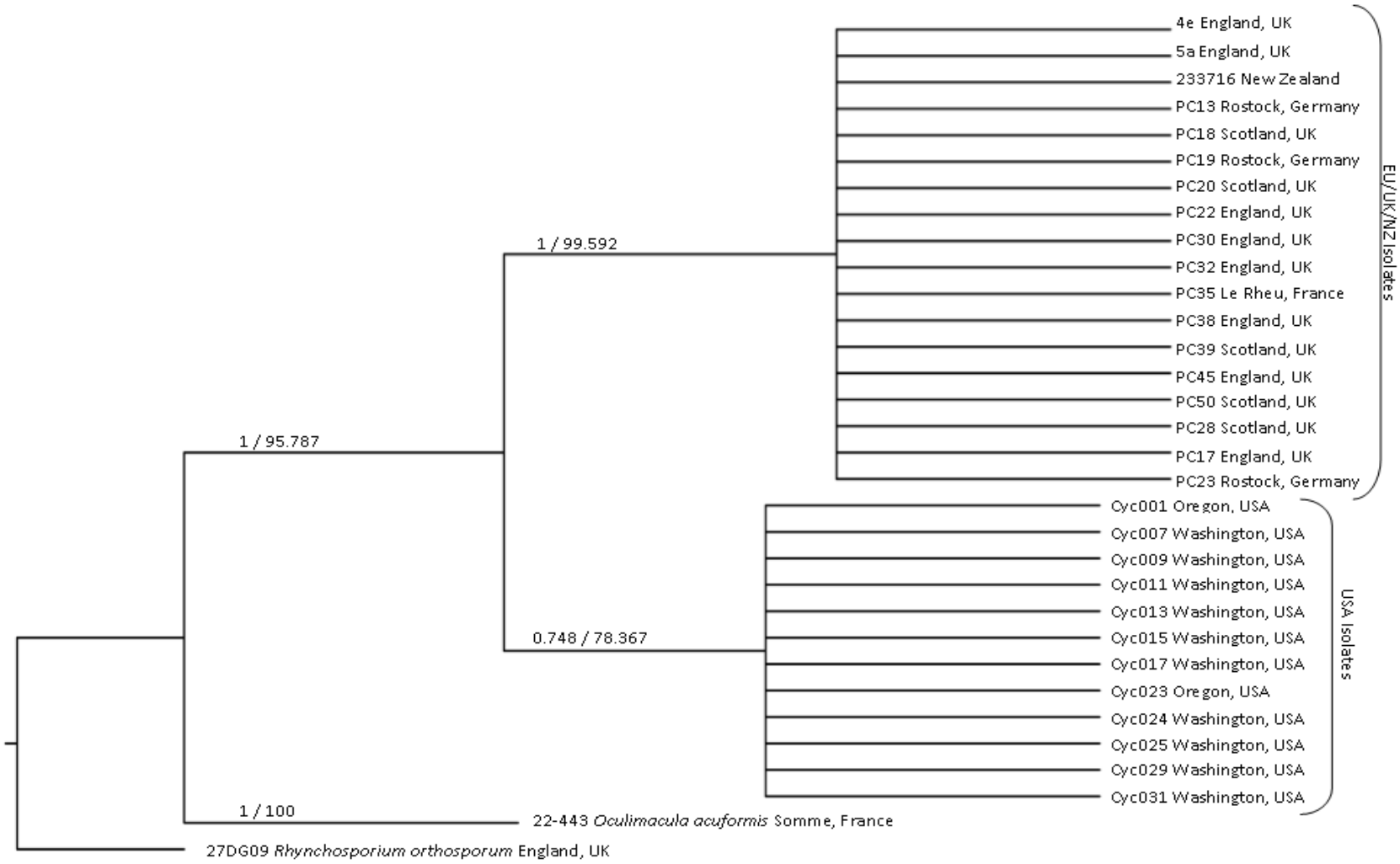

Fig. 3.8. Maximum likelihood phylogenetic tree with the Bayesian posterior probability (BPP) support indicated on the branches of the translation elongation factor (TEF) 1-α gene of isolates of *Pyrenopeziza brassicae* from the EU, New Zealand (NZ), and the UK, and *P.* cf. *brassicae* isolates from the USA. BPP and maximum likelihood bootstrap support (MLBS) values are indicated at the nodes (BPP/MLBS). Putative species clades are delimited as shown. The tree was rooted to *Rhynchosporium orthosporum* isolate 27DG09. Refer to Table 3.2.

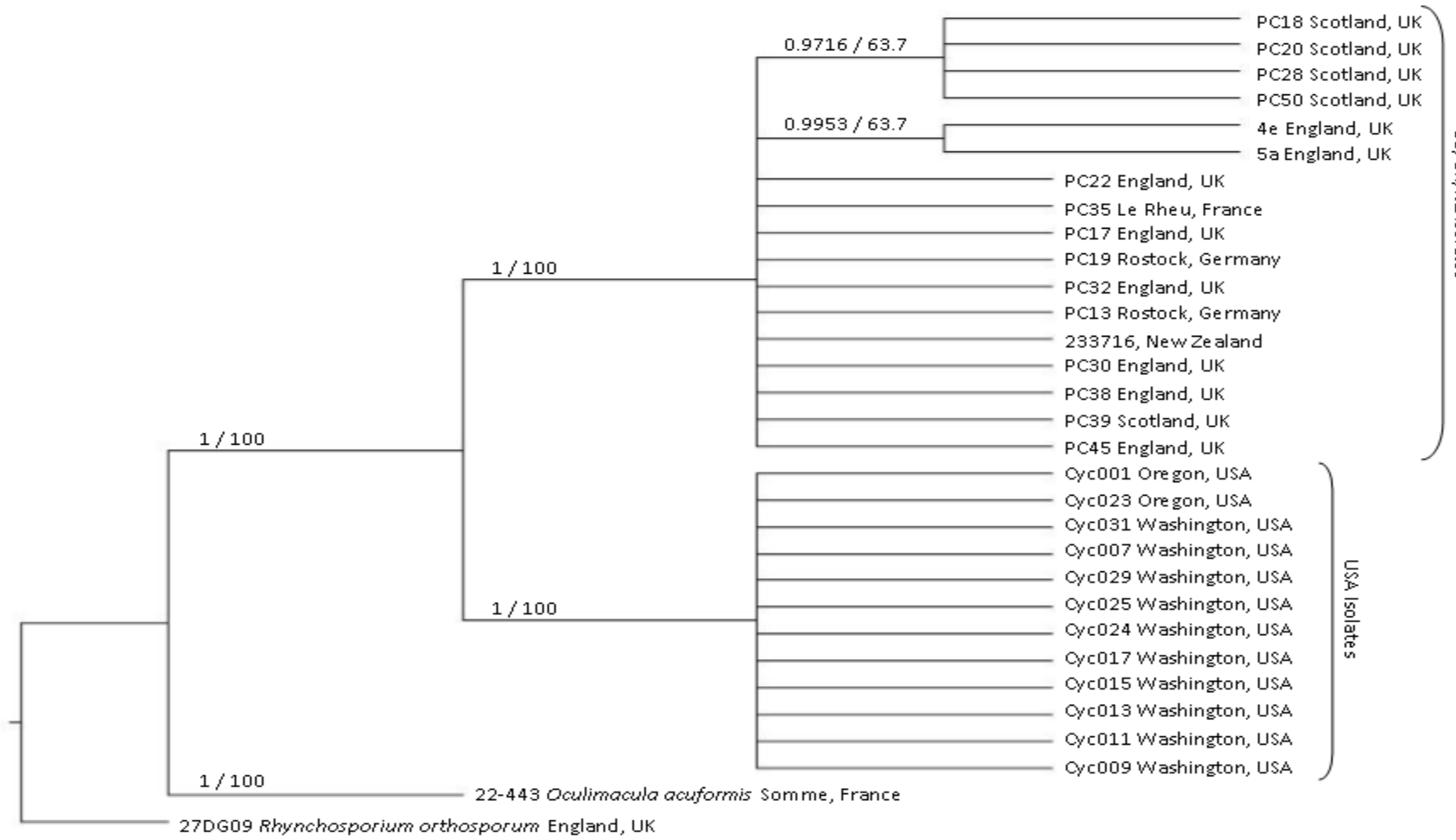

Fig. 3.9. Maximum likelihood phylogenetic tree with the Bayesian posterior probability (BPP) support indicated on the branches of the combined alignment of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), β tubulin gene, and translation elongation factor (TEF) 1-α gene sequences of isolates of *Pyrenopeziza brassicae* from the EU, New Zealand (NZ), and the UK, and isolates of *P.* cf. *brassicae* from the USA. BPP and maximum likelihood bootstrap support (MLBS) values are indicated at the nodes (BPP/MLBS). Putative species clades are delimited as shown. The tree was rooted to *Rhynchosporium orthosporum* isolate 27DG09. Refer to Table 3.2 for details.

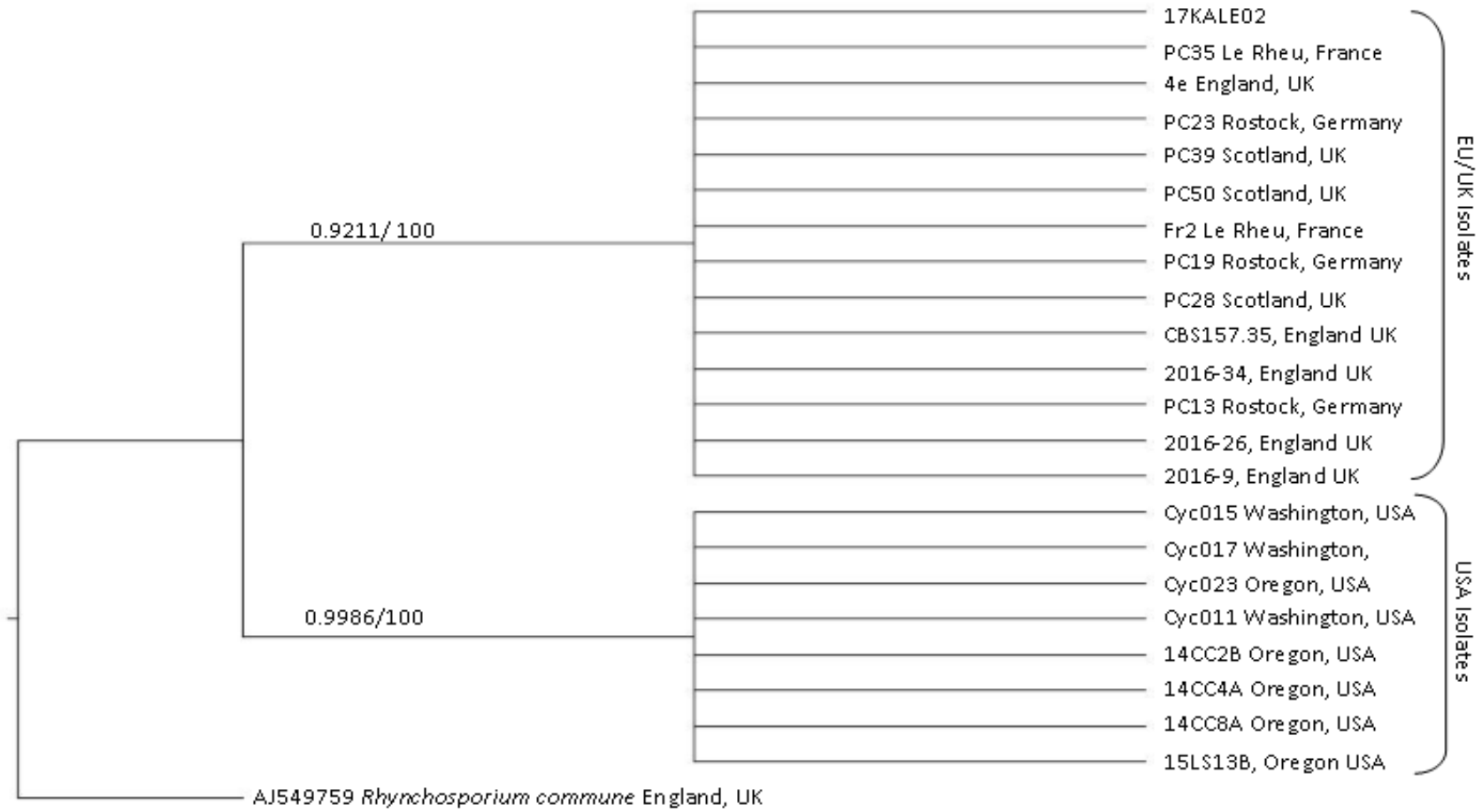

Fig. 3.10. Maximum likelihood phylogenetic tree with the Bayesian posterior probability (BPP) support indicated on the branches for the *MAT1-3* mating type gene of isolates of *Pyrenopeziza brassicae* from the EU and UK, and *P.* cf. *brassicae* isolates from the USA. BPP and maximum likelihood bootstrap support (MLBS) values are indicated at the nodes (BPP/MLBS). Isolate names and geographic origin are labeled at the tip of the trees. Putative species clades are delimited as shown. The tree was rooted to *Rhynchosporium commune* isolate AJ549759. Refer to Table 3.2 for details of the isolates.

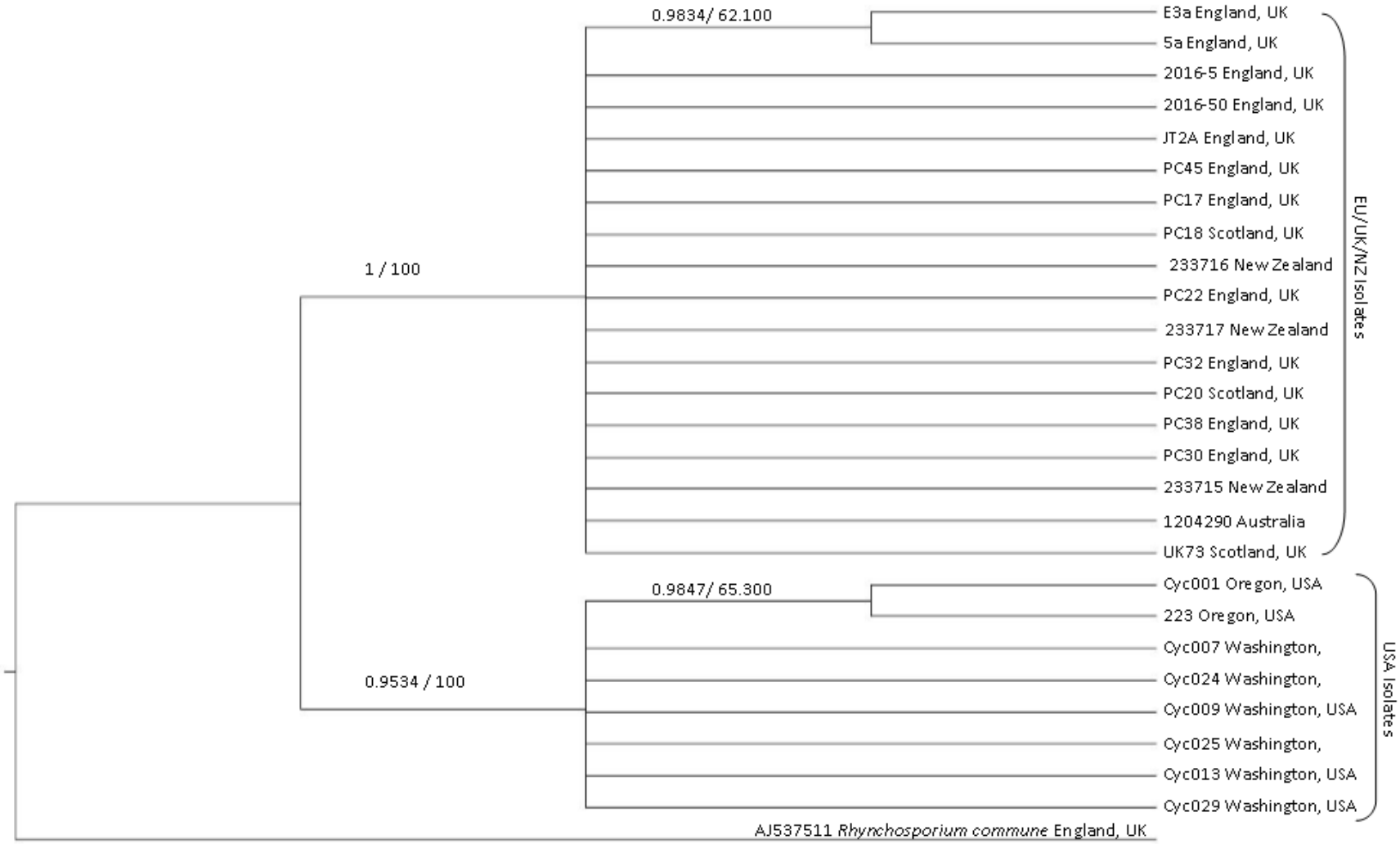

Fig. 3.11. Maximum likelihood phylogenetic tree with the Bayesian posterior probability (BPP) support indicated on the branches for the MAT1-2 mating type gene of isolates of *Pyrenopeziza brassicae* from the EU, New Zealand (NZ), and the UK, and *P.* cf. *brassicae* isolates from the USA. BPP and maximum likelihood bootstrap support (MLBS) values are indicated at the nodes (BPP/MLBS). Isolate names and geographic origin are labeled at the tip of the trees. Putative species clades are delimited as shown. The tree was rooted to *Rhynchosporium commune* isolate AJ537511. Refer to Table 3.2 for details of the isolates.

CHAPTER 4

EFFICACY OF SEED TREATMENTS FOR ERADICATION OF THE LIGHT LEAF SPOT PATHOGEN FROM MUSTARD (*BRASSICA JUNCEA*) SEED

4.1 Introduction

Brassica crops are grown in the Pacific Northwest USA for many markets and purposes. These include conventional and organic fresh market and processing crops, cover crops, seed crops, and oilseed and biofuel crops (Inglis et al. 2013). Potential seedborne pathogens of brassica crops are not only a concern for brassica seed producers, because of the risk of planting infected stock seed and/or disseminating infected harvested seed, but to all brassica crop producers who want to ensure they are purchasing pathogen-free seed. The most common seed transmitted pathogens of brassica crops include *Alternaria brassicae, A. brassicicola, Botrytis cinerea, Phoma lingam,* and *Xanthomonas campestris* pv. *campestris* (Cappelli et al. 1999; Richardson 1990; Rimmer et al. 2007). Growing pathogen-free brassica crops in the Pacific Northwest can be challenging with the diversity of types and cultivars grown, year-round cropping of brassicas, and weedy and volunteer brassica plants (Inglis et al. 2013; Pscheidt and Ocamb 2016). In the 1970s, the western Washington and Oregon brassica seed industry was impacted significantly following black leg outbreaks, caused by *Phoma lingam*, in the Midwest and the northeast USA that were traced back to brassica seed crops grown in the Pacific Northwest (Jacobsen and Williams 1971; Jenkins 2015; Neergaard 1997). Neergaard (1977) estimated that, in 1973, black leg and black rot (latter caused by the bacterial pathogen *X. campestris* pv. *campestris*) caused estimated yield losses of 10% in the USA, valued at $20 million. Williams (1980) considered black rot to be the most important vegetable brassica disease worldwide.

From the 1970s until 2011, following intensive research and implementation of strict black leg management practices by the Pacific Northwest brassica seed industry and seed growers, the Pacific

Northwest was considered “free” of black leg (Inglis et al. 2013; Paulitz et al. 2017). However, black leg was detected in northern Idaho in 2011 (Agostini et al. 2013), in eastern Washington in 2015 (Paulitz et al. 2017), in west-central Idaho and north-central Oregon in 2015 (Paulitz et al. 2017; L. J. du Toit Washington State University, *personal communication*) and widespread in the Willamette Valley of western Oregon in 2014 (Ocamb 2016a; Ocamb et al. 2015). In 2014, in addition to black leg, light leaf spot (caused by *Pyrenopeziza brassicae*) was detected in multiple types of brassica crops and on Brassicaceae weeds in the Willamette Valley of Oregon (Ocamb 2014a; Ocamb 2014b). Light leaf spot had never been reported before in North America (Ocamb 2014a; Farr and Rossman 2017). This pathogen has since persisted and become widespread in the Willamette Valley (C. M. Ocamb, Oregon State University, *personal communication*). In the spring of 2016, light leaf spot was detected in *Brassica juncea* (mustard) cover crops and *B. rapa* weeds (bird’s rape mustard) in northwestern Washington (see Chapter 3). In 2017, a Willamette Valley grower reported nearly 100% incidence of light leaf spot in each of a *B. juncea* seed crop and a *B. rapa* seed crop, estimating >50% yield losses (L. J. du Toit, Washington State University, *personal communication*). Interestingly, adjacent *B. napus* and *B. oleracea* seed crops on that farm had very little infection. Because light leaf spot is new to the Pacific Northwest USA, the impacts of this disease are not yet well understood in the context of the diverse brassica crop production systems in this region.

Internationally accepted seed health assays have been established by the International Seed Health Initiative (ISHI) of the International Seed Federation (ISF) for *P. lingam* and *X. campestris* pv. *campestris* (International Seed Health Initiative-Veg. 2011). The Washington State Department of Agriculture (WSDA) implemented a quarantine ruling in 2006, WAC 16-301-490-580, at the request of brassica vegetable seed growers and seed companies, to protect six counties in northwestern Washington that are the primary region for biennial brassica vegetable seed crops in that state (Inglis et al. 2013; WSDA 2015). The black leg part of the regulation was extended in 2015 to include all counties

east of the Cascade Mountains (WSDA 2015). The WSDA rule requires that any brassica seed lot to be planted in the protected area of Washington State must be certified as *P. lingam*-free, and the lot must be tagged as having met this condition. Seed treatments can play an important role in keeping areas protected by such quarantines disease-free and as a management tool to eradicate seedborne pathogens from infected seed. Extensive research has been done on the efficacy of seed treatments to eradicate *P. lingam* and *X. campestris* pv. *campestris* from infected brassica seed (Jacobsen and Williams 1971, Neergaard 1977; Rimmer et al. 2007). As detailed in Chapter 2, *P. brassicae* can be seedborne and seed transmitted. However, it is unknown whether the management tools used to control black leg, including seed treatments used to eradicate *P. lingam* from seed or reduce the risk of seed transmission, are also effective at managing *P. brassicae*.

Seed treatments are used to prevent the transmission of seedborne pathogens from seed to the seedlings that develop when the seed is plated (Neergaard 1977). Maude (1996) described the various ways seed can be treated, such as subjecting seed to a compound (fungicide, chlorine, etc.), a form of energy (heat, radiation, etc.), or a process (e.g., drying). Fungicides are the most form of common chemical seed treatment used and have largely replaced other methods of seed treatments in conventional agriculture (Maude 1996). For example, nearly all commercial hybrid corn (*Zea mays*), canola (*Brassica* spp.), and sugar beet (*Beta vulgaris* subsp. *vulgaris*) seed planted in the USA is treated with at least one fungicide (Mueller et al. 2013). Fungicide seed treatments can be applied by growers using a slurry mixer or drill-box seed treater, but the commercial treatment of seed is becoming more common (Mueller et al. 2013). Chemical, physical, and other seed treatments may be limited in the ability to eradicate deep, internally infected seed, unless the treatment includes a fungicide with some systemic activity, or the energy such as heat can penetrate the seed deep enough to kill the pathogen without damaging the embryo (Maude 1996; Mueller et al. 2013; Neergaard 1977). Conventional fungicide seed treatments are not permitted in certified organic agriculture in the USA under the United

States Department of Agriculture (USDA) National Organic Program (NOP) regulation 7 CFR 205.204 (USDA NOP 2017); however, disinfectant treatments, in which seeds are soaked in a disinfectant such as NaOCl, can be used, as can hot water or other physical treatments (Agarwal and Sinclair 1997).

Neergaard (1977) referred to seed treatments that subject seed to different forms of energy or processes as physical seed treatments that usually are applied in a manner to inhibit the development of, or kill, seedborne pathogens without killing or damaging the seed. Examples of physical seed treatments include hot water, steam, dry heat, and radiation (Neergaard 1977). Physical seed treatments often are used for internally infected seed. For example, hot water seed treatments are commonly used to control bacterial seedborne pathogens, particularly for specialty crops for which there are limited pesticide seed treatment registrations (Maude 1996; Neergaard 1997), and in organic agricultural systems (Finckh et al. 2015). Seed is subjected to hot water treatment by immersing the seed in water heated to a precisely-controlled temperature for a duration that damages the target microorganisms in or on the seed but is not harmful to the seed (Baker 1962). In 1888, Danish seedsman J. L. Jensen established a hot water treatment for control of smut pathogens in cereals (Maude 1996; Neergaard 1977; Freeman and Johnson 1909). This method of seed treatment also has been proven effective at eradicating *P. lingam* and *X. campestris* pv. *campestris* from crucifer seed (Maude 1996; Neergaard 1977), as well as other pathogens such as *A. brassicae* and *A. brassicicola* (Maude 1996). The most common brassica hot water seed treatment entails treating seed for 15 to 30 min in water heated to approximately 50°C (Miller and Lewis Ivey 2005).

The principle of steam-treating seed, or aerated-steam seed treatment, uses pressure to treat dry seed in moving vapor, for which different types of equipment have been developed (Baker 1962; Finckh et al. 2015; Maude 1996; Neergaard 1977). Although not a new technology, there has been a resurgence in the use of steam seed treatments in organic agriculture because the method can be used to treat seed of some types of plant species that cannot be treated with hot water, e.g., the seed coat of

arugula (*Eruca sativa*) seed and flax (*Linum usitatissumum*) seed becomes mucilaginous and hygroscopic when steeped in water (Neergaard 1977; T. Stearns, High Mowing Seed Co., *personal communication*). Maude (1996) wrote that aerated steam-treated seeds are drier, with less damage to the seed coat and to seed germination than seeds treated with hot water. However, higher temperatures of steaming and longer durations of treatment are needed for steam treatments compared to hot water treatment because the heating capacity of aerated steam is only 0.5 times that of hot water but 2.5 times greater than that of dry, hot air. Recently developed technologies have had better results for treating vegetable seed with continuous, humidified hot air (steam) when treatment duration, air flow rate, and temperature are controlled precisely (Finckh et al. 2015). Navaratnam et al. (1980) showed that aerated steam treatment at 54°C for 30 min was effective at eradicating *X. campestris* pv. *campestris* from brassica seed. In addition, a proprietary steam treatment developed by ThermoSeed (BioAgri AB, Uppsala, Sweden) at 53°C for 10 min was effective at eradicating *X. campestris* pv. *campestris* (Finckh et al. 2015). Depending on the technology used, steam seed treatments also can be completed more rapidly than hot water seed treatment because of the shorter, post-treatment drying time (T. Stearns, High Mowing Seed Co., *personal communication*).

Though there are many ways to treat infected seed, complete eradication of seedborne pathogens using seed treatments is not always possible (Maude 1996). Nonetheless, seed treatments can help reduce the incidence of infection of a seed lot to below a known threshold for a target pathogen that might result in commercially significant disease outbreaks (Maude 1996). As demonstrated in Chapter 2, *P. brassicae* can infect brassica seed and be transmitted to seedlings grown from infected seed. The objective of this study was to assess the efficacy of chlorine, hot water, steam, and fungicide seed treatments for eradicating the light leaf spot pathogen from an infected mustard seed lot. The infected mustard seed lot was produced as described in Chapter 2, using a USA isolate of the light leaf pathogen that, subsequently, was demonstrated to be a different species of *Pyrenopeziza*

than *P. brassicae*, as detailed in Chapter 3. The pathogen is hereafter referred to as *P. cf.* brassicae. The overall objective was to identify effective conventional and organic seed treatments that brassica growers and the brassica seed industry can use to manage seedborne *P.* cf. *brassicae.*

4.2 Materials and Methods

For each type of seed treatment evaluated, the treated seed were subjected to three assays: a seed health assay to quantify the incidence of seed with viable *P.* cf. *brassicae*, a seed germination assay to assess the effects of the treatments on seed quality, and a seed transmission assay to determine if the treatments reduced or prevented seed transmission of *P.* cf. *brassicae*. Mustard seed of 'Caliente 199' was used for this study. The seed lot was grown, inoculated, and harvested at the Washington State University (WSU) Mount Vernon Northwestern Washington Research and Extension Center (NWREC) as described in Chapter 2. Using the 4°C NP-10 agar seed health assay described in that chapter, the mustard seed lot was determined to be infected with *P.* cf. *brassicae* at an incidence ranging from 10.50 to 19.75%.

4.2.1 Chlorine seed treatments. To determine the efficacy of chlorine seed treatments on eradication of *P.* cf. *brassicae* from mustard seed, five durations of seed treatment (0, 10, 20, 30, and 40 min) in 1.2% NaOCl were evaluated using a randomized complete block (RCB) design with four replications of each treatment. For each replication of each seed treatment, a mesh tea strainer containing 500 *P.* cf. *brassicae*-infected mustard seed was soaked in 100 ml of 1.2% NaOCl in a 250 ml glass beaker on a gyratory shaker at 220 rpm for the appropriate duration. The entire beaker was covered with aluminum foil during the treatment to minimize NaOCl breaking down in light, and the opening of the beaker and the mesh tea strainer and aluminum foil were covered with Parafilm (Pechiney Plastic Packaging, Menasha, WI). After the appropriate duration of treatment, the seed in the

tea strainer was triple-rinsed in sterilized, deionized water. The seed was then dried on sterilized coffee filter paper (Melitta, Clearwater, FL) in a laminar flow hood for at least 1 h, and placed in a disposable Petri dish. The dishes were each sealed with Parafilm and stored at room temperature (24 ± 2°C) in the dark. The five replications of the non-treated control seed were stored similarly. A sample of 100 seed of each replicate of the seed subjected to each treatment was then evaluated using the 4°C NP-10 agar seed health assay, a second sample of 100 seed/replicate/treatment was subjected to the blotter seed germination assay of the Association of Official Seed Analysts (AOSA) (Yaklich 1985), and a third sample of 200 seed/replicate/treatment was subjected to a seed transmission assay in a greenhouse, as described in Chapter 2. The entire trial was repeated (Trial 2). Each assay was completed using an RCB design.

The seed transmission assay was performed as described in Chapter 2, with the following modifications. The assay was 4 weeks long and completed in greenhouse Bay 103 at the WSU Mount Vernon NWREC, starting on 21 October 2016. The repeat assay was initiated on 29 November 2016. The seed for each treatment was planted into three 72-cell flats (1 seed/cell, with each cell 3.8 cm diameter x 5.7 cm deep; Steuber's Distributing Co., Snohomish, WA) for each of four replications. For every replication of each treatment, the number of days after planting at which symptoms of light leaf spot were first detected was recorded, as well as the incidence of seedlings that developed symptoms of light leaf spot and from which *P*. cf. *brassicae* was isolated (Chapter 2). Temperature, relative humidity, and leaf wetness were recorded every 15 min using a WatchDog data logger and leaf wetness sensor (Model 425, Spectrum Technologies, Inc., Plainfield, IL).

4.2.2. Hot water seed treatments. To determine the efficacy of hot water seed treatments on eradication of *P.* cf. *brassicae* from mustard seed, a trial was set up in an RCB design with four replications of each of three durations (0, 15, and 30 min) of hot water treatment at 50°C (Miller and Lewis Ivey 2005). These durations of treatment were selected to cover the durations of hot water

treatments typically used by the brassica seed industry and brassica growers (Jahn et al. 2007). For each of the four replications, two mesh tea strainers, one for each of the 15- and 30-min hot water treatment, and each containing 500 *P.* cf. *brassicae*-infected mustard seed, were immersed in deionized water heated to 50°C in a programmable circulating water bath (Fisher Scientific International, Inc., Hampton, NH). In Trial 1, a pre-heating seed treatment for 10 min at 40°C was used to attempt to minimize heat stress on the seed, based on the recommendation of Miller and Lewis Ivey (2005). However, this pre-heating step was not used in the repeat trial (Trial 2) based on organic seed industry practices (F. Morton, Wild Garden Seed Co., Corvallis, OR, *personal communication*) and observation of slight phytotoxicity to mustard seedlings caused by the 30 min hot water treatment in the seed germination and seed transmission assays for Trial 1 (see Results). Each tea strainer was removed from the water bath after the appropriate duration of treatment, and the seed triple-rinsed in cold, sterilized, deionized water. The seed was then dried on sterilized coffee filters in a laminar flow hood for at least 1 h, and placed in disposable Petri dishes. The dishes were each sealed with Parafilm and stored at room temperature. The five replications of the non-treated control seed were stored similarly. A sample of 100 seed for each replication of each treatment was then plated using the 4°C NP-10 agar seed health assay the following day, 100 seed of each replication of each treatment were subjected to the AOSA blotter seed germination assay, and 200 seed of each replication of each treatment were subjected to the seed transmission assay as described for the chlorine seed treatments. An RCB design was used for each assay and the entire trial was repeated (Trial 2).

4.2.3. Steam seed treatments. To determine the efficacy of steam seed treatments on eradication of *P.* cf. *brassicae* from the mustard seed lot, a trial was set up in an RCB design with four replications of each of five temperatures of steam treatment (no treatment, 62.8, 65.6, 68.3, and 71.2°C). The proprietary steam treatment equipment and the protocol were developed by High Mowing Seed Co. (Wolcott, VT). Four replications of five packets of 500 seed of the *P.* cf. *brassicae*-infected

mustard seed were shipped to High Mowing Seed Co. on 8 April 2016. For each stream treatment, the four replicate samples of seed were steamed for 90 s at the appropriate temperature using the proprietary equipment. Post-steaming, the seed were placed into paper envelopes and dried at 26.7°C and 20% RH for 6 h (T. Stearns, High Mowing Seed Co., *personal communication*). The four replicate samples of non-treated control seed were also put into the drier in envelopes. Seed for one of the four replications of the 65.6°C steam treatment was steamed accidentally at 64.4°C. The steam-treated seed was sent back to the WSU Mount Vernon NWREC to be tested with the AOSA blotter seed germination assay, 4°C NP-10 agar seed health assay, and seed transmission assay. For the repeat trial (Trial 2), another set of 20 samples of 500 seed of the mustard seed lot was shipped to High Mowing Seed Co. on 17 August 2016, subjected to the same steam treatments, and tested using the three assays described above.

The 4°C NP-10 agar seed health assay and AOSA blotter seed germination assay were performed as described in Chapter 2. For the seed health assay in Trial 1, the seed for the first replication of the treatments were plated on 3 June 2016, and seed for subsequent replications were plated weekly thereafter. Seed health assays were initiated similarly on 21 September 2016 for Trial 2. The seed transmission assay for each replication of each treatment was completed in greenhouse West Bay 1 at the WSU Mount Vernon NWREC for Trial 1, starting on 7 October 2016 (first replication), 14 October (second and third replications), and 21 October 2016 (fourth replication); and in greenhouse Bay 103 for Trial 2, starting all four replications of the assay on 29 November 2016. The seed transmission assay was run for four weeks for each replication of the treatments.

4.2.4. Fungicide seed treatments. Ten fungicide seed treatments were evaluated to determine the efficacy of each treatment at eradicating *P.* cf. *brassicae* from infected mustard seed and preventing seed transmission of the pathogen. The 10 treatments are used commonly on seed of various *Brassica* species for other seedborne fungal pathogens (Pesticide Information Center Online 2016). In addition,

two control treatments were included in the trial, seed treated with a proprietary colorant or dye, and seed not treated with fungicides or dye (Table 4.1). Seed colorants or dyes are required by USDA federal law 40 CFR 153.155(c) for any seed treated with pesticides (American Seed Trade Association 2017), as the dye functions both to identify treated seed lots and as a formulated adherent to ensure the chemical adheres to the seed. For each of the fungicide seed treatments, seed were placed in a slurry of a proprietary blue polymer-colorant (Spectrum Polymer Coating, Pawne, Navi, Mumbai, India) at 4% by seed weight (mixed 1:1 with water), to which the appropriate fungicide was added. The dye control seed was treated with water + blue polymer-colorant, while the non-treated control seed received no dye or fungicide.

A freeze-blotter seed health assay was used instead of the 4°C NP-10 agar seed health assay because fungicides on seed dissipate into agar media, reducing the effective rate of product applied to the seed, as has been documented for several seed health assays of the International Seed Trade Association (ISTA 2017). Acrylic boxes, each 110 mm x 110 mm x 35 mm deep (Hoffman Manufacturing, Inc., Jefferson, OR), and the lids were sterilized with 70% ethyl alcohol in a laminar flow hood, followed by exposure to ultraviolet light (UV) for 20 min. Steel blue germination blotters (each 105 mm x 105 mm, 38 lb, Anchor Paper Co., Plymouth, MN) were sterilized by autoclaving twice at 1.05 kg/cm^2 and 121°C for 20 min, with 24 h between the two autoclavings. Each blotter was added to a sterilized box and moistened with 10 ml sterilized, deionized water. Using sterilized forceps, 100 seed for each replication of each treatment were plated onto the blotters in three boxes (34, 34, and 32 seed/box), and the seeds incubated in the dark at room temperature to imbibe for at least 8 h or until the first evidence of a radicle emerging from any seed in the boxes. The boxes were then placed at -20°C for 12 to 14 h to freeze the seed and prevent further germination. The boxes of seed were covered with a towel immediately after removal from the freezer to slow the thawing process, and placed in the dark at 4°C in cardboard boxes, as for the 4°C NP-10 seed health assay. Microscopic examination of the seed

was done similarly to the NP-10 seed health assay described above, except that delays in the development of fungi on the seed caused by the dye and/or fungicides necessitated extending the assay to an 8-week duration, with microscopic readings done 21, 28, 35, 42, 49, and 56 days after plating the seed. In Trial 2, microscopic readings were only done 28, 42, and 56 days after plating the seed. The AOSA blotter seed germination assay and seed transmission assay were performed as for the chlorine, hot water, and steam seed treatments. The entire trial was repeated (Trial 2). Each assay was set up in an RCB design. Non-treated control seed was not included in the Trial 1 seed health assay and seed germination assay, but was included in the seed transmission assay for Trial 1 and in all three assays for Trial 2.

Based on the results of the seed health assay for Trial 1, either the freeze blotter seed health assay and/or the dye control treatment affected the ability to detect *P.* cf. *brassicae* on the seed (see Results). Therefore, a seed health assay was set up to compare non-dyed, non-treated seed to seed treated with the blue dye, using both the 4°C NP-10 agar seed health assay and the 4°C freeze blotter seed health assay. Samples of 100 non-treated seed and 100 seed treated with the dye were plated on NP-10 agar medium and on blotters, as described above, and stored at 4°C for 3 weeks with microscopic examination 21, 28, and 35 days after plating.

4.2.5. Statistical analysis. For each seed treatment trial, the data collected for the seed health assay, seed germination assay, and seed transmission assay were subjected to analyses of variance (ANOVA), and treatment means were compared using Fisher's protected least significant difference (LSD) at $P < 0.05$ using SAS University (SAS Institute, Cary, NC). Replications were treated as a random effect, and seed treatments as a fixed effect in the ANOVA model for each trial. When assumptions of normality and/or variance heterogeneity were not met, the data were transformed or Friedman's non-parametric rank test was used, as detailed in Tables 2 to 9.

4.3 Results

4.3.1 Chlorine seed treatments. None of the durations of seed treatment in 1.2% NaOCl had a significant adverse effect on seed quality in Trial 1 or Trial 2, as measured by the percentage of seed with normal germination (*P* = 0.062 and 0.611, respectively), non-germination (*P* = 0.056 and 0.562, respectively), abnormal germination (*P* = 0.194 and 0.523, respectively), or the percentage of rotten seed (*P* = 0.127 and 0.722, respectively) (Table 4.2). Averaged across all five chlorine seed treatments in Trial 1, the seed lot had 96.20 ± 0.50% normal germination, 1.55 ± 0.29% non-germinated seed, 0.75 ± 0.16% abnormally germinated seed, and 1.50 ± 0.25% rotten seed (Table 4.3). In Trial 2, the seed lot averaged 97.30 ± 0.44% normal germination, 0.90 ± 0.19% non-germination, 0.55 ± 0.18% abnormal germination, and 1.25 ± 0.32% rotten seed (Table 4.3).

The chlorine seed treatments significantly reduced the incidence of *P.* cf. *brassicae* detected on the mustard seed lot in Trial 1 and Trial 2 (*P* <0.001) (Table 4.2). In Trial 1, the incidence of seed on which *P.* cf. *brassicae* was observed was reduced from 16.50% for the non-treated seed to ≤4.50% for seed treated with 1.2% NaOCl for durations ranging from 10 to 40 min (Table 4.3). Similarly, in Trial 2, the incidence of *P.* cf. *brassicae* that developed on the seed was reduced from 12.50% for control seed to ≤4.00% for the four durations of chlorine seed treatment (Table 4.3). In both trials, significantly more seed was infected with *P.* cf. *brassicae* if the treatment was for 10 or 20 min (4.50 and 3.25%, respectively in Trial 1; 3.35 and 4.00%, respectively in Trial 2), compared to seed treated for 30 or 40 min (1.00 and 0.50%, respectively in Trial 1; and 1.25 and 0.75%, respectively in Trial 2) (Table 4.3). In Trial 1, the incidence of seed on which *Alternaria* spp. were detected in the 4°C NP-10 agar seed health assay was reduced significantly (*P* = 0.001) from 8.50% for the non-treated control seed to 2.25, 2.75, 3.50, and 1.00% for seed treated for 10, 20, 30, and 40 min, respectively, with no significant differences among the chlorine treatment durations. Likewise, in Trial 2, the incidence of seed on which *Alternaria*

spp. were detected was significantly (P = 0.002) greater on the non-treated control seed (8.00%) than on the seed treated with chlorine for 10 min (4.75%), 20 min (2.50%), 30 min (3.50%), and 40 min (1.25%). In that trial, there was no significant difference in the incidence of seed with these fast-growing fungi between the non-treated control seed and the seed treated with 1.2% NaOCl for 10 min, but significantly fewer seed treated with 1.2% NaOCl for 20, 30, and 40 min had *Alternaria* spp. than that of the non-treated seed (Table 4.3).

The chlorine seed treatments also significantly reduced the incidence of seedlings on which *P.* cf. *brassicae* was detected in the seed transmission assay in both Trials 1 and 2 (P <0.001 for both trials) (Table 4.2). Light leaf spot symptoms were observed on the cotyledons, as described in Chapter 2. In Trial 1, *P.* cf. *brassicae* was isolated from 34 of 47 suspect seedlings sampled; and in Trial 2, the pathogen was isolated from 29 of 34 symptomatic seedlings sampled. The incidence of seedlings infected with *P.* cf. *brassicae* in Trial 1 was reduced from 3.88% for plants that grew from the non-treated seed to 0.25, 0.13, and 0.00% for seedlings that grew from seed treated with 1.2% NaOCl for 20, 30, or 40 min, respectively; and in Trial 2 from 3.50% for seedlings that grew from non-treated seed to 0.13 and 0.00% for seedlings that grew from seed treated for 30 min or 10, 20, and 40 min, respectively (Table 4.3). There were no significant differences in seed transmission rates detected on seedlings that developed from the seed treated with the different durations of chlorine treatment, and seed transmission was not detected on seedlings grown from seed treated for 30 or 40 min in 1.2% NaOCl in Trial 1, or from seed treated for 10, 20, or 40 min in Trial 2. In both trials, symptoms were observed first on the seedlings that grew from non-treated control seed. The minimum number of days after planting (dap) when symptoms were detected on seedlings in Trial 1 was 19 to 29 dap for seedlings that grew from the non-treated control seed, 24 to 26 dap for seedlings that grew from seed treated for 10 min, and 29 dap for seedlings that grew from seed treated for 20 min (Table 4.3). In Trial 2, symptoms were

first detected 15 to 17 dap for seedlings in flats planted with the non-treated control seed (4 days sooner than in Trial 1), and 21 dap for seedlings in flats planted with seed treated for 30 min in chlorine.

In Trial 1, the daytime (7 am to 7 pm) average greenhouse temperature was 16.71 ± 1.02°C, average leaf wetness was 9.77 ± 5.95 min/h, and average RH was 71.20 ± 14.82%; compared to nighttime (7 pm to 7 am) averages of 15.81 ± 1.12°C, 10.80 ± 5.61 min/h, and 83.97 ± 14.16% RH, respectively. In Trial 2, the average daytime temperature in the greenhouse was 16.73 ± 1.00°C, average leaf wetness was 9.71 ± 5.64 min/h, and average RH was 81.85 ± 12.25%; compared to nighttime averages of 14.97 ± 0.82°C, 11.04 ± 5.08 min/h, and 89.63 ± 11.65% RH, respectively.

4.3.2 Hot water seed treatments. In Trial 1 of the hot water seed treatments, normal seed germination was affected significantly (P = 0.023) by the duration of hot water seed treatment at 50°C (Table 4.4), with only 89.75% normal germination for seed treated with hot water for 30 min compared to 98.00% for the non-treated control seed and 95.75% for seed subjected to 15 min of hot water treatment (Table 4.5). Abnormal seed germination also was affected significantly by the hot water treatments in Trial 1 (P = 0.008), with 5.50% abnormally germinated seed treated in 50°C water for 30 min compared to 1.00 and 1.75% for the non-treated seed and seed treated for 15 min at 50°C. The latter two treatments did not differ in the incidence of seed with abnormal germination. There was not a significant main effect of hot water treatment for the percentage of non-germinated seed (P = 0.308) or rotten seed (P = 0.292) in Trial 1 (Table 4.4), with an average of 0.42 ± 0.52% non-germinated seed and 2.33 ± 1.51% rotten seed across all three treatments (Table 4.5). Hot water seed treatments did not have a significant effect on seed quality in Trial 2, with 96.08 ± 2.47% normally germinated seed 0.83 ± 0.38% non-germinated seed, 1.58 ± 1.62% abnormally germinated seed, and 1.83 ± 0.52% rotten seed (Tables 4 and 5). The repeat trial did not include the pre-heating step of 40°C prior to the 50°C hot water treatment, which appeared to account for the differences in phytotoxicity observed for the 30 min hot water treatment in Trial 1 vs. Trial 2.

Hot water seed treatments significantly reduced the incidence of seed on which *P.* cf. *brassicae* ($P < 0.001$) and *Alternaria* spp. ($P < 0.001$) were detected in the seed health assay for both trials (Table 4.4). *P.* cf. *brassicae* was observed on 16.50% of the non-treated seed and none of the seed treated for 15 or 30 min in hot water at 50°C in Trial 1; and on 12.50% of the non-treated control seed, 0.25% of the seed subjected to hot water treatment for 15 min, and none of the seed treated for 30 min in Trial 2 (Table 4.5). The incidence of seed on which *Alternaria* spp. were detected was reduced significantly from 8.50 and 8.00% of the non-treated seed in Trials 1 and 2, respectively, to 0.0% for both durations of hot water treatment in both trials (Table 4.5).

The incidence of seedlings infected with *P.* cf. *brassicae* was affected highly significantly by the hot water seed treatments in both trials ($P <0.001$) (Table 4.4). In Trial 1, 31 of the 37 suspect symptomatic seedlings were confirmed to be infected with *P.* cf. *brassicae* microscopically; and in Trial 2, 28 of 29 sampled seedlings developed *P.* cf. *brassicae* when plated onto half strength, chloramphenicol-amended potato dextrose agar (see Chapter 2). The incidence of seed transmission detected in Trial 1 was reduced from 3.88% for plants that grew from non-treated seed to 0.00% for plants that grew from hot water treated seed, regardless of the duration of treatment (15 or 30 min); and similarly was reduced in Trial 2 from 3.50% for control seed to 0.00% from seed treated at 50°C for 15 or 30 min (Table 5). Symptoms were first detected 19 to 29 dap on seedlings that grew from the non-treated control seed in Trial 1, and 15 to 17 dap in Trial 2. Temperature, leaf wetness, and RH in the greenhouse were as described for the chlorine seed transmission trials, as the seed transmission assays were carried out at the same time for the chlorine and hot water seed treatment trials.

4.3.3 Steam seed treatments. The steam seed treatments had a significant main effect on seed quality as measured by normal seed germination ($P <0.001$) and abnormal seed germination ($P = 0.004$) in the first trial (Table 4.6). Significantly less of the seed steamed at 71.1°C displayed normal germination (90.00%) than that of non-treated control seed (98.25%) and of seed steam-treated at temperatures

<71.1°C (97.75 to 98.25% germination) (Table 4.7). There was no significant difference in normal germination for the control seed and the seed steamed at 62.8, 65.6, and 68.3°C (Table 4.7). The incidence of seed with abnormal germination was 0.50% for the non-treated seed vs. 8.00% for seed treated with steam at the highest temperature evaluated, 71.1°C; compared to 0.00% for seed steamed at 62.8°C, and 0.75% for seed steamed at 65.6 or 68.3°C (Table 4.7). No significant differences were detected in the incidence of non-germinated seed (P = 0.376) or rotten seed (P = 0.507) among any of the steam treatments or control seed in Trial 1 (Table 4.6). Averaged over all five treatments in Trial 1, the incidence of non-germinated seed was 0.50 ± 0.18%, and the incidence of rotten seed was 1.20 ± 0.30%.

In Trial 2, steam seed treatments had no significant effects on the incidence of non-germinated seed (P = 0.461) and abnormally germinated seed (P = 0.445). Averaged across all five treatments, the incidence of non-germinated seed was 1.15 ± 0.21% and the incidence of abnormally germinated seed was 0.45 ± 0.14%. However, the steam treatments had a significant effect on normal seed germination (P = 0.009) and the incidence of rotten seed (P = 0.030) (Table 4.6). Normal seed germination was reduced significantly for seed steamed at 62.8°C (95.0%) or 71.1°C (93.75%) compared to non-treated seed (97.75%) or seed steamed at 65.6°C (98.50%) or 68.3°C (97.75%) (Table 4.7). The incidence of rotten seed was significantly greater for seed steamed at 71.1°C (3.75%) compared to non-treated seed (1.00%), with no significant differences among the other (cooler) steam treatments and the control seed (Table 4.7).

In both trials, steam treatments significantly affected the incidence of seed on which *P.* cf. *brassicae* (P <0.001) and *Alternaria* spp. (P = 0.001 and P <0.001 in Trials 1 and 2, respectively) were detected in the 4°C NP-10 agar seed health assay (Table 4.6). In both trials, *P.* cf. *brassicae* was eradicated from all of the seed by all steam treatments, except for seed treated at 71.1°C in Trial 1, for which *P.* cf. *brassicae* was detected on 0.54% of the seed, which was still significantly less than that of

the control seed (14.48%) (Table 4.7). The mean incidence of control seed infected with *P.* cf. *brassicae* in Trial 2 was 10.50% compared to 0.00% for all of the steam-treated seed (Table 4.7). In Trial 1, the incidence of seed on which *Alternaria* spp. were detected in the seed health assay was reduced from 8.90% for the control seed to 0.26, 0.26, 0.25, and 0.00% for seed steamed at 62.8, 65.6, 68.3, and 71.1°C, respectively, with no significant differences among the steam treatment durations (Table 4.7). Likewise, in Trial 2, the incidence of seed on which *Alternaria* spp. were detected was 4.75% on the non-treated control seed compared to none detected on any of the steam-treated seed.

Steam treatments significantly reduced the rate of *P.* cf. *brassicae* seed transmission detected ($P \leq 0.001$ in both trials) (Table 4.6). The mean incidence of seed transmission of *P.* cf. *brassicae* for the control seed was 1.50% in Trial 1 and 2.88% in Trial 2 (Table 4.7). *P.* cf. *brassicae* was not detected on any seedlings that developed from seed subjected to any of the steaming temperatures, except for seed steamed at 68.3°C in Trial 2, for which the seed transmission rate of *P.* cf. *brassicae* was 0.125%, which was not significantly different than that of the other steam treatments (Table 4.7). In both trials, a 2- to 3-day delay in seedling emergence was noted for seed steamed at 71.1°C (*data not shown*). In addition, slight differences in symptom expression were observed between the two trials. As described in Chapter 2, symptom development in greenhouse West Bay 1, where the Trial 1 seed transmission assay was completed, was first observed on the first true leaves (Fig. 2.5B), while expression of light leaf spot symptoms in the seed transmission assay for Trial 2, completed in greenhouse Bay 103, was first observed on the cotyledons and first true leaves (Fig. 2.5A). In Trial 1, symptoms only were detected on seedlings that grew from non-treated seed, with the first symptoms observed 33 to 39 dap (Table 4.7). In Trial 2, symptoms first were observed on seedlings 16 to 29 dap for plants that grew from the non-treated seed, and 22 dap for very few plants that grew from seed steamed at 68.3°C (Table 4.7). In Trial 1, there were 12 confirmed isolates of *P.* cf. *brassicae* from 16 seedlings sampled that had symptoms suspected to be caused by *P.* cf. *brassicae*; and in Trial 2, *P.* cf. *brassicae* was isolated from 24 of 25

seedlings sampled. Greenhouse microclimate data were not collected during Trial 1 because of a data logger error. In Trial 2, the daytime average greenhouse temperature was 16.73 ± 1.00°C, average leaf wetness was 9.71 ± 5.64 min/h, and average RH was 81.85 ± 12.25%; compared to nighttime averages of 14.97 ± 0.82°C, 11.04 ± 5.08 min/h, and 89.63 ± 11.65% RH, respectively.

4.3.4 Fungicide seed treatments. Fungicide seed treatments did not have a significant effect on any measurement of seed quality (percentage normally germinated, non-germinated, abnormally germinated, or rotten seed) in Trial 1 or Trial 2 (Table 4.8). The seed germination assay for Trials 1 and 2 revealed 97.45 ± 0.25 and 97.70 ± 0.24% normally germinated seed, 0.77 ± 0.14 and 1.13 ± 0.13% rotten seed, 1.23 ± 0.16 and 0.67 ± 0.12% non-germinated seed, and 0.55 ± 0.11 and 0.50 ± 0.12% abnormally germinated seed, respectively. However, there were statistically significant main effects of fungicide treatments on the incidence of seed on which *P.* cf. *brassicae* ($P = 0.050$ and $P < 0.001$ in Trials 1 and 2, respectively), and *Alternaria* spp. ($P < 0.001$ and $P = 0.001$ in Trials 1 and 2, respectively) were observed in the seed health assay (Table 4.8). Significantly fewer seed developed signs of *P.* cf. *brassicae* in Trial 1 for seed with the following fungicide treatments compared to the dye control seed (latter had 2.25% infection): Helix Vibrance (0.00%), Maxim 4FS (0.25%), Mertect 340F (0.25%), Obvius (0.25%), Coronet (0.50%), and Metlock (0.50%) (Table 4.9). In contrast, Farmore FI400 (0.75% seed infection), Dynasty (1.25%), Rovral 4 Flowable Fungicide (1.25%), and Vibrance (1.50%) did not reduce the seed infection rate significantly from that of the dye control seed (Table 4.9). Significantly fewer seed treated with the following fungicides had *Alternaria* spp. than that observed on the dye control seed (2.50%): Coronet (0.00%), Farmore FI400 (0.00%), Helix Vibrance (0.00%), Obvius (0.25%), Maxim 4FS (0.50%), and Metlock (0.75%) (Table 4.9). In contrast, the incidence of seed on which *Alternaria* spp. was detected did not differ significantly for seed treated with Dynasty (1.00%), Rovral 4F (1.50%), Vibrance (1.75%), and Mertect 340F (2.25%) compared to that of the dye control seed (Table 4.9).

In Trial 2, 8.50% of the non-treated control seed developed *P.* cf. *brassicae* in the 4°C NP-10 agar seed health assay. In contrast, the incidence of seed on which *P.* cf. *brassicae* was observed was reduced to 0.50% of the seed treated with dye only; to 0.00% of the seed treated with Coronet, Farmore FI400, Helix Vibrance, or Mertect 340F; 0.25% of the seed treated with Dynasty; 0.50% of the seed treated with Maxim 4FS; 0.75% of the seed treated Metlock, Obvius, and Rovral 4F Flowable; and 1.25% of the seed treated with Vibrance (Table 4.9). All 10 fungicide seed treatments reduced the incidence of seed infection by *Alternaria* spp. from 33.75% of the control seed to: 0.00% for seed treated with Helix Vibrance, Metlock, or Rovral 4F Flowable; 0.25% for the Coronet-treated seed; 0.50% for the dye control seed and seed treated with Obvius or Farmore FI400; 1.00% for seed treated with Dynasty; 1.25% for seed treated with Maxim 4FS or Vibrance; and 1.75% for seed treated with Mertect 340F (Table 4.9).

Fungicide treatments significantly affected the rate of seed transmission of *P.* cf. *brassicae* detected in both trials ($P < 0.001$) (Table 4.8). The rate of seed transmission was reduced in Trial 1 from 5.25% for the non-treated control seed to ≤0.38% for all fungicide treatments, including the dye control seed; and in Trial 2 from 2.63% for the non-treated control seed to ≤0.25% for all fungicide treatments (Table 4.9). In Trial 1, the following treatments did not affect the seed transmission rate of *P.* cf. *brassicae* any more than that of the dye alone (0.38% incidence of transmission): Metlock (0.13% seed transmission), Obvius (0.13%), Rovral 4F (0.13%), Farmore FI400 (0.25%), and Vibrance (0.38%). In contrast, seed transmission was not observed on any seedlings that grew from seed treated with Coronet, Dynasty, Helix Maxim 4FS, Mertect 340F, or Vibrance. In Trial 2, seed treated with Maxim 4FS (0.13% seed transmission) and Obvius (0.13%) did not affect the seed transmission rate significantly compared to that of the dye treatment alone (0.25%); whereas seed transmission was prevented completely for all of the seed that had been treated with Coronet, Dynasty, Farmore FI400, Helix Vibrance, Metlock, Mertect 340F, Rovral 4F Flowable, or Vibrance (Table 4.9). Seedlings that developed from seed treated with metconazole (Metlock) were stunted and darker green than seedlings from any

other seed treatment in both trials, demonstrating a phytotoxic effect of this seed treatment. In Trial 1, *P.* cf. *brassicae* was isolated from 52 of 65 seedlings sampled that had symptoms suspected to be caused by this fungus; and in Trial 2, the fungus was isolated from 25 of 46 seedlings sampled. In Trials 1 and 2, symptoms first were observed on seedlings 17 to 28 and 16 to 29 dap, respectively, for non-treated control seed; and 18 to 26 and 22 to 25 dap, respectively, for seedlings that grew from the dye control seed, respectively (Table 4.9). In Trial 1, symptoms were detected 28 dap on plants that grew from seed treated with Metlock, 21 dap for the Rovral 4F Flowable treatment, 20 to 26 dap for the Farmore FI400 seedlings, 19 to 21 dap for the Vibrance seedlings, and 21 dap for the plants that grew from the Obvius-treated seed. In Trial 2, symptoms were detected 23 dap for seedlings that grew from seed treated with Maxim 4FS, and 27 dap for plants from the Obvius-treated seed.

During the seed transmission assay for the first fungicide seed treatment trial, average day and night greenhouse temperatures were 15.86 ± 1.19 and 14.74 ± 0.70°C, respectively; average leaf wetness readings were 7.37 ± 5.73 and 12.03 ± 3.08 min/h, respectively; and average RH was 85.56 ± 7.45 and 94.30 ± 4.52%, respectively. In Trial 2, average day and night greenhouse temperatures were 15.51 ± 1.52 and 14.88 ± 0.71°C, respectively; average leaf wetness was 11.17 ± 3.70 and 13.30 ± 1.66 min/h, respectively; and average RH was 79.22 ± 13.62 and 93.39 ± 3.92%, respectively.

Comparison of the 4°C NP-10 agar seed health assay with the 4°C freeze blotter seed health assay for non-treated seed vs. dye-treated seed revealed that *P.* cf. *brassicae* was detected on 12.00% of the non-treated seed and 2.00% of the dye-treated seed plated onto NP-10 agar medium, and on 8.00% of the non-treated seed and 2.00% of the dye-treated seed plated onto blotters. Therefore, the dye alone appeared to have caused a 33% reduction in the incidence of seed on which *P.* cf. *brassicae* was detected on NP-10 agar medium, but did not affect the incidence of seed on which the fungus was detected using the freeze-blotter assay. For non-treated seed, *P.* cf. *brassicae* was detected on 67% fewer seed with the 4°C freeze blotter assay than the 4°C NP-10 agar assay. However, these assays were

not replicated so the results could not be compared statistically for the two assays and two seed treatments.

4.4 Discussion

Using a mustard seed lot infected with *P.* cf. *brassicae,* chlorine, hot water, steam, and fungicide seed treatments evaluated in this study revealed highly effective conventional and organic seed treatments that brassica growers and the brassica seed industry potentially could use to manage seedborne *P.* cf. *brassicae.* The most efficacious organic seed treatments were the steam and hot water treatments which reduced the seed infection and seed transmission rates of *P.* cf. *brassicae* from mustard seed to 0 or almost 0%. Likewise, all of the chemical (fungicide and chlorine) seed treatments evaluated reduced the incidence of mustard seed infection and seed transmission rates of *P.* cf. *brassicae*, as did the polymer coating (dye) treatment alone that was used to apply each of the fungicide treatments to the seed. All of the chlorine, hot water, steam, and fungicide seed treatments evaluated can be used in conventional agricultural production systems for those *Brassica* species on which the treatments are registered for use. In addition, the chlorine, hot water and steam seed treatments also can be used in certified organic agriculture in the USA (USDA NOP 2017). Each type of treatment has advantages and disadvantages. For example, the development of new proprietary equipment for steam seed treatments has led to more efficiency in steam-treating vegetable seed, i.e., better treatment of the seed coat which protects the embryo, faster drying times after treatment, and shorter treatment durations than equivalent hot water seed treatments (Finckh et al. 2015; T. Stearns, High Mowing Seed Co., *personal communication*). However, the new steam seed treatment equipment and methods usually are patented and can be expensive for custom seed treatments (Forsberg and Sanchez-Sava 2009). Equipment for hot water treatment (i.e., a circulating hot water bath) is less expensive than that

required for steam-treating seed, and more treatment protocols and information have been developed for are access by growers and seed companies (Morton et al. 2016; Neergaard 1977; Miller and Lewis Ivy 2005). Chlorine seed treatments also do not require specialized equipment and can be done readily by growers, although chlorine seed treatments evaluated in this study were not as efficacious as hot water and steam seed treatments against *P.* cf. *brassicae*.

Both 15 and 30 min durations of hot water seed treatment at 50°C, as well as the steam seed treatment for 90 s at 62.8, 65.6, 68.3, and 71.1°C were highly effective at reducing the incidence of seed infected with *P.* cf. *brassicae*, as detected with the 4°C NP-10 agar seed health assay. For both hot water seed treatment durations, *P.* cf. *brassicae* was not observed in the seed health assay except on one seed treated for 15 min in the seed health assay of the repeat trial. Likewise, seed transmission of *P.* cf. *brassicae* was not detected in the seed transmission assay for seed subjected to hot water treatment for either duration. Similar efficacy was observed with the steam seed treatments as *P.* cf. *brassicae* seed infection was only observed on two seeds (steamed at 71.1°C), and only one seed transmission event (a single seedling infected with *P.* cf. *brassicae* from seed steam-treated at 68.3°C) was detected for all four temperatures of the steam treatments evaluated. Although the chlorine seed treatments were effective at reducing the incidence of mustard seed on which *P.* cf. *brassicae* developed, *P.* cf. *brassicae* was still detected on some seed subjected to each of the durations of bleach treatment (≤4.75% for all chlorine-treated seed compared to ≥8.00% for the non-treated control seed). However, significantly fewer seed developed *P.* cf. *brassicae* conidiomata in the seed health assay for seed treated in 1.2% NaOCl for the longer durations (30 or 40 min) compared to that of seed treated for only 10 or 20 min. *P.* cf. *brassicae* also was detected on 4 seedlings that grew from chlorine-treated seed in the seed transmission assays, with a seed transmission rate of 0.25 and 0.13% for seed treated for 10 and 20 min, respectively, in Trial 1; and 0.13% for seed treated for 30 min in Trial 2. This was significantly less than the seed transmission rate from the non-treated seed (3.88 and 3.50% in Trials 1 and 2, respectively).

The results from these trials suggest that *P.* cf. *brassicae* infection of the mustard seed lot used in this study was more internal than just on, or in, the seed coat as the pathogen was still detected on a very limited incidence of some of the seed subjected to each of the chlorine seed treatments, and even on a few of the seed treated at temperatures as great as 71°C. Neergaard (1977) found that physical seed treatments such as steam and hot water are more effective than some chemical or disinfectant seed treatments against seedborne pathogens that have infected seed internally. Similar results with greater efficacy of hot water vs. chlorine seed treatments were found by du Toit and Hernandez-Perez (2005) for two seedborne pathogens of spinach, *Cladosporium variabile* and *Stemphylium botryosum*, that can infect seed internally and externally. Knox-Davies (1979) showed that *A. brassicicola* could infect cabbage and Brussels sprout seed both internally and externally, and that the infection rates could be affected by damage to the testas or maturity of the seed. Jacobsen and Williams (1971) established that the outer epidermis, subepidermal parenchyma, and sometimes the embryo of seed coats of *Brassica* spp. could become infected by *P. lingam*. For both *A. brassicicola* and *P. lingam,* which can infect seed internally, Nega et al. (2003) found hot water treatments of cabbage seed to be very effective at killing the seedborne inoculum of both pathogens. Cook et al. (1952) found evidence that the bacterial black rot pathogen, *X. campestris* pv. *campestris*, can infect cabbage seed through the funiculus from where the pathogen moves into the seed coat. Despite the internal nature of seed infection by this pathogen, Babadoost et al. (1996) found that, after 12 brassica seed lots infected with *X. campestris* pv. *campestris* were immersed in 0.525% NaOCl for 5 min, the pathogen was not detected in 8 of the seed lots with pathogen counts ≤5.3 x 10^6 CFU/100 seed. The remaining 4 seed lots had greater pathogen counts, ≥ 9.0 x 10^6 CFU/10,000 seeds, and required a longer, 15 min treatment in which *X. campestris* pv. *campestris* was reduced but not eradicated.

Significantly fewer mustard seed developed sporulation of *P.* cf. *brassicae* in the seed health assay for all 10 fungicide seed treatments evaluated in this study, including the dye control seed,

compared to the non-treated control seed. Likewise, seed transmission of *P.* cf. *brassicae* was significantly greater from the non-treated control seed than from seed with any of the 10 fungicide treatments evaluated and for the seed treated with dye only. Coronet, Dynasty, Helix Vibrance, and Mertect 340F completely prevented seed transmission of *P.* cf. *brassicae* from the mustard seed lot in both trials. If results of both the seed health assay and the seed transmission assay are considered, Coronet, Helix Vibrance, and Mertect 340F appeared to be the most efficacious seed treatments for *P.* cf. *brassicae*. In contrast, the following fungicide seed treatments, each applied with the proprietary dye, did not differ from the seed treated with dye alone in terms of both the percentage of seed infected with *P.* cf. *brassicae* and the rate of seed transmission of *P.* cf. *brassicae*: Farmore FI400, Rovral 4F Flowable, and Vibrance in Trial 1; and Maxim 4FS and Obvius in Trial 2. To try and understand the potential effect of the dye on *P.* cf. *brassicae* on mustard seed samples of the mustard seed were tested using the 4°C NP-10 agar seed health assay and the 4°C freeze blotter seed health assay, with and without dye on the seed. Fewer seed treated with the dye developed conidiomata of *P.* cf. *brassicae* than seed without dye, regardless of the seed health assay used, i.e., the dye alone limited the development of *P.* cf. *brassicae* on mustard seed, supporting results of the fungicide seed treatment trials. The primary purpose of the dye (polymer seed coating) was to ensure the active ingredient(s) in each treatment or product adhered to the seed. However, polymer seed coatings are used for other agricultural purposes based on the fact that polymer seed coatings can impact the physiology of seed, e.g., in winter canola (*B. napus*) crops to delay germination of seed, allowing growers to avoid difficult, late winter seeding conditions (Clayton et al. 2004). Interestingly, *P.* cf. *brassicae* was detected on 67% fewer non-treated seed with the 4°C freeze blotter assay than with the 4°C NP-10 agar assay, but, there was no difference in the incidence of the dye-treated seed on which *P.* cf. *brassicae* developed with the two assays. A previous study comparing NP-10 and freeze blotter assays for detection and quantification of the incidence of spinach seed infected with *Verticillium dahliae* in spinach did not find significant

differences between the two seed health assays, except when seed had been treated with fungicides (du Toit 2011). This reflected the fact that fungicides can readily diffuse into agar media compared to blotters diminishing the efficacy of the fungicide(s) against particular fungi, and resulting in increased levels of pathogen detection (International Seed Health Initiative-Veg. 2015).

The FRAC groups of the active ingredients (a.i.'s) in the most efficacious fungicide seed treatments evaluated in this study included methyl benzimidazole carbamates (FRAC group 1), demethylation inhibitors (also called triazoles or imidazoles, FRAC group 3), succinate dehydrogenase inhibitors (carboxamides, FRAC group 7), quinone outside inhibitors (strobilurins, FRAC group 11), and phenylpyrroles (FRAC group 12). Coronet, which contains pyraclostrobin (FRAC group 11) and boscalid (FRAC group 7), was registered in 2017 in Washington State as a seed treatment for canola seed, stock seed for brassica vegetable seed crops, and seed for brassica vegetable crops (Pesticide Information Center Online 2016). This was one of the three most effective fungicide seed treatments evaluated in this study for control of seedborne *P.* cf. *brassicae*. In addition, Coronet has also been demonstrated to be highly effective for control of seedborne *P. lingam* (du Toit et al. 2005; du Toit et al. 2006; Ocamb 2015). However, Obvious, which also has a.i.'s in FRAC groups 7 (fluxapyroxad) and 11 (pyraclostrobin), was not as effective at preventing development of *P.* cf. *brassicae* on the seed and preventing seed transmission of *P.* cf. *brassicae* as Coronet. This suggests boscalid has greater efficacy than fluxapyroxad against *P.* cf. *brassicae,* despite being in the same FRAC group. Furthermore, treatment of seed with Vibrance (a.i. sedaxane, FRAC group 7) did not prevent development of *P.* cf. *brassicae* on seed in either trial, or prevent seed transmission in one of two fungicide trials. The active ingredient azoxystrobin in Dynasty (FRAC group 11) did not completely prevent *P.* cf. *brassicae* from developing on seed, but did prevent seed transmission in both trials. Individual a.i.'s could be evaluated in future studies in order to understand the efficacy of specific products in FRAC groups 7 and 11 for the management of seedborne *P.* cf. *brassicae.*

Helix Vibrance has four fungicide a.i.'s: difenoconazole (FRAC group 3), fludioxonil (FRAC group 12), metalaxyl (M and S isomers) (FRAC group 4), and sedaxane (FRAC group 7); as well as the insecticide thiamethoxam [Insecticide Resistance Action Committee (IRAC) group 4]. Therefore, although this fungicide seed treatment was one of the three most effective against *P.* cf. *brassicae* in this study, it was not possible to assess the relative contribution of the four a.i.'s against *P.* cf. *brassicae,* except by comparing results of this product with the results for other seed treatments that included some of the same a.i.'s. Vibrance also contains sedaxane, but was no more effective against *P.* cf. *brassicae* on mustard seed than the dye control treatment, suggesting that sedaxane was not a major component of Helix Vibrance that prevent seed transmission of *P.* cf. *brassicae*. The metalaxyl isomers in Helix Vibrance are only effective against oomycetes, not ascomycetes such as *P.* cf. *brassicae* (Mueller et al. 2013). Fludioxonil (FRAC group 12) is in Helix Vibrance, Maxim 4FS, and Farmore FI400. The latter two had intermediate efficacy overall against seedborne *P.* cf. *brassicae* in this study. Difenoconazole has been demonstrated to be highly effective for control of light leaf spot when used as foliar applications in rapeseed crops in the UK (McCartney and Doughty 2007) and, therefore, probably accounted for at least some of the highly efficacious results observed in this study with Helix Vibrance. As of 2017, Helix Vibrance was only registered in Washington State as a seed treatment for canola production, i.e., additional registrations will be required to use this product on other types of brassica seed. Although Metlock also has a FRAC group 3 a.i., metconazole, and demonstrated good efficacy against seedborne *P.* cf. *brassicae,* seed treatment with this fungicide caused phytotoxicity to the mustard cultivar used in this study. Mertect 340F, with the a.i. thiabendazole (FRAC group 1), was registered as a seed treatment in Washington State in 2016 for use on canola seed, stock seed for brassica vegetable seed crops, and seed for brassica vegetable crops (Pesticide Information Center Online 2016). This was one of the three most efficacious fungicide seed treatments against seedborne *P.* cf. *brassicae*. For many years, researchers have demonstrated that methyl benzimidazole carbamates (MBCs) (FRAC group 1) (Cheah

et al. 1981; Sutherland 2001) and demethylation inhibitors (FRAC group 3) [Agriculture and Horticulture Development Board (AHDB) Cereals & Oilseeds Fungicide Project 2015; McCartney and Doughty 2007] are effective against *P.* cf. *brassicae*, so the results with Helix Vibrance and Mertect 340F seed treatments in this study were not surprising.

Resistance of isolates of *P.* cf. *brassicae* to benzimidazole fungicides (FRAC group 1) was reported by Carter et al. (2013) in the UK, and by Karolewski and Kosiada (2010) in Poland. Fungicide resistance has led to increasing brassica crop losses in the UK to light leaf spot in recent years (Rothamsted Research 2017). Fungicide resistance detected in the EU is to fungicides in FRAC groups 1 and 3 (Carter et al. 2013), i.e., the FRAC groups in fungicide seed treatments that were the most effective against *P.* cf. *brassicae* in this study. However, preliminary research on sensitivity of isolates of *P.* cf. *brassicae* from the USA to carbendazim, a widely used, broad-spectrum benzimidazole fungicide, has shown that none of 10 USA isolates tested to date had the key mutations known to confer resistance to carbendazim, including 3 isolates from Oregon and 7 isolates from Washington (K. King, Rothamsted Research, *personal communication*). While this is helpful information for brassica growers in the USA to know they can use fungicides in FRAC group 1 for control of light leaf spot, it is a valuable reminder that effective cultural control practices, rotation of fungicides with different FRAC groups, and use of appropriate fungicide application rates are very important to keep fungicides in FRAC group 1 and 3 efficacious in the USA by minimizing the risk of isolates of *P.* cf. *brassicae* developing resistance to these fungicides in this region (Mueller et al. 2013). Using efficacious fungicide seed treatments can prevent the introduction into the USA and other areas of new isolates of *P.* cf. *brassicae*, and limit the risk of fungicide resistance developing because seed treatments expose the pathogen to a fraction of the fungicide compared to foliar applications, and many seed treatments consist of combinations of a.i.'s from multiple FRAC groups, such as Coronet, Helix Vibrance, etc.

Phytotoxic effects were not detected from the fungicide seed treatments or the chlorine seed treatments evaluated in this study based on the incidences of normally germinated seed, non-germinated seed, abnormally germinated seed, and/or rotten seed in the seed germination assay. However, stunted seedlings with darker green cotyledons developed from seed treated with Metlock compared to seedlings that developed from seed treated with any of the other fungicide treatments and from control seed in the seed transmission assay. For all fungicide seed treatments evaluated in this study, the highest labeled rate of application to seed was used to assess the efficacy against *P.* cf. *brassicae*. Evaluating lower labeled rates of application for Metlock (e.g., as little as 29.3 μl/kg seed), could be informative to assess for reduced phytotoxicity to brassica seed while maintaining efficacy against *P.* cf. *brassicae*.

A limited degree of phytotoxicity also was observed for mustard seed treated with some of the hot water and steam treatments evaluated in this study. The incidence of normally germinated seed was reduced slightly, and the incidence of abnormally germinated seed was increased, when mustard seed was subjected to a pre-treatment of 40°C water for 10 min, followed by 30 min in water heated to 50°C in Trial 1, but these adverse effects were not detected in the repeat trial in which the preheat treatment at 40°C was excluded from the hot water treatment protocol. There was no difference detected among the two durations of hot water seed treatment for percentage seed infection or seed transmission of *P.* cf. *brassicae,* and both completely prevented seed transmission of the pathogen. Therefore, 15 min of seed treatment in 50°C water, without a 10 min preheat treatment at 40°C, was a highly effective, non-phytotoxic method of hot-water treating *P.* cf. *brassicae* -infected mustard seed. Seed germination was affected adversely by the hottest steam seed treatment evaluated, i.e., 71.1°C. This temperature of steaming also increased the incidence of abnormally germinated seed in Trial 1, and rotten seed in Trial 2. In summary, although hot water and steam seed treatments were highly efficacious at reducing seedborne *P.* cf. *brassicae* inoculum, both types of seed treatments can damage mustard (brassica) seed

if not carried out at precisely controlled temperatures for the appropriate durations. Nega et al. (2003) found hot water seed treatments to be very effective against *A. brassicicola* and *P. lingam* on cabbage seed, and that the treatment duration must be reduced if the hot water temperature is increased in order to avoid reducing seed germination. Similarly, Babadoost et al. (1996) found that the average cabbage seed germination rates ranging from 85 to 90% after chlorine seed treatments (0.52% NaOCl for up to 15 min) were greater than the seed germination rate, 80%, following hot water seed treatment at 50° for 20 min.

In a study of spring barley (*Hordeum vulgare*) seed infected with *Ustilago nuda,* minor changes in the combination of temperature and duration of steam seed treatments had a significant effect on seed germination, making it difficult to find the treatment window or the precise combination of steam seed treatment parameters that provided sufficient control of this seedborne pathogen (Finckh et al. 2015). Forsberg and Sanchez-Sava (2009) noted that the sensitivity of seed to aerated steam treatments varies among crops and even among seed lots of the same cultivars. This variation was demonstrated to be associated with seed moisture content, as seed lots with lower moisture content were less sensitive to steam treatment than seed lots with greater seed moisture content. Forsberg and Sanchez-Sava (2009) advised using pre-tests to find the best parameters for treatment of individual seed lots before treating entire seed lots (Forsberg et al. 2003).

The mustard seed lot used in this study was infected with other fungi besides *P.* cf. *brassicae*, mostly saprophytic species of *Alternaria* (Chapter 2). Therefore, the effects of the various seed treatments on fungi of this genus were recorded to assess the potential effects on other seedborne fungi of brassicas. *Alternaria* spp. were not detected in the seed health assays on any of the seed subjected to the hot water seed treatments. This is consistent with the literature on hot water treatments for small-seed vegetables, e.g., *A. dauci* and *A. radicina* on carrot seed, and *A. alternata* and *A. brassicicola* on brassica seed were controlled by hot water seed treatment at 50°C for 20 to 30 min;

and various *Alternaria* spp. were controlled on cabbage, carrot, celery (*Apium graveolens*), parsley (*Petroselinum crispum*), and lamb's lettuce (*Valerianella locusta*) seed by hot water seed treatment at 53°C for 10 to 30 min (Nega et al. 2003). For the range of steam treatments evaluated in this study, *Alternaria* spp. were detected in the seed health assay on only one seed at each of the 62.8, 65.6, and 68.3°C steam treatments in the first trial, but were not detected on seed for any of the steam temperatures evaluated in the repeat trial. Baker (1969) found aerated-steam to be effective at reducing the percentage of cabbage seed infected with *A. brassicae* when steam was applied at 56°C for 30 min. For the chlorine seed treatment durations evaluated in this study, *Alternaria* spp. were detected on seed treated for 10, 20, 30, and 40 min in 1.2% NaOCl (≤4.75% for all chlorine-treated seed compared to ≥8.00% for the non-treated control seed). du Toit and Hernandez-Perez (2005) similarly found more chlorine-treated spinach seed to be infected with *Alternaria* spp. compared to hot water-treated spinach seed of the same lots. Results of the seed treatments evaluated in this study for control of seedborne *Alternaria* are consistent with previous work on various *Alternaria* spp. on brassicas (Maude et al. 1984). All 10 of the fungicide seed treatments evaluated reduced the incidence of seed infected by *Alternaria* spp., from 33.75% of the control seed to <1.75%, including seed treated only with the dye. The seed treatment labels for Coronet, Dynasty, Helix Vibrance, Obvius, and Rovral 4F Flowable each state efficacy of the product against *Alternaria,* which the results of this study supported. If growers or seed companies are treating brassica seed with fungicides for control of seedborne infection by *A. brassicae* or *A. brassicicola*, the treatments might also help manage potential *P.* cf. *brassicae* infection if the products contains a.i.'s in FRAC groups 1, 3, 7, 11, and/or 12.

Environmental conditions can have an important influence on seed transmission of plant pathogens and subsequent disease development (Neergaard 1977). Previous studies have found that 16°C is optimal for conidial germination of *P.* cf. *brassicae*, and 15 to 20°C is optimal for mycelial growth of this fungus (Hartill and Cheah 1984; Rawlinson et al. 1978; Staunton 1967; Sutherland 2001). In a

greenhouse with 80% RH, the severity of light leaf spot averaged 45% on cauliflower (*B. oleracea* var. *botrytis*) plants maintained at 18°C, whereas symptoms of light leaf spot did not develop on plants held at 80% RH and 10, 22, or 26°C (Hartill and Cheah 1984), i.e., a temperature difference as little as 4°C (18 vs. 22°C) had a major influence on the development of light leaf spot. The seed transmission trials in this study consistently had cool temperatures (14.74 to 16.73°C), relatively long periods of leaf wetness (7.37 to 13.30 min/h) and high RH (71.20 to 94.30%) that should have been favorable for light leaf spot development (Hartill and Cheah 1984; Rawlinson et al. 1978; Staunton 1967). A study in the UK (Gilles et al. 2001a) and a study in Poland (Karolewski et al. 2002) found ideal conditions for *P. brassicae* infection on oilseed rape plants to be 80% RH for 24 to 48 h. Maddock and Ingram (1981) recorded an average incubation period of 3 weeks before light leaf spot symptoms were observed when infection of oilseed rape occurred at optimum temperatures and RH. The seed transmission assay for the first mustard steam treatment trial in this study was conducted in a different greenhouse than the seed transmission assays for all of the other seed treatment trials. Symptoms of light leaf spot did not develop on the cotyledons, only on the first true leaves, in the first assay, whereas distinct symptoms were observed on both the cotyledons and first true leaves in all the other seed transmission assays. Additionally, in the seed transmission assay for the second steam treatment trial, the rate of seed transmission of *P.* cf. *brassicae* was greater (2.88% compared to 1.50%), and the duration after planting when symptoms were first observed was shorter (16 to 29 dap vs. 33 to 39 dap), than for the first steam treatment trial. The 2- to 4-week incubation period detected for light leaf spot in the seed transmission assays conducted in the second greenhouse was consistent with the average incubation period of 3 weeks for *P. brassicae* reported by Maddock and Ingram (1981) on oilseed rape. The slight variations in microclimate in the two greenhouses used in this study, as well as other aspects of the trials, impacted the seed transmission rates of *P.* cf. *brassicae* and expression of light leaf spot symptoms, e.g., greenhouse lighting and time of

the year (latter has a significant influence on day length and radiation intensity at the latitude at which the greenhouse is located, 48°26'20.9").

This study demonstrated that effective organic and conventional seed treatments are available that can be used to manage seedborne *P.* cf. *brassicae*. Some of these treatments are used currently to treat brassica seed lots for other seedborne pathogens, e.g., *P. lingam, A. brassicae,* and *A. brassicicola*. For example, organic brassica seed growers in the Pacific Northwest, and seed companies selling organic brassica seed for planting in regulated areas of Oregon and Washington States, rely on hot water seed treatment to control seedborne *P. lingam* (F. Morton, Wild Garden Seed, *personal communication;* T. Stearns, High Mowing Seed Co., *personal communication*). Gabrielson et al. (1977) also found benomyl, benzimidazole fungicide, to be highly effective against *P. lingam.* This fungicide was the standard seed treatment used to manage *P. lingam* on brassica seed in the USA until all agricultural uses of benomyl were terminated in 2006 (Environmental Protection Agency 2002). For growers of conventional brassica crops, Coronet and Mertect 340F were subsequently identified as efficacious replacements for benomyl as seed treatments for *P. lingam* (du Toit et al. 2005; du Toit et al. 2006) and were labelled for use as seed treatments in canola crops, brassica seed crops, and brassica vegetable crops in both states in 2016 (Pesticide Information Center Online 2016). The Oregon Department of Agriculture recommended Coronet, Mertect 340F, Rovral 4F Flowable, or hot water as seed treatments to control seedborne *P. lingam* (Ocamb 2016). Helix Vibrance, which was very effective against *P.* cf. *brassicae* in this study, is labeled to control seedborne *P. lingam* on canola crops only. Potentially, this registration could be expanded to cover seed used to plant additional types of brassica crops, such as cover crops, brassica vegetable seed crops, and cover crop seed crops. None of the fungicide seed treatments evaluated in this study was registered for use as a seed treatment for planting brassica cover crops in Oregon or Washington in 2017, representing an important gap in seed treatment options for brassica growers in

this region, particularly given the recent rapid increase in cover cropping in the USA based on the numerous benefits of cover crops (Watts 2016).

A major limitation of this study was that the efficacy of seed treatments for control of seedborne *P.* cf. *brassicae* was evaluated using only one seed lot of one *Brassica* species, *B. juncea*. It will be important to expand this research to look at *P.* cf. *brassicae* -infected seed lots of multiple *Brassica* species of different cultivars, and to assess the seed treatment efficacy under field conditions in different regions where brassica crops are grown. Light leaf spot is a newly introduced disease to the USA, and as the potential effects of this disease on the diverse brassica cropping systems in the Pacific Northwest become evident, the potential risks associated with *P.* cf. *brassicae* -infected seed, and the importance of using effective seed treatments, will become apparent.

4.5 Literature Cited

Agarwal, V. K., and Sinclair, J. B. 1997. Principles of Seed Pathology, 2nd Ed. CRC Press, Boca Raton, FL.

Agostini, A., Johnson, D. A., Hulbert, S., Demoz, B., Fernando, W. G. D., and Paulitz, T. 2013. First report of blackleg caused by *Leptosphaeria maculans* on canola in Idaho. Plant Dis. 97:842.

Agriculture and Horticulture Development Board (AHDB) Cereals & Oilseeds Fungicide Project. 2015. Fungicides for light leaf spot control in winter oilseed rape: 2015 Summary. Warwickshire, UK.

American Seed Trade Association. 2017. The guide to seed treatment stewardship: Seed treatment application. www.seed-treatment-guide.com [Accessed on 17 April 2017].

Babadoost, M., Derie, M. L., and Gabrielson, R. L. 1996. Efficacy of sodium hypochlorite treatments for control of *Xanthomonas campestris* pv. *campestris* in Brassica seeds. Seed Sci. Technol. 24:7-15.

Baker, K. F. 1962. Principles of heat treatment of soil and planting material. J. Instit. Agric. Sci. 28:118-126.

Baker, K. F. 1969. Treatment of seed and planting material. Pages 25-29 in: New South Wales (Australia) Association of Nurserymen's Seminar, 26-27 February 1969, New South Wales, Australia.

Cappelli, C., Buonaurio, V., and Paul, V. H. 1999. Traditional and innovative methods to detect seed-transmitted pathogens in linseed and rape. Pages 55–61 In: Integrated Control of Oilseed Crops. V.H. Paul, I. Foller, N. Evans, and I. Williams, eds. International Organisation for Biological and Integrated Control (IOBC), Prague.

Carter, H. E., Cools, H. J., West, J. S., Shaw, M. W., and Fraaije, B. A. 2013. Detection and molecular characterisation of *Pyrenopeziza brassicae* isolates resistant to methyl benzimidazole carbamates. Pest Manag. Sci. 69:1040–1048.

Cheah, L. H., Corbin, J. B., and Hartill, W. F. T. 1981. Control of light leaf spot of brassicas (*Pyrenopeziza brassicae* Sutton & Rawlinson) with fungicides. N. Z. J. Agric. Res. 24:391–395.

Clayton, G. W., Harker, K. N., O'Donovan, J. T., Blackshaw, R. E., Dosdall, L., Stevenson, F. C., Johnson, E. N., and Ferguson, T. 2004. Polymer seed coating of early- and late-fall-seeded herbicide-tolerant canola (*Brassica napus* L.) cultivars. Can. J. Plant Sci. 84:971-979.

Cook, A. A., Larson, R. H., and Walker, J. C. 1952. Relation of the black rot pathogen to cabbage seed. Phyopathology 42:316-320.

du Toit, L.J. 2011. ISHI Spinach Verticillium Ring Test. Method validation report. International Seed Health Initiative – Vegetable (ISHI – Veg), ISF. 6 pp.

du Toit, L. J., Derie, M. L., and Morrisen, R. H. 2005. Evaluation of fungicide seed treatments for control of black leg of cauliflower, 2004. Fung. Nemat. Tests 60:ST011.

du Toit, L. J., Derie, M. L., and Brissey, L. M. 2006. Evaluation of fungicides for control of black leg of cabbage, 2005. Fung. Nemat. Tests 61:VO41.

du Toit, L. J., and Hernandez-Perez, P. 2005. Efficacy of hot water and chlorine for eradication of *Cladosporium variable*, *Stemphylium botryosum*, and *Verticillium dahliae* from spinach seed. Plant Dis. 89:1305-1312.

Environmental Protection Agency. 2002. Benomyl; Proposed Revocation of Tolerances. Fed. Regist. 67:1917–1925.

Farr, D. F, and Rossman, A. Y. 2017. Fungal Databases, U.S. National Fungus Collections, Agricultural Research Service, United States Department of Agriculture. https://nt.ars-grin.gov/fungaldatabases. [Accessed on 24 April 2017].

Finckh, M. R., van Bruggen, A. H. C., and Tamm, L. 2015. Plant Diseases and Their Management in Organic Agriculture. American Phytopathological Society, St. Paul, MN.

Forsberg, G., and Sanchez-Sava, V. 2009. ThermoSeed treatment – A novel disinfection technology for vegetable seeds. Proc. Symp. Seed Prod. Treat. Changing Environ. British Crop Protection Council, 24-25 February, 2009, Alton, UK.

Forsberg, G., Kristensen, L., Eibel, P., Titone, P., and Hartl, W. 2003. Sensitivity of cereal seeds to short duration treatment with hot, humid air. J. Plant Dis. Prot. 110:1-16.

Freeman, E. M., and Johnson, E. C. 1909. The loose smuts of barley and wheat. U. S. Dep. Agric. Washington, Bur. Pl. Industry Bull. 152:48.

Gabrielson, R. L., Mulanax, M. W., Matsuoka, K., Williams, P. H., Whiteaker, G. P., and Maguire, J. D. 1977. Fungicidal eradication of seedborne *Phoma lingam* of crucifers. Plant Dis. Rep. 61:118-121.

Gilles, T., Fitt, B. D., and Jeger, M. J. 2001. Effects of environmental factors on development of *Pyrenopeziza brassicae* (light leaf spot) apothecia on oilseed rape debris. Phytopathology 91:392–398.

Hartill, W. F. T., and Cheah, L.-H. 1984. Some effects of climate and plant growth on the development of light leaf spot in cauliflowers. N. Z. J. Agric. Res. 27:441–449.

Inglis, D. A., du Toit, L.J., and Miller, T. W. 2013. Production of Brassica Seed Crops in Washington State: A Case Study on the Complexities of Coexistence. Washington State Univ. Ext. Man. EMO062E.

International Seed Health Initiative-Veg. 2011. Methods for the detection of *Phoma lingam* on *Brassica* spp. seed. International Seed Federation, Zurich. http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/ [Accessed on 26 April 2017].

International Seed Health Initiative-Veg. 2015. Methods for the detection of *Verticillium dahliae* on spinach seed. International Seed Federation, Zurich. http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/ [Accessed on 31 March 2017].

International Seed Testing Association. 2017. Seed Health Methods. International Seed Federation, Zurich. https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html [Accessed on 28 April 2017].

Jacobsen, B. J., and Williams, P. H. 1971. Histology and control of *Brassica oleracea* seed infection by *Phoma lingam.* Plant Dis. Rep. 55:10.

Jahn, M., Koch, E., Blum, H., Nega, E., and Wilbois, K. P. 2007. Guideline for seed health in organic farming – vegetable crops. Forschungsinstitut fur biologischen Landbau e.V., Frankfurt. http://orgprints.org/11675/ [Accessed on 14 May 2017].

Jenkins, D. 2015. WSDA extends battle against black leg to E. Washington. Cap. Press. 23 July 2015. http://www.capitalpress.com/Washington/20150723/wsda-extends-battle-against-black leg-to-e-washington.

Karolewski, Z., and Kosiada, T. 2010. Sensitivity of *Pyrenopeziza brassicae* (light leaf spot) populations to fungicides recommended for the fungus control. Phytopathologia 56:59–65.

Karolewski, Z., Evans, N., Fitt, B. D. L., Todd, A. D., and Baierl, A. 2002. Sporulation of *Pyrenopeziza brassicae* (light leaf spot) on oilseed rape (*Brassica napus*) leaves inoculated with ascospores or conidia at different temperatures and wetness durations. Plant Pathol. 51:654–665.

Knox-Davies, P. S. 1979. Relationship between *Alternaria brassicicola* and *Brassica* seeds. Trans. Brit. Myco. Soc. 73:235-248.

Maddock, S. E., and Ingram, D. S. 1981. Studies of survival and longevity of the light leaf spot pathogen of Brassicas, *Pyrenopeziza brassicae*. Trans. Br. Mycol. Soc. 77:153–159.

Maude, R. B. 1996. Seedborne Diseases and their Control: Principles and Practice. Centre for Agriculture and Biosciences, Warwick, UK.

Maude, R. B., Humpherson-Jones, F. M., and Shuring, C. G. 1984. Treatments to control *Phoma* and *Alternaria* infections of brassica seeds. Plant Pathol. 33:525-535.

McCartney, H. A., and Doughty, K. J. 2007. Light leaf spot. Pages 31-35 In: Compendium of Brassica Diseases. S. Roger Rimmer, V. I. Shattuck, and L. Buchwaldt, eds. American Phytopathological Society, St. Paul, MN.

Miller, S., and Lewis Ivey, M. 2005. Hot water treatment of vegetable seeds to eradicate bacterial plant pathogens in organic production systems. Extension FactSheet HYG-3086-05, Ohio State University, Columbus, OH.

Morton, F., Stearns, T., and Andrews, N. 2016. Small-Scale Cost-Effective Hot Water Seed Treatment Presentation. Seminar presented at Washington State University Mount Vernon Northwestern Washington Research and Extension Center on 2 February 2016 http://mtvernon.wsu.edu/path_team/Small-scale%20Hot%20Water%20Treatment%202016-02-29.pdf [Accessed on 30 April 2017].

Mueller, D. S., Wise, K. A., Dufault, N. S., Bradley, C. A., and Chilvers, M. I. 2013. Fungicides for Field Crops. American Phytopathological Society, St. Paul, MN.

Navaratnam, S. J., Shuttleworth, D., and Walker, D. 1980. The effect of aerated steam on six seed-borne pathogens. Austral. J. Exper. Agric. Animal Husb. 20:97-101.

Neergaard, P. 1977. Seed Pathology, Vol. 1. Halsted Press, New York.

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.

Ocamb, C. M. 2014a. Disease Alert – Light Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/Light%20leaf%20spot%20OSU%20Disease%20Alert%20in%20crucifers%2017%20June.pdf [Accessed on 12 December 2016]

Ocamb, C. M. 2014b. Disease Alert – White Leaf Spot in Crucifer Seed Fields in the Willamette Valley. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/White%20leaf%20spot%20OSU%20Disease%20Alert%20in%20Brassiceae.pdf [Accessed on 12 December 2016].

Ocamb, C. M. 2016. A Clinic Close-Up: Black leg, light leaf spot, and white leaf spot in western Oregon. Oregon State University, Corvallis, OR. https://pnwhandbooks.org/sites/pnwhandbooks/files/insect/document/turnip-rutabaga-brassica-sp-light-leaf-spot/black-leg-light-leaf-spot-white-leaf-spot-crucifers16.pdf [Accessed on 12 December 2016].

Ocamb, C. M. 2015. A Clinic Close-Up: Management of black leg in Oregon on Vegetable *Brassica* crops and seed crops. Oregon State University, Corvallis, OR. http://mtvernon.wsu.edu/path_team/Clinic%20closeup%20Black%20leg%20management%20in%20Brassica%20vegetable%20seed%20Jan%202015%20version%20II.pdf [Accessed 11 May 2017].

Ocamb, C. M., Mallory-Smith, C., Thomas, W. J., Serdani, M., Putman, M. 2015. New and re-emerging fungal pathogens affecting Brassicaceae plants in western Oregon: black leg, light leaf spot, and white leaf spot. Phytopathology 105:S4.103. (Abstract).

Paulitz, T. C., Knerr, A. J., Carmody, S. M., Schlatter, D., Sowers, K., Derie, M., and du Toit, L. J. 2017. First report of *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, causal agents of black leg, on canola in Washington State. Plant Dis. 101:504.

Pesticide Information Center Online. 2016. Washington State University, Pullman WA. http://cru66.cahe.wsu.edu/labels/Labels.php?SrchType=C. [Accessed on 12 April 2017].

Pscheidt, J. W., and Ocamb, C. M. 2016. Pacific Northwest Plant Disease Management Handbook. http://pnwhandbooks.org/plantdisease. Oregon State University, Corvallis, OR.

Rawlinson, C. J., Sutton, B. C., and Muthyalu, G. 1978. Taxonomy and biology of *Pyrenopeziza brassicae* sp. nov. (*Cylindrosporium concentricum),* a pathogen of winter oilseed rape *(Brassica napus* spp. *oleifera*). Trans. Br. Mycol. Soc. 71:425-439.

Richardson, M. L. 1990. An Annotated List of Seed-Borne Diseases, 4th Ed. International Seed Testing Assoc., Zurich.

Rimmer, S. R., Shattuck, V. I., and Buchwaldt, L., Editors. 2007. Compendium of Brassica Diseases. American Phytopathological Society, St. Paul, MN.

Rothamsted Research. Historical Trends - Light Leaf Spot. http://rothamsted.ac.uk/light-leaf-spot-forecast/historical-trends-light-leaf-spot [Accessed on 1 January 2017].

Staunton, W. P. 1967. Studies on light leaf spot (*Gloeosporium concentricum*) of Brassica. Irish J. Agric. Res. 6:203–211.

Sutherland, K. G. 2001. Technical note: light leaf spot in winter oilseed rape. The Scottish Agricultural College, Edinburgh.

United States Department of Agriculture National Organic Program. 2017. National Organic Program Handbook. Washington, D.C. www.ams.usda.gov/rules-regulations/organic/handbook [Accessed on 15 May 2017].

Washington State Department of Agriculture. 2015. Crucifer Seed Quarantine. http://agr.wa.gov/Inspection/SeedInspection/docs/478-CruciferSeedQuarantineFactSheet-Web.pdf. [Accessed on 1 January 2017].

Watts, C. 2016. Annual Report. 2015-2016 Cover Crop Survey. Sustainable Agriculture Research and Education/Conservation Technology Information Center, College Park, MD.

Williams, P. H. 1980. Black rot: a continuing threat to world crucifers. Plant Dis. 64:736–742.

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 4.1. Fungicide seed treatments evaluated for efficacy at eradication of *Pyrenopeziza* cf. *brassica* on mustard (*Brassica juncea*) seed[a]

	Active ingredient					
Product	**Fungicide**	**Concentration**	**FRAC group[b]**	**Registrant**	**EPA No.[c]**	**Brassica crop registration in Washington State[d]**
Coronet	Pyraclostrobin +	9.0%	11	BASF Corp.	7969-274	Canola, seed, vegetable
	Boscalid	18.0%	7			
Dynasty	Azoxystrobin	9.6%	11	Syngenta	100-1159	Canola, seed, vegetable
Farmore FI400	Fludioxonil +	40.3%	12	Syngenta	100-758	Canola
	Azoxystrobin +	9.6%	11		100-1159	
	Mefenoxam +	33.3%	4		100-799	
	Thiamethoxam +	47.6%	4 IRAC		100-941	
Helix Vibrance	Difenoconazole +	16 g/liter	3	Syngenta	31454	Canola
	Fludioxonil +	1.7 g/liter	12			
	Metalaxyl-M and S isomers +	5.0 g/liter	4			
	Sedaxane +	3.4 g/liter	7			
	Thiamethoxam	269.0 g/liter	4 IRAC			
Maxim 4FS	Fludioxonil	40.3%	12	Syngenta	100-758	Canola, seed, vegetable
Mertect 340F	Thiabendazole	42.3%	1	Syngenta	100-889	Canola, seed, vegetable
Metlock	Metconazole	40%	3	Valent BioSciences LLC	59636-171	Canola
Obvius	Fluxapyroxad +	1.58%	7	BASF Corp.	7969-371	Canola
	Pyraclostrobin +	1.58%	11			
	Metalaxyl	95.58%	4			
Rovral 4 Flowable	Iprodione	41.6%	2	Bayer/Monsanto	264-482	Canola, seed
Vibrance	Sedaxane	43.7%	7	Syngenta	100-1374	Canola
Dye only control	Proprietary blue colorant	4% by seed weight	-	-	-	-
Non-treated control[e]	-	-	-	-	-	-

[a] Fungicide seed treatments approved for use on canola, brassica vegetables, and/or other brassica seed crops in 2016 in Washington State, USA (Pesticide Information Center Online 2016). Except for the non-treated control seed, all treatments included a proprietary blue colorant (Spectrum Polymer Coating, Pawne, Navi Mumbai, India) applied at 4.0% by seed weight (mixed 1:1 with water) to adhere the product(s) to the seed. Seed treated with the dye only served as the dye control treatment.

[b] The Fungicide Resistance Action Committee (FRAC) is an international organization developed to address the issue of fungicide resistance by distinguishing different fungicide groups based on fungicide modes of action; IRAC = Insecticide Resistance Action Committee (Mueller et al. 2013).

[c] The Environmental Protection Agency (EPA) number is assigned to pesticide products for identification purposes under USA environmental regulations.

[d] Brassica crops on which the fungicide was registered for use as a seed treatment in 2016 in Washington State, USA (Pesticide Information Center Online 2016). Seed crops include any brassica grown as oilseed, vegetable seed, or cover crop seed production.

[e] The non-treated control seed was not treated with fungicides or dye.

Table 4.2. Analyses of variance for a seed germination assay, seed health assay, and seed transmission assay of mustard (*Brassica juncea*) seed infected with *Pyrenopeziza* cf. *brassicae* and treated with chlorine (1.2% NaOCl) for durations ranging from 0 to 40 minutes[a]

	Trial 1				Trial 2			
	Mean square (*P* > F)				Mean square (*P* > F)			
Dependent variable[b]	**Replication**	**Treatment duration**	**CV (%)**	**R^2**	**Replication**	**Treatment duration**	**CV (%)**	**R^2**
Seed germination assay (% seed)								
Normally germinated[c]	0.087	0.062	1.76	0.630	1.000	0.611	53.47	0.188
Non-germinated seed	0.187	0.056	67.41	0.602	0.362	0.562	95.69	0.355
Abnormally germinated	0.291	0.194	86.07	0.487	0.261	0.523	146.59	0.398
Rotten seed	0.077	0.127	95.24	0.596	0.553	0.722	390.83	0.263
Seed health assay (% seed infected)[d]								
P. cf. *brassicae*	1.000	<0.001	13.18	0.951	1.000	<0.001	13.18	0.951
Alternaria spp.	0.323	0.001	52.89	0.772	1.000	0.002	30.99	0.731
Seed transmission assay (% seedlings infected)[e]								
P. cf. *brassicae*	1.000	<0.001	24.06	0.784	1.000	<0.001	14.43	0.902

[a] Each trial was set up as a randomized complete block design with four replications of each duration of treatment in 1.2% NaOCl (0, 10, 20, 30, and 40 min). CV = coefficient of variation. R^2 = coefficient of determination for the analysis of variance.

[b] Assumptions of homogeneous variances and normally distributed residuals were met for parametric analysis for each dependent variable except percentage *P.* cf. *brassicae* seed infection in Trials 1 and 2, percentage seed transmission in Trials 1 and 2, normal seed germination in Trial 2, and percentage of seed infected with *Alternaria* spp. in Trial 2, which were each analyzed using Friedman's non-parametric rank test because of heterogeneous variances. The data for rotten seed in Trials 1 and 2 were log-transformed to generate homogeneous variances.

[c] Four replications of 100 seed for each treatment were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[d] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) and incubated at 4°C for five weeks in the dark.

[e] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in flats in a greenhouse, and each seedling that developed was examined daily for symptoms of light leaf spot, as described in the main text.

Table 4.3. Efficacy of chlorine seed treatments for eradication of *Pyrenopeziza* cf. *brassicae* from a mustard seed lot[v]

Trial and treatment[w]	Germination assay (% of seed)[x]				Seed health assay (% seed infected)[y]		Seed transmission assay (% seedlings infected with *P.* cf. *brassicae*)[z]	Days after planting symptoms observed
	Normally germinated	Non-germinated	Abnormally germinated	Rotten	*P.* cf. *brassicae*	*Alternaria* spp.		
Trial 1								
0	98.00	0.25	1.00	0.75	16.50 a	8.50 a	3.88 a	19 to 29
10	97.50	1.00	0.75	0.75	4.50 b	2.25 b	0.25 b	24 to 26
20	95.00	1.75	1.00	2.25	3.25 b	2.75 b	0.13 b	29
30	94.75	2.25	1.00	1.75	1.00 c	3.50 b	0.00 b	-
40	95.75	2.50	0.00	2.00	0.50 c	1.00 b	0.00 b	-
LSD (*P* <0.05)	NS	NS	NS	NS (Log)	Rank	2.93	Rank	
Trial 2								
0	97.25	0.75	0.00	2.00	12.50 a	8.00 a	3.50 a	15 to 17
10	98.25	0.50	0.50	0.75	3.25 b	4.75 ab	0.00 b	-
20	96.50	1.50	1.00	1.00	4.00 b	2.50 cd	0.00 b	-
30	96.50	1.00	0.75	1.75	1.25 c	3.50 bc	0.13 b	21
40	98.00	0.75	0.50	0.75	0.75 c	1.25 d	0.00 b	-
LSD (*P* <0.05)	NS (Rank)	NS	NS	NS (Log)	Rank	Rank	Rank	

[v] Original means are presented. Means separations were calculated using Fisher's protected least significant difference (LSD) at *P* <0.05. Data for the incidence of seed infected with *P.* cf. *brassicae* in the seed health assay and the incidence of seedlings infected in the seed transmission assay in both trials, normally germinated seed in Trial 2, and incidence of seed infected with *Alternaria* spp. in Trial 2 were subjected to Friedman's non-parametric rank test (Rank) because the data could not be normalized and/or variances made homogeneous. Data for the incidence of rotten seed in Trials 1 and 2 were log transformed (Log) to meet assumptions for parametric analysis. Means followed by different letters within a column for each trial are significantly different.

[w] Seed were treated for the appropriate duration in 1.2% NaOCl, triple-rinsed in sterilized, deionized water, and dried as described in the main text. The entire trial was repeated (Trial 2).

[x] Four replications of 100 seed for each lot were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[y] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) in square acrylic boxes (32 to 34 seeds/box) and incubated at 4°C for five weeks in the dark.

[z] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in 72-cell flats in a greenhouse, and the seedlings that developed were observed daily for symptoms of light leaf spot.

Table 4.4. Analyses of variance for a seed germination assay, seed health assay, and seed transmission assay of mustard (*Brassica juncea*) seed infected with *Pyrenopeziza* cf. *brassica* and treated with hot water (50°C) for 15 or 30 min[a]

	Trial 1				Trial 2			
	Mean square (*P* > F)				Mean square (*P* > F)			
Dependent variable[b]	Replication	Treatment Duration	CV (%)	R^2	Replication	Treatment Duration	CV (%)	R^2
Seed germination assay (% seed)								
Normally germinated[c]	1.000	0.023	29.76	0.717	0.608	0.132	2.67	0.564
Non-germinated seed	0.455	0.308	207.85	0.495	0.591	0.523	107.70	0.370
Abnormally germinated	0.566	0.008	50.34	0.815	1.000	0.053	27.95	0.625
Rotten seed	0.582	0.292	104.74	0.463	0.886	0.803	118.88	0.153
Seed health assay (% seed infected)[d]								
P. cf. *brassicae*	-	<0.001	0	1.000	1.000	<0.001	12.50	0.942
Alternaria spp.	-	<0.001	0	1.000	-	<0.001	0	1.000
Seed transmission assay (% seedlings infected)[e]								
P. cf. *brassicae*	-	<0.001	0	1.000	-	<0.001	0	1.000

[a] Each trial was set up as a randomized complete block design with four replications of each duration of treatment in hot water (50°C) for 15 min and 30 min. CV = coefficient of variation. R^2 = coefficient of determination for the analysis of variance.

[b] Assumptions of homogeneous variances and normally distributed residuals were met for parametric analysis for each dependent variable except for percentage *P.* cf. *brassicae* and *Alternaria* spp. seed infection in Trials 1 and 2, percentage seed transmission in Trials 1 and 2, total normal germination in Trial 1, and abnormal germination in Trial 2 which were analyzed using Friedman's non-parametric test (rank) because transformations did not generate homogenous variances and/or normality.

[c] Four replications of 100 seed for each treatment were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[d] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) and incubated at 4°C for five weeks in the dark.

[e] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in flats in a greenhouse, and each seedling that developed was examined daily for symptoms of light leaf spot, as described in the main text.

Table 4.5. Efficacy of hot water seed treatments for eradication of *Pyrenopeziza* cf. *brassicae* from a mustard seed lot[v]

Trial and treatment[w]	Germination assay (% of seed)[x]				Seed health assay (% seed infected)[y]		Seed transmission assay (% seedlings infected with *P.* cf. *brassicae*)[z]	Days after planting symptoms observed
	Normally germinated	Non-germinated	Abnormally germinated	Rotten	*P.* cf. *brassicae*	*Alternaria* spp.		
Trial 1								
0	98.00 a	0.25	1.00 b	0.75	16.50 a	8.50 a	3.88 a	19 to 29
15	95.75 ab	0.00	1.75 b	2.50	0.00 b	0.00 b	0.00 b	-
30	89.75 b	1.00	5.50 a	3.75	0.00 b	0.00 b	0.00 b	-
LSD (*P* <0.05)	Rank	NS	2.40	NS	Rank	Rank	Rank	
Trial 2								
0	97.25	0.75	0.00	2.00	12.50 a	8.00 a	3.50 a	15 to 17
15	96.75	0.50	1.50	1.25	0.25 b	0.00 b	0.00 b	-
30	93.25	1.25	3.25	2.25	0.00 b	0.00 b	0.00 b	-
LSD (*P* <0.05)	NS	NS	NS (Rank)	NS	Rank	Rank	Rank	

[v] Original means are presented. Means separations were calculated using Fisher's protected least significant difference (LSD) at *P* <0.05. Friedman's non-parametric test (rank) was used for the incidence of seed infected with *P.*cf. *brassicae* in the seed health assay and the incidence of seedlings infected in the seed transmission assay in both trials, and abnormal germination in Trial 2 because the data did not meet assumptions of normality and/or equal variances. Means followed by different letters within a column are significantly different.

[w] Seed were treated for the appropriate duration in a circulated water bath at 50°C for 15 min and 30 min, triple-rinsed in sterilized, deionized water, and dried as described in the main text. The entire trial was repeated (Trial 2).

[x] Four replications of 100 seed for each lot were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[y] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) and incubated at 4°C for six weeks in the dark.

[z] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in flats in a greenhouse, and the seedlings that developed were observed daily for symptoms of light leaf spot.

Table 4.6. Analyses of variance for a seed germination assay, seed health assay, and seed transmission assay of mustard (*Brassica juncea*) seed infected with *Pyrenopeziza brassica* and treated with steam for 90 s at 62.8, 65.6, 68.3, and 71.1°C vs. non-steamed control seed[a]

	Trial 1				Trial 2			
	Mean square (*P* > F)				Mean square (*P* > F)			
Dependent variable[b]	**Replication**	**Treatment temperature**	**CV (%)**	**R^2**	**Replication**	**Treatment temperature**	**CV (%)**	**R^2**
Seed germination assay (% seed)								
Normally germinated[c]	0.149	<0.001	2.18	0.813	0.231	0.009	1.80	0.695
Non-germinated seed	1.000	0.376	37.88	0.279	0.809	0.461	86.23	0.287
Abnormally germinated	1.000	0.004	29.85	0.699	0.120	0.445	121.72	0.482
Rotten seed	0.068	0.507	96.53	0.515	0.024	0.030	115.99	0.709
Seed health assay (% seed infected)[d]								
*P.*cf. *brassicae*	1.000	<0.001	14.43	0.902	-	<0.001	0	1.000
Alternaria spp.	1.000	0.001	24.77	0.759	-	<0.001	0	1.000
Seed transmission assay (% seedlings infected)[e]								
*P.*cf. *brassicae*	-	<0.001	0	1.000	1.000	<0.001	14.43	0.902

[a] Each trial consisted of a randomized complete block design with four replications of each temperature of steam treatment (62.8, 65.6, 68.3, and 71.1°C) and no steam. CV = coefficient of variation. R^2 = coefficient of determination.

[b] Friedman's non-parametric rank test was used for percentage seed infected with *P.*cf. *brassicae* and *Alternaria* spp. for both trials, percentage seed transmission in both trials, and non-germinated and abnormally germinated seed in Trial 1 because transformations did meet assumptions for parametric analysis. The data for rotten seed in Trial 2 was log-transformed (Log) to meet assumptions for parametric analysis.

[c] Four replications of 100 seed for each treatment were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[d] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) in square acrylic boxes (32 to 34 seeds/box) and incubated at 4°C for five weeks in the dark.

[e] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in flats in a greenhouse, and each seedling that developed was examined daily for symptoms of light leaf spot, as described in the main text.

Table 4.7. Efficacy of steam seed treatments for eradication of *Pyrenopeziza brassicae* from a mustard seed lot[v]

Trial and treatment[w]	Germination assay (% of seed)[x]				Seed health assay (% seed infected)[y]		Seed transmission assay (% seedlings infected with *P.*cf. *brassicae*)[z]	Days after planting symptoms observed
	Normally germinated	Non-germinated	Abnormal	Rotten	*P.*cf. *brassicae*	*Alternaria* spp.		
Trial 1								
Control	98.25 a	0.75	0.50 a	0.50	14.48 a	8.90 a	1.50 a	33 to 39
62.8°C	98.25 a	0.00	0.00 a	1.75	0.00 b	0.26 b	0.00 b	-
65.6°C	97.25 a	1.00	0.75 a	1.00	0.00 b	0.26 b	0.00 b	-
68.3°C	97.75 a	0.50	0.75 a	1.00	0.00 b	0.25 b	0.00 b	-
71.1°C	90.00 b	0.25	8.00 b	1.75	0.54 b	0.00 b	0.00 b	-
LSD (*P* <0.05)	2.231	NS (Rank)	Rank	NS	Rank	Rank	Rank	
Trial 2								
Control	97.75 a	1.00	0.25	1.00 b	10.50 a	4.75 a	2.88 a	16-29
62.8°C	95.00 b	1.50	0.75	2.75 ab	0.00 b	0.00 b	0.00 b	-
65.6°C	98.50 a	0.50	0.25	0.75 b	0.00 b	0.00 b	0.00 b	-
68.3°C	97.75 a	1.00	0.25	1.00 b	0.00 b	0.00 b	0.13 b	22
71.1°C	93.75 b	1.75	0.75	3.75 a	0.00 b	0.00 b	0.00 b	-
LSD (*P* <0.05)	2.680	NS	NS	Log	Rank	Rank	Rank	

[v] Original means are presented. Means separations were calculated using Fisher's protected least significant difference (LSD) at *P* <0.05. Friedman's non-parametric (rank) test was used for the incidence of seed infected with *P.*cf. *brassicae* and *Alternaria* spp. in the seed health assay and the incidence of seedlings infected in the seed transmission assay in both trials, and total non-germinated and abnormally germinated seed in Trial 1, because the data did not meet assumptions of normality and/or equal variances. The data for rotten seed in Trial 2 was log-transformed (Log) to meet assumptions for parametric analysis. Means followed by different letters within a column are significantly different.

[w] Seed were steam treated at 62.8, 65.6, 68.3, and 71.1°C, or not steamed, and dried as described in the main text. The entire trial was repeated (Trial 2).

[x] Four replications of 100 seed for each lot were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[y] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) and incubated at 4°C for six weeks in the dark.

[z] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in 72-cell flats in a greenhouse, and the seedlings that developed were observed daily for symptoms of light leaf spot.

Table 4.8. Analyses of variance for a seed germination assay, seed health assay, and seed transmission assay of mustard (*Brassica juncea*) seed infected with *Pyrenopeziza brassica* and treated with fungicide seed treatments[a]

	Trial 1				Trial 2			
	Mean square (*P* > F)				Mean square (P > F)			
Dependent variable[b]	**Replication**	**Fungicide**	**CV (%)**	**R^2**	**Replication**	**Fungicide**	**CV (%)**	**R^2**
Seed germination assay (% seed)								
Normally germinated[c]	0.718	0.734	1.84	0.214	0.317	0.838	1.75	0.232
Non-germinated seed	1.000	0.889	54.77	0.138	0.733	0.960	142.62	0.139
Abnormally germinated	0.194	0.516	125.85	0.324	0.076	0.847	37.99	0.295
Rotten seed	0.080	0.274	97.06	0.406	0.405	0.246	74.07	0.350
Seed health assay (% seed infected)[d]								
*P.*cf. *brassicae*	1.000	0.050	43.95	0.419	1.000	<0.001	31.16	0.679
Alternaria spp.	1.000	<0.001	37.35	0.590	1.000	0.001	36.32	0.571
Seed transmission assay (% seedlings infected)[e]								
*P.*cf. *brassicae*	1.000	<0.001	31.82	0.576	1.000	<0.001	20.59	0.738

[a] Each trial consisted of a randomized complete block design with four replications of each fungicide treatment for Trials 1 and 2. CV = coefficient of variation. R^2 = coefficient of determination.

[b] Results for percentage seed infected with *P.*cf. *brassicae* and *Alternaria* spp., and percentage seed transmission for both trials; and non-germinated seed in Trial 1 were analyzed using Friedman's non-parametric rank test because transformations did not meet assumptions for parametric analysis. The data for rotten seed in Trial 1 was log-transformed (Log), and the data for abnormally germinated seed in Trial 2 was square root-transformed (SqRt) to meet assumptions for parametric analysis.

[c] Four replications of 100 seed for each treatment were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[d] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) and incubated at 4°C for six weeks in the dark.

[e] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in flats in a greenhouse, and each seedling that developed was examined daily for symptoms of light leaf spot as described in the main text.

Table 4.9. Efficacy of fungicide seed treatments for eradication of *Pyrenopeziza brassicae* from a mustard seed lot[v]

Trial and treatment[w]	Germination assay (% of seed)[x]				Seed health assay (% seed infected)[y]		Seed transmission assay (% seedlings infected with *P.*cf. *brassicae*)[z]	Days after planting symptoms observed
	Normally germinated	Non-germinated	Abnormally germinated	Rotten	*P.*cf. *brassicae*	*Alternaria* spp.		
Trial 1								
Non-treated control	-	-	-	-	-	-	5.25 a	17 to 28
Dye-only control	97.25	1.25	0.75	0.75	2.25 a	2.50 a	0.38 bc	18 to 26
Coronet	98.50	1.00	0.25	0.25	0.50 bcd	0.00 c	0.00 c	-
Dynasty	98.50	1.25	0.25	0.00	1.25 abc	1.00 abc	0.00 c	-
Farmore FI400	98.00	0.75	0.50	0.75	0.75 abcd	0.00 c	0.25 bc	20 to 26
Helix Vibrance	96.50	1.25	0.75	1.50	0.00 d	0.00 c	0.00 c	-
Maxim 4FS	96.50	1.25	0.75	1.50	025 cd	0.50 c	0.00 c	-
Mertect 340F	97.25	1.00	1.00	0.75	0.25 cd	2.25 ab	0.00 c	-
Metlock	97.00	2.00	0.00	1.00	0.50 bcd	0.75 bc	0.13 bc	28
Obvius	96.75	2.00	0.50	0.75	0.50 bcd	0.25 c	0.13 bc	21
Rovral 4F Flowable	98.00	0.75	1.00	0.25	1.25 abc	1.50 ab	0.13 bc	21
Vibrance	97.75	1.00	0.25	1.00	1.50 ab	1.75 ab	0.38 b	19 to 21
LSD (*P* <0.05)	NS	NS (Rank)	NS	NS (Log)	Rank	Rank	Rank	
Trial 2								
Non-treated control	98.25	0.25	0.50	1.00	8.50 a	33.75 a	2.63 a	16 to 29
Dye-only control	96.25	0.75	0.75	2.25	0.50 cd	0.50 bcd	0.25 b	22 to 25
Coronet	98.50	0.50	0.25	0.75	0.00 d	0.25 cd	0.00 c	-
Dynasty	97.50	0.50	0.25	1.75	0.25 cd	1.00 bcd	0.00 c	-
Farmore FI400	97.50	0.75	0.50	1.25	0.00 d	0.50 bcd	0.00 c	-
Helix Vibrance	98.00	0.50	1.00	0.50	0.00 d	0.00 d	0.00 c	-
Maxim 4FS	98.00	1.00	0.25	0.75	0.50 cd	1.25 bcd	0.13 bc	23
Mertect 340F	98.00	1.00	0.00	1.00	0.00 d	1.75 ab	0.00 c	-
Metlock	97.75	1.00	0.25	1.00	0.75 bc	0.00 d	0.00 c	-
Obvius	96.75	1.00	1.00	1.25	0.75 bc	0.50 bcd	0.13 bc	27
Rovral 4F Flowable	98.25	0.25	0.75	0.75	0.75 cd	0.00 d	0.00 c	-
Vibrance	97.75	0.50	0.50	1.25	1.25 ab	1.25 bc	0.00 c	-
LSD (*P* <0.05)	NS	NS	NS (SqRt)	NS	Rank	Rank	Rank	

[v] Original means are presented. Means separations were calculated using Fisher's protected least significant difference (LSD) at *P* <0.05. Data for the incidence of seed infected with *P.*cf. *brassicae* and *Alternaria* spp., the percentage seedlings infected with *P.*cf. *brassicae* in the seed transmission assay in both trials, and non-germinated seed in Trial 1 were analyzed using Friedman's non-parametric rank test because transformations did not meet assumptions required for parametric analysis. The data for rotten seed in Trial 1 was log-transformed (Log) and the data for abnormal seed in Trial 2 was

square root-transformed (SqRt) to meet assumptions for parametric analysis. Means followed by different letters within a column for each trial are significantly different.

[w] Seed were treated with each fungicide as described in Table 4.1 and the main text. The entire trial was repeated (Trial 2).

[x] Four replications of 100 seed for each lot were subjected to the blotter germination assay of the Association of Official Seed Analysts (Yaklich, 1985), as described in the main text.

[y] An NP-10 agar seed health assay was used as described in the main text. For each assay, four replications of 100 seed/treatment were plated onto NP-10 agar medium (Sorensen et al. 1991) in square acrylic boxes (32 to 34 seeds/box) and incubated at 4°C for six weeks in the dark.

[z] Details of the seed transmission assay are described in the main text. For each assay, four replications of 200 seed/treatment were planted in 72-cell flats in a greenhouse, and the seedlings that developed were observed daily for symptoms of light leaf spot.