Improving Integrated Pest Management of Stemphylium Leaf Blight of Onion

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ABSTRACT

IMPROVING INTEGRATED PEST MANAGEMENT OF
STEMPHYLIUM LEAF BLIGHT OF ONION

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Stemphylium leaf blight (SLB), caused by *Stemphylium vesicarium*, has become an important disease of onion in Ontario, Canada and the north-eastern USA in recent years. This is the first study in Canada to confirm the species of *Stemphylium* isolates collected in onion fields using molecular methods and to investigate several elements of the life cycle in North America. The research confirmed that sexual reproduction and survival of overwintering structures on onion leaves occurred in the field and identified six weed species that are alternative hosts: redroot pigweed (*Amaranthus retroflexus*), marshcress (*Rorippa palustris*), yellow nutsedge (*Cyperus esculentus*), perennial sowthistle (*Sonchus arvensis*), bull thistle (*Cirsium vulgare*) and purslane (*Portulaca oleracea*). Using the forecasting models TOMcast and BSPcast to trigger foliar fungicide application reduced applications by one or two sprays. Foliar sprays in combination with fungicide seed treatments reduced SLB severity by 33–48%, but seed treatments or foliar sprays alone did not suppress symptoms. The efficacy of foliar fungicides for the management of SLB has declined over the past 10 years. Isolates of *S. vesicarium* collected in southern Ontario from onion, asparagus, and leek in 2012–2019 were assessed for insensitivity to the active ingredients of commonly used fungicides via mycelial growth and conidial germination assays. Of the isolates collected in southern Ontario in 2018–2019, 94% were insensitive to azoxystrobin, 61% to pyrimethanil, and 18% to fluopyram. Difenoconazole did not inhibit conidial germination and 1% of *S. vesicarium* isolates were insensitive in the mycelial growth

assay. Weather variables relating to moisture (high daily average relative humidity, low vapour pressure deficit, and increased leaf wetness) were correlated with an increase in air-borne *S. vesicarium* spores. A model was developed to predict the concentration of air-borne spores but requires validation with more data, especially for years with high SLB severity. Other abiotic factors such as drought may increase disease severity, but a controlled environment experiment did not find a relationship between SLB susceptibility and drought. Future research should focus on new fungicides, alternative products, biocontrol agents, and modifying the integrated pest management program to better indicate when no fungicides are required.

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Nevertheless, she persisted.

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LIST OF ABBREVIATIONS AND ACRONYMS

a.i – active ingredient
AOX – alternative oxidase
AP – anilinopyrimidine
AUDPC – area under disease progress curve
BLAST – Basic Local Alignment Search Tool
BSP – brown spot of pear
CDSI – cumulative disease severity values
cytB – cytochrome *b*
DMI – demethylation inhibitors
DSI – disease severity index
DSV – disease severity value
EC_{50} – effective fungicide concentration that caused 50% inhibition of growth or germination
FRAC – Fungicide Resistance Action Committee
gapdh – glyceraldehyde-3-phosphate dehydrogenase
GSWC – gravimetric soil water content
HCl – hydrochloride
HHK – hybrid histidine kinase
HK – histidine kinase
HST – host specific toxins
IPM – integrated pest management
ISR – induced systemic resistance
ITS – internal transcribed spacer
MAP – mitogen-activated protein
MCRS – Muck Crops Research Station
PDA – potato dextrose agar
ATP – adenosine triphosphate
PP – phenylpyrrole fungicides
QoI – quinone outside inhibitor
qPCR – quantitative polymerase chain reaction
SAR – systemic acquired resistance
sAUDPC– standardized area under disease progress curve
sdh – succinate dehydrogenase complex iron sulfur subunit (sdhA, sdhB, sdhC, sdhD)
SDHI- succinate dehydrogenase inhibitor
SHAM – salicylhydroxamic acid
SLB – Stemphylium leaf blight
TEf-A – translation elongation factor 1α
UV – ultraviolet
VPD – vapour pressure deficit
vmaA-vpsA – intergenic spacer between the vacuolar membrane ATPase catalytic subunit A gene and the vacuolar protein sorting-associated protein
VSWC – volumetric soil water content

Chapter 1 Literature review

1.1 Introduction

Vegetables and herbs from the genus *Allium* have been cultivated for over 4000 years for their characteristic flavors, medicinal properties, and ornamental uses (Fritsch and Friesen, 2002). Onion (*Allium cepa* L.) is one of the most valuable *Allium* crops, with a total world production of approximately 125 million tonnes in 2019 (FAOSTAT, 2020). The excellent storage properties of onion allows for year-around availability in many countries. Stemphylium leaf blight (SLB), caused by *Stemphylium vesicarium* (Wallr.) E. G. Simmons, is a devastating foliar disease that can cause complete defoliation of onion plants leading to small or unmarketable bulbs, reduced capacity for long-term storage, and greater susceptibility to bacterial diseases (Wright, 1993; Paibomesai et al., 2012).

Research on SLB is important because the disease is difficult to manage using cultural methods and fungicide applications are often ineffective (Tayviah, 2017; Stricker et al., 2020). There are reported cases of insensitivity to dicarboximide, dithiocarbamate, and copper oxychloride fungicides in SLB populations (Alberoni et al., 2005; Hussein et al., 2007). Some of the difficulty in managing SLB may be due to the general lack of knowledge concerning the life cycle and overwintering strategy of *S. vesicarium*. The literature on this pathogen is largely focused on diseases it causes on other host plants, particularly brown spot of pear (BSP) in Europe. The aim of this study was to develop effective disease management practices to improve yields and produce onion crops in Ontario that are suitable for long-term cold storage.

1.2 Onion production

1.2.1 Onion taxonomy and description

Plants in the genus *Allium* are typically suited to arid conditions, but onions have adapted to many ecological conditions and are grown around the world. The taxonomy of *Allium* has changed frequently, but is currently in the Class Monocotyledonae, sub-family Amaryllidaceae (formerly Alliaceae), within the order Asparagales (Chase et al., 2009). The genus is monophyletic. It has been proposed that evolution proceeded along three evolutionary lines, which can be further divided into 15 subgenera (Li et al., 2010). The subgenus *cepa* is further

divided into five sections: *cepa, annuloprason, sacculiferum, schoenoprasum,* and *condensatum* (Friesen et al., 2006). Section *cepa* includes Welsh onion (*Allium fistulosum* L.), French gray shallot (*Allium oschaninii* O. Fedtsch), and onion (*Allium cepa* L.). Wild species within the subgenus *cepa* occur in central and south-east Asia, which suggests that this may be the origin of onion, but the exact origin is unknown (Fritsch and Friesen, 2002). In Canada, common commercially produced *Allium* crops include dry onion (*A. cepa*), green onion (*A. cepa*), French red shallots (*A. cepa* var. *aggregatum*), and garlic (*A. sativum* L.) (Pesticide Risk Reduction Program, 2012).

Allium cepa is a highly variable species made up of hundreds of cultivars, hybrids, and landraces worldwide that vary greatly in their adaptation to photoperiod and temperature, bulb storage life, dry-matter content, flavour, and skin colour (Fritsch and Friesen, 2002; Brewster, 2008). Onions are biennial plants characterized by distichous leaves that are cylindrical, flattened, and pointed at the tip (Fritsch and Friesen, 2002). If onions are not harvested in the fall, the bulb overwinters and will flower in the following spring and set seed, a process that requires vernalization (Brewster, 2008; Lee et al., 2013). The inflorescence stalk of common onion is 1–2 m long and hollow (Brewster, 2008). The developing inflorescence is covered by a leafy spathe, which splits to reveal clusters of white flowers in a round umbel (Brewster, 2008). Premature or unwanted flowering, called bolting, can result from unseasonable cold temperatures when the plants are at a young stage, or stressful environmental conditions, and result in lower yield and unmarketable bulbs (Brewster, 2008).

Cultivars of onion are grouped in two general horticultural categories, the Common Onion group and the *Aggregatum* group (Hanelt, 1990). Plants in the *Aggregatum* group form clusters of smaller bulbs encased in a dry outer bulb skin (Brewster, 2008). The Common Onion group includes the most economically important cultivars, which form single globose bulbs that are composed of layers of leaf-bases with membranous skins (Fritsch and Friesen, 2002). Onion bulbs can range in size from 5 mm to over 100 mm diameter at harvest (Lancaster et al., 1996). The bulbs of onion cultivars vary in shape, size, firmness, susceptibility to bolting, colour (ranging from white, light yellow, dark yellow / brown, pink, to red and dark purplish-red), pungency, sweetness, and juiciness (Bosch Serra and Currah, 2002).

1.2.2 Onion production and cultivation

Onion, also known as the common onion, dry onion, or bulb onion, is valued for its aromatic, flavourful bulbs. In 2019, onion represented 8.8% of the total world vegetable production quantity and 8.7% of area harvested (FAOSTAT, 2020). China and India are typically the two greatest producers of onion, totalling over 22 million metric tonnes (T) of dry onion each (FAOSTAT, 2020). The average yield of onion is 23 T ha^{-1}, and countries with the highest yield per hectare were Guyana, Korea, and the USA, with 60–77 T ha^{-1} in 2019 (FAOSTAT, 2020).

In Canada, approximately 5,700 ha of onion are planted, yielding a total production of 250,000 T (Statistics Canada, 2020). The farm gate value of the onion industry in Canada is $86.4–113.5 million. Most onions and shallots grown in Canada are sold domestically in the fresh market, and a small proportion is exported (Pesticide Risk Reduction Program, 2012). In 2016–2020, Ontario produced an average of 102,604 T of dry onion, which represents 40% of national production, with an average yield of 42,900 kg ha^{-1} (Statistics Canada, 2020). Growers in Ontario mainly produce yellow globe onion and some red onion (McDonald et al., 2016b). The current research program focused on yellow onions grown in the Holland Marsh, an area in Ontario that is characterized by organic muck soil (50–75% organic matter, pH 5.5–6.5), where the majority of Ontario onion crops are produced.

There are several methods that can be used to establish the onion crop: direct sowing, transplants, or sets (Brewster, 2008). In most onion-growing regions of Canada, onion seed is sown using a pneumatic precision seeder at 35–45 seeds per m of row at 2 cm depth (Brewster, 2008; Pesticide Risk Reduction Program, 2012). Transplanting is less cost-effective, but results in a more uniform crop with desirable spacing compared to direct seeding, and can be used to lengthen the growing season (Brewster, 2008). Bare-root transplanting involves planting seeds in nursery beds at high densities, removing the plants at the 3- to 4-leaf stage from the soil, trimming the roots and shoots, and transplanting the plants to the field (Bosch Serra and Currah, 2002). Plug transplants are established as single- or multiple-seeded cells grown in greenhouses or under rain shelters and the entire plug, consisting of plant and growth media, is transplanted to the field (Bosch Serra and Currah, 2002). The third option is to plant using sets; sets are initially seeded at a high density and harvested when the bulbs are less than 25 mm in diameter, for planting in the following growing season (Brewster, 2008). Onions planted as sets can grow very

quickly, but are prone to bolting and can transmit pests and diseases (Bosch Serra and Currah, 2002; Brewster, 2008).

Onions destined for long-term storage are commonly sprayed with maleic hydrazide, a growth-regulator that disrupts cell division and inhibits bolting in storage (Isenberg et al., 1974). This product is applied to green onion leaves when the onions begin to lodge, and it is translocated to the bulb (Brewster, 2008). Onions should have 5-8 green leaves in order to absorb maleic hydrazide (Ilić et al., 2011; Arysta LifeScience Canada, 2020).

1.2.3 Growing conditions

The cultivation and distribution of onions are influenced by photoperiod length, light intensity and quality, and temperature (Khokhar, 2017). The optimal temperature for onion seed germination is 25 °C, but can occur between 10–35 °C (Maynard and Hochmuth, 2006). The ideal temperatures for onions are 15–20 °C during early growth and 20–27 °C for bulb development (Khokhar, 2017). The temperature must exceed 12 °C for optimal bulb development, and growth rates decrease at temperatures over 32 °C (Brewster, 2008). However, if the day length is inappropriate for the cultivar, no bulbing will occur even at ideal temperatures (Khokhar, 2008). Seed companies often classify onion cultivars based on the minimum day length required to initiate bulb development into ‘short-day’, ‘intermediate-day’, ‘long-day’, and ‘very long-day’ types (Brewster, 2008). Short-day onions require a minimum of 11 h of light, intermediate-day types require greater than 13 h, and long-day types initiate bulbs only when day length exceeds 16 h (Brewster, 2008). Growers must select the appropriate type of onion cultivar to grow based on their latitude, altitude, growing season, and consumer market. Commonly used commercial cultivars grown in the Holland Marsh are ‘long-day’ and mature 92–116 days after seeding (McDonald et al., 2016a).

In the Holland Marsh, it is recommended to seed barley as a nurse crop to decrease wind erosion in the early spring, followed by the application of a slow-kill selective herbicide 3 to 4 weeks after barley emergence to kill the barley once the onion crop has established to prevent competition effects (Souza Machado and Ali, 1992).

Onions can be cultivated in many types of soils, so long as they are fertile and well-drained (Pesticide Risk Reduction Program, 2012). The soil should be homogeneous, free of debris and stones, not prone to compaction, and have a pH in the range of 5.0–8.4 (Bosch Serra and Currah, 2002; Pesticide Risk Reduction Program, 2012). Onions are susceptible to drought

stress because the roots are relatively shallow, with main roots reaching only about 18 cm deep, so water that has moved below 76 cm from the soil surface is inaccessible to the crop in mineral soil (Drinkwater and Janes, 1955). However, mature onions can have root systems in sandy loam mineral soil reaching as deep as 99 cm if the soil is not compacted (Weaver and Bruner, 1927).

The soil in the Holland Marsh is classified as muck soil, also known as a sapric soil, which is a type of histosoil that is high in decomposed organic matter. Muck soil typically occurs in a topographical depression, and is characterized by a dark brown or black surface layer (Hoffman and Richards, 1955). The Holland Marsh was a marsh that was first drained in 1925, and is now a hub for vegetable production (Hoffman and Richards, 1955). The soil in this region was relatively high in nitrogen but low in potassium, phosphorus, copper, boron, and manganese when it was first drained (Hoffman and Richards, 1955). The soil pH may be slightly acidic to slightly alkaline, the drainage is poor, and the area is prone to flooding in extremely wet years (Hoffman and Richards, 1955). The pH near the Muck Crops Research Station in the Holland Marsh is approximately 6.5 (Al-Daoud et al., 2020). An extensive tile drainage network has been put into place in the Holland Marsh to improve drainage (Grenon et al., 2021).

1.2.4 Foliar diseases of onion

All plants are susceptible to pathogen attack and unfavourable environmental conditions that may cause disease-like symptoms. A disease is an abnormality in appearance or function, caused by the invasion by an infectious agent or pathogen (Agrios, 2005). There are many diseases and disorders of onions in North America caused by fungi, bacteria, nematodes, viruses, and abiotic factors (Table 1.1). Commercial onion cultivars differ in their resistance or tolerance to pests, diseases, and environmental conditions (Brewster, 2008). Environmental conditions such as hail, rain, and ozone can damage leaves and increase susceptibility to foliar diseases (Pesticide Risk Reduction Program, 2012). Common foliar diseases of onion in Ontario include Botrytis leaf blight, downy mildew, and purple blotch (Chaput, 1995). Stemphylium leaf blight (SLB) is a relatively new disease that was first observed in Ontario in 2008, and is now a major concern for onion production (Tesfaendrias et al., 2014). SLB causes leaf dieback, which affects the uptake of maleic hydrazide into onion bulbs and so could affect the post-harvest storage properties of the crop (McDonald et al., 2016b).

Table 1.1 Common diseases of onion in North America and causal pathogens (Adapted from Pesticide Risk Reduction Program, 2012).

Disease name	Pathogen or causal agent
Bacterial soft rot	*Erwinia carotovora* (Jones) Bergey et al.
Botrytis leaf blight	*Botrytis squamosa* J.C.Walker
Botrytis neck rot	*Botrytis aclada* Fresen.
Onion smut	*Urocystis cepulae* Frost
Downy mildew	*Peronospora destructor* (Berk.) Casp.
Purple blotch	*Alternaria porri* (Ellis) Cif.
Stemphylium leaf blight	*Stemphylium vesicarium* (Wallr.) E.G. Simmons
Damping-off	*Pythium* spp., *Rhizoctonia* spp., *Fusarium* spp.
Fusarium basal rot	*Fusarium oxysporum* Schltdl.
White rot	*Sclerotium cepivorum* Berk.
Aster yellows	Aster yellows phytoplasma
Stem and bulb nematode	*Ditylenchus dipsaci* Kuhn
Root-lesion nematode	*Pratylenchus penetrans* (Cobb) Filipjev and Schuurmans Stekhoven

1.3 Stemphylium leaf blight

Stemphylium leaf blight (SLB), sometimes called stalk rot, is caused by the fungal pathogen *Stemphylium vesicarium* (Sharma and Sharma, 1999). Depending on the environmental conditions and location, SLB can be an aggressive foliar disease of onion and garlic around the world (Gupta et al., 1994; Hassan et al., 2007; Mishra and Singh, 2017). SLB can lead to premature defoliation of the crop, thus making it more susceptible to post-harvest losses (Paibomesai et al., 2012). Concern is mounting in North America that applications of registered fungicides do not provide adequate SLB suppression (Pethybridge et al., 2016).

1.3.1 Symptoms of Stemphylium leaf blight

Conidia of *S. vesicarium* infect onion leaves when there is moisture on the leaves and temperatures between 10–25 °C, and SLB severity increases in response to extended leaf wetness (Suheri and Price, 2000). SLB symptoms are characterized by brown, oval lesions up to 7 cm in diameter at the tip and center of outer leaves (Ideta, 1911), or yellow, mottled lesions 0.5–4.0 cm in diameter on inner leaves (Misawa, 2008). *Stemphylium vesicarium* may produce host-specific toxins that cause leaf dieback (Singh et al., 1999), reduce yield, and may increase susceptibility to other diseases (Tesfaendrias et al., 2014). Lesions on garlic may initially be small and white, which can enlarge and coalesce until the leaves have withered or died (Zheng et al., 2008). SLB symptoms can sometimes be confused with purple blotch caused by *Alternaria porri* (Suheri and Price, 2000a).

A closely related species, *Stemphylium solani* G.F. Weber, also causes symptoms similar to SLB on garlic in China (Zheng et al., 2008).

Symptoms on asparagus are elliptical, slightly sunken lesions (0.8–1.6 mm) are formed on the spears and ferns (Falloon et al., 1984). The spots are initially purple but may become tan in the centre with a purple edge as the disease progresses. On pear, necrotic brown spots (2.5–10 mm) develop on pear fruit, leaves, and twigs (Moragrega et al., 2018). Diseased asparagus spears and pear fruits are unmarketable due to cosmetic damage. This pathogen is prevalent on asparagus in Ontario but has not yet been reported on pear in North America (Foster, 2018).

1.3.2 Origin, distribution, and host range of *Stemphylium vesicarium*

Stemphylium vesicarium has been described as a plant pathogen worldwide. On onion, SLB has been reported in Canada (Paibomesai et al., 2012), the United States (Miller et al., 1978), Egypt (Hassan et al., 2007), Japan (Misawa and Yasuoka, 2012), India (Gupta et al., 1994), Korea (HyeSun and SeungHun, 1998), Portugal (Tomaz and Lima, 1988), New Zealand (Wright et al., 2019), and Venezuala (Cedeño et al., 2003). On garlic, SLB has been reported in Australia (Suheri and Price, 2000a), Brazil (Boiteux et al., 1994), Ethiopia (Gedefaw et al., 2019), Korea (HyeSun and SeungHun, 1998), South Africa (Aveling and Naude, 1992), Spain (Basallote-Ureba et al., 1999), Turkey (Polat et al., 2012), and Canada (M.R. McDonald, *unpublished data*). SLB has also been reported affecting leeks in Australia (Suheri and Price, 2001) and Canada (M.R. McDonald, *unpublished data*) and spinach in New York (Spawton et al., 2020).

The mycologist Christiaan Hendrik Persoon first described this fungus in Europe as *Sphaeria herbarum* in 1801, although this name has since changed many times (Table 1.2). The accepted name for the anamorph of this fungus is now *Stemphylium vesicarium* (Wallr.) E.G. Simmons. The teleomorph is *Pleospora allii* (Rabenh.) Ces. & De Not. However, the ‘one fungus - one name’ resolution of the International Biological Congress has eliminated the dual nomenclature system for fungi (McNeill et al., 2012). The use of the anamorph name (*Stemphylium*) over the teleomorph name is recommended by the International Committee on the Taxonomy of Fungi (Rossman et al., 2015). A recent study has suggested to synonymise phylogenetically identical species under the name *S. vesicarium* (Table 1.2) (Woudenberg et al., 2017).

Table 1.2 Synonyms of *Stemphylium vesicarium*. See Index Fungorum for additional synonyms (http://www.speciesfungorum.org).

Anamorphs	**Telomorphs**
Alternaria putrefaciens (Fuckel) E.G. Simmons	*Ampullina herbarum* (Pers.) Quél.
Clasterosporium putrefaciens (Fuckel) Sacc.	*Phoma albicans* Roberge ex Desm.
Exormatostoma herbarum (Pers.) Gray	*Pleospora albicans* Fuckel
Helminthosporium vesicarium Wallr.	*P. alfalfae* E.G. Simmons
Macrosporium commune Rabenh.	*P. allii* (Rabenh.) Ces. & De Not.
M. echinellum Berk. & M.A. Curtis	*P. asparagi* Rabenh.
M. parasiticum Thüm.	*P. cheiranthi* Cocc. & Morini
M. sarcinula Berk.	*P. denotata* (Cooke & Ellis) Sacc.
M. vesicarium (Wallr.) Sacc.	*P. euonymi* Fuckel
Sphaeria denotata Cooke & Ellis	*P. excavata* var. *basitricha* (Durieu & Mont.) Sacc.
Sporidesmium putrefaciens Fuckel	*P. frangulae* Fuckel
Stemphylium alfalfae E.G. Simmons	*P. gymnocladi* Bagnis
Stemphylium brassicicola Y.F. Pei & X.G. Zhang	*P. herbarum* (Pers.) Rabenh. ex Ces. & De Not.
S. commune (Rabenh.) N.F. Buchw	*P. labiatarum* Cooke & Harkn.
S. cremanthodii Y.F. Pei & X.G. Zhang	*P. leguminum* (Wallr.) Rabenh.
S. herbarum E.G. Simmons	*P. lolii* P. Karst. & Har.
S. mali Yong Wang bis & X.G. Zhang	*P. mali* Hesler
S. parasiticum (Thüm.) J.A. Elliott	*P. meliloti* Rabenh.
S. sedicola E.G. Simmons	*P. pisi* (Sowerby) Fuckel
S. tomatonis E.G. Simmons	*P. pomorum* A.S. Horne
***S. vesicarium* (Wallr.) E.G. Simmons**	*P. putrefaciens* A.B. Frank
	P. salsolae Fuckel
	P. samarae Fuckel
	P. sedicola E.G. Simmons
	P. tomatonis E.G. Simmons
	P. typhae Pass. ex Brunaud
	Sphaeria brassicae Lasch
	S. excavata var. *basitricha* Durieu & Mont.
	S. herbarum Pers.
	S. leguminum Wallr

Stemphylium vesicarium can infect a range of plant species from many families. The host plants include several crops from the *Allium* genus, fruit trees, legumes, and ornamentals (Table 1.3). However, cross-inoculation studies have shown that isolates from one host are not always pathogenic on another host. For example, isolates collected from onion were able to infect wounded, but not unwounded, pear fruit and were sometimes less aggressive on asparagus spears (Foster, 2018). Köhl et al. (2009) also reported that *S. vesicarium* isolates from symptomatic onion and asparagus, or pear leaf litter, were not pathogenic on European pear. *Stemphylium vesicarium* isolates from pear leaf debris required a period of time after inoculation but were able to re-infect pear leaves, whereas isolates collected from lesions on pear fruits or leaves exhibited higher pathogenicity and aggressiveness on pear (Llorente et al., 2010a; Moragrega et al., 2018).

Interestingly, *S. vesicarium* isolates from asparagus were pathogenic on Japanese pear but not on European pear (Singh et al., 1999). In another study, *S. vesicarium* isolates from asparagus, onion, and garlic were pathogenic to all three crops, although this was not always the case (Basallote-Ureba et al., 1999).

Isolates from parsley were pathogenic to celery, carrot, and pear, but not to leek, onion, spinach, or tomato (Koike et al., 2013). *Stemphylium vesicarium* isolated from lupin (*Lupinus angustifolius* L) was pathogenic on lupin but not on faba bean, lentil, or alfalfa, whereas isolates collected from lentil only caused symptoms on lentil and not on faba bean, lupin, or alfalfa (Vaghefi et al., 2020). In addition, isolates collected from faba bean, chickpea, and alfalfa did not cause symptoms on any of the crops tested (Vaghefi et al., 2020).

Of 44 *S. vesicarium* isolates collected as air-borne spores in a pear orchard, only 5 (11%) were pathogenic on pear (Moragrega et al., 2018). These reports show that variation exists within the *S. vesicarium* species, resulting in a range of saprophytic and necrotrophic pathogenic activities on specific plant tissues. *Stemphylium vesicarium* isolated from onion also exhibited high variability in aggressiveness when re-inoculated on onion (Hassan et al., 2020). *Stemphylium* spp. often colonize dead or dying plant tissues, and some isolates of *S. vesicarium* can be saprophytic (Hudson, 1971; Bansal et al., 1991). In addition, *S. vesicarium* can develop as an endophyte in the living tissues of asymptomatic plants (Misawa and Yasuoka, 2012).

Table 1.3 Host plants of Stemphylium vesicarium. For a more extensive list, refer to the USDA Agricultural Research Service fungal database (https://nt.ars-grin.gov/fungaldatabases/).

Common name	Latin name	Source
Fir	*Abies* sp.	(Woudenberg et al., 2017)
Leek	*Allium ampeloprasum* L.	(Suheri and Price, 2001)
Common onion	*Allium cepa* L.	(Raghavendra Rao and Pavgi, 1975)
Welsh onion	*Allium fistulosum* L.	(Misawa and Yasuoka, 2012)
Garlic	*Allium sativum* L.	(Aveling and Naude, 1992)
Common ragweed	*Ambrosia artemisiifolia* L.	(Hanlin, 1963)
Giant reed	*Arundo donax* L.	(Pantidou, 1973)
Oats	*Avena* sp.	(Brahmanage et al., 2019)
Asparagus	*Asparagus officinalis* L.	(Falloon et al., 1987)
Beet	*Beta vulgaris* L.	(Hanse et al., 2015)
Wild bishop	*Bifora radians* M. Beib.	(Brahmanage et al., 2019)
Carrot	*Daucus carota* L.	(Mulenko et al., 2008)
Canola	*Brassica napus* L.	(Mulenko et al., 2008)
Chinese cabbage	*Brassica rapa* L. ssp. *pekinensis*	(Woudenberg et al., 2017)
Butterfly bush	*Buddleja davidii* Franch.	(Dudka et al., 2004)
Chili pepper	*Capsicum chinense* Jacq.	(Vitale et al., 2017)
Common knapweed	*Centaurea nigra* L.	(Kirk and Spooner, 1964)
Citrus	*Citrus* sp.	(Woudenberg et al., 2017)
White goosefoot	*Chenopodium album* L.	(Babuschkina, 1995)
Chrysanthemum	*Chrysanthemum* sp.	(Pantidou, 1973)
Daisy spp.	*Cremanthodium discoideum* Maxim.	(Woudenberg et al., 2017)
Dahlia	*Dahlia pinnata* Cav.	(Woudenberg et al., 2017)
Carnation	*Dianthus caryophyllus* L.	(Woudenberg et al., 2017)
Carnation spp.	*Dianthus pseudarmeria* M. Beib.	(Brahmanage et al., 2019)
Viper's bugloss	*Echium* sp.	(Riley, 1960)
Common ash	*Fraxinus excelsior* L.	(Mulenko et al., 2008)
Cleavers	*Galium aparine* L.	(Brahmanage et al., 2019)
Soybean	*Glycine max* L.	(Pande and Rao, 1998)
Sunflower	*Helianthus annuus* L.	(Arzanlou et al., 2012)
Sweet potato vine	*Ipomoea batatas* L. Lam.	(Gorter, 1977)
Lettuce	*Lactuca sativa* L.	(Liu et al., 2019)
Sweet pea	*Lathyrus odoratus* L.	(Köhl et al., 2009)
Lentil	*Lens culinaris* Medikus	(Sinha and Singh, 1993)
Flax	*Linum usitatissimum* L.	(Woudenberg et al., 2017)
Perennial ryegrass	*Lolium* sp.	(Thambugala et al., 2017)
Honeysuckle	*Lonicera* sp.	(Woudenberg et al., 2017)
Bird's-foot trefoil	*Lotus purshianus* Clem & E.G. Clem	(French, 1989)
Annual honesty	*Lunaria annua* L.	(Woudenberg et al., 2017)
Lupin	*Lupine* sp.	(Ahmad, 2014)
Apple	*Malus* sp.	(Woudenberg et al., 2017)
Mango	*Mangifera indica* L.	(Ahmad, 2014)
Alfalfa	*Medicago sativa* L.	(Díaz-Valderrama et al., 2021)
Lemon balm	*Melissa officinalis* L.	(Brahmanage et al., 2019)
Grape hyacinth	*Muscari* sp.	(Pantidou, 1973)
Parsley	*Petroselinum crispum* [Mill.] Fuss	(Koike et al., 2013)
Green bean	*Phaseolus vulgaris* L	(Câmara et al., 2002)

Common name	Latin name	Source
Common reed	*Phragmites australis* (Cav.) Trin. ex Steud.	(Peláez et al., 1998)
Pea	*Pisum sativum* L.	(Woudenberg et al., 2017)
Poplar	*Populus* spp.	(Woudenberg et al., 2017)
Pear	*Pyrus* spp.	(Rossi et al., 2008)
Radish	*Raphanus raphanistrum* subsp. *sativus* (L.) Domin	(Belisario et al., 2008)
Black locust	*Robinia pseudoacacia* L.	(Unamuno, 1941)
Snake plant	*Sansevieria trifasciata* Prain	(Ahmadpour and Poursafar, 2018)
Ice plant	*Sedum spectabile* Boreau	(Moslemi et al., 2017)
Tomato	*Solanum lycopersicum* L.	(Woudenberg et al., 2017)
Spinach	*Spinacia oleracea* L.	(Misawa et al., 2017)
Marigold	*Tagetes erecta* L.	(Unamuno, 1941)
Alsike clover	*Trifolium hybridum* L.	(Richardson, 1990)
Fenugreek	*Trigonella foenum-graecum* Linn.	(Woudenberg et al., 2017)
Cattail	*Typha latifolia* L.	(French, 1989)
Valerian	*Valeriana* sp.	(Brahmanage et al., 2019)
Smooth vetch	*Vicia villosa* Roth	(Yan et al., 2019)
Grape	*Vitis vinifera* L.	(Kranz, 1965)
Corn	*Zea mays* L.	(Unamuno, 1941)

Diseases caused by *S. vesicarium* can result in considerable economic loss. For instance, the symptoms on asparagus spears include unsightly blighting that reduces the marketable yield. Purple spot of asparagus caused by *S. vesicarium* can result in a loss of 24–29% yield (spear weight) on unsprayed compared to fungicide-treated crops (Meyer and Hausbeck, 2000). In New Zealand, severe symptoms of Stemphylium leaf spot resulted in defoliation and up to 52% yield loss in unsprayed asparagus (Menzies, 1983). In extreme cases, purplish lesions can occur on 90% of harvested spears (Hausbeck et al., 1999).

Loss in onion production and quality caused by SLB can also be severe. In 2002, the Vidalia onion crop in Georgia was exposed to warm fall temperatures and heavy spring frost that was associated with an outbreak of SLB (du Toit and Inglis, 2002). Afterwards, the diseased onions were infected with sour skin (*Burkholderia cepacia* complex [Palleroni and Holmes] Yabuuchi et al.), resulting in an overall loss of approximately 60% of the marketable crop ($50 million) (du Toit and Inglis, 2002). In a particularly high disease year, SLB caused 80–85% loss in the onion crop in Portugal (Tomaz and Lima, 1986). Following rainy periods, onion crop loss in onion can be as high as 90% (Miller et al., 1978). Also, application of fungicides to reduce SLB using fungicides increased bulb weight by 33–40%, and increased the number of jumbo grade (> 7.6 cm dia.) bulbs by 29% in New York (Hoepting, 2018a; b).

SLB was first reported in New York in 1985 (Shishkoff and Lorbeer, 1989) and spread across most onion fields in the state by 1990, where it resulted in severe foliar dieback in some fields (Lorbeer, 1993). SLB was considered of minor importance in North America until its re-emergence in the last 10 years as the dominant foliar disease affecting broad-acre onion production (Hay et al., 2019). The focus of the current research program was on management of *S. vesicarium* on onion in Ontario.

1.3.3 Identification

Stemphylium vesicarium is in the phylum Ascomycota. It reproduces sexually by forming ascospores within pseudothecia, and asexually via conidia produced on conidiophores. *Stemphylium* spp. require exposure to ultraviolet light to sporulate (Raghavendra Rao and Pavgi, 1975). The pseudothecia of *S. vesicarium* are small, black, and pinhead-like (100–500 µm dia.) and form near the end of the growing season on both diseased and symptomless leaves and inflorescence scapes (Raghavendra Rao and Pavgi, 1975; Misawa and Yasuoka, 2012). Within the pseudothecia are asci, which are bitunicate and club-shaped (110–170 × 24–38 µm) and contain eight ascospores each (Figure 1.1) (Raghavendra Rao and Pavgi, 1975; Basallote-Ureba et al., 1999; Misawa and Yasuoka, 2012). At maturity, the ascospores are yellow to olive-brown, ellipsoid but slightly tapered (33–44 × 15–20 µm), and muriform with 3–7 transverse septa and 6–14 longitudinal septa (Simmons, 1969; Raghavendra Rao and Pavgi, 1975). Ascospores can initiate primary infections on new growth of onions in the spring (Hausbeck et al., 1999).

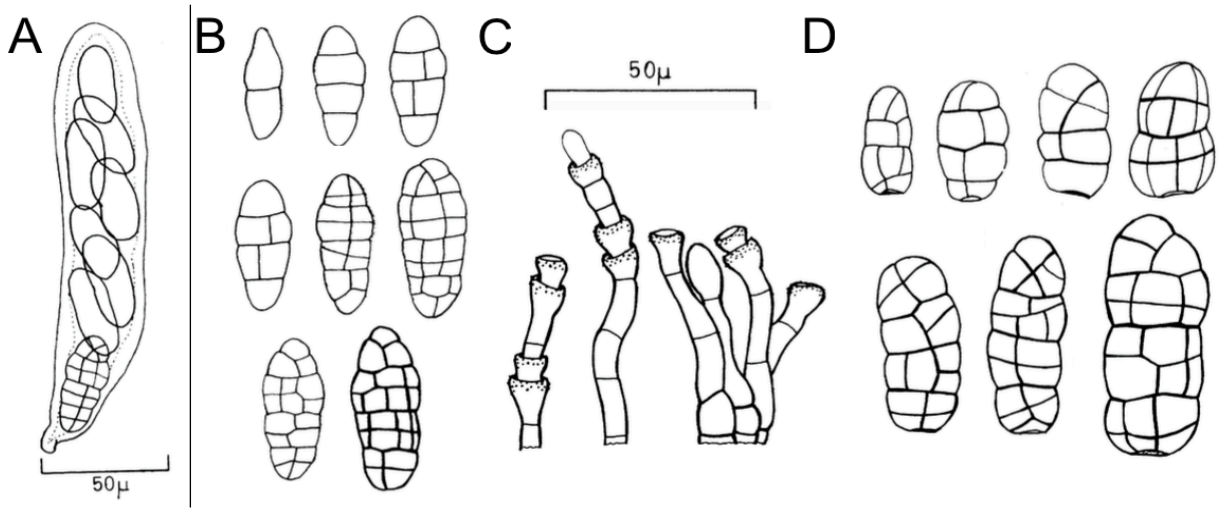

Figure 1.1 *Stemphylium vesicarium* A) ascus, B) ascospores, C) conidiophores, and D) conidia (Simmons, 1969).

Ascospores take time to mature. On garlic debris in Spain, undifferentiated ascocarps were present within 30 days in the field, but it took 4 months for ascospores to develop in mature pseudothecia (Basallote-Ureba et al., 1999). In New York, pseudothecia on onion leaves which that had overwintered in the field did not contain ascospores (Hay et al., 2019; Leach et al., 2020), but mature pseudothecia containing ascospores were observed on artificially inoculated and symptomatic onion leaves that had been maintained in cold storage (~4 °C) for 2–3 months. However, ascospores were not present on onion leaf tissue collected from the field that had been maintained under the same conditions (Dr. S.J. Pethybridge, Cornell University, *personal communication*). Pseudothecia have not previously been documented at the Holland Marsh, but air-borne ascospores have been collected in the region (Gossen et al., 2021).

In Italy, *S. vesicarium* produced mature ascospores under field conditions on detached leaves of pear and several alternative hosts such as grasses and weeds (Kentucky bluegrass [*Poa pratensis* L.], red fescue [*Festuca rubra* L.], sheep fescue [*Festuca ovina* L.], perennial ryegrass [*Lolium perenne* L.], tall crabgrass [*Digitaria sanguinalis* L. Scoop.], yellow foxtail [*Setaria glauca* L. Beauv.], and white clover [*Trifolium repens* L.]) (Rossi et al., 2005b). Pseudothecia developed earlier and in greater density on the alternative hosts, but those produced on pear leaves were larger and ascospore maturation and ejection was highest from pear leaves (Rossi et al., 2005b). Ascospore release typically occurred between April–June in Ontario onion fields (Gossen et al., 2021), from February–April for garlic fields in Spain (Prados-Ligero et al., 2003), and September–January and then again in May for asparagus in New Zealand (Menzies et al., 1992). In addition, ascospore production on pear leaves in Italy occurred from February–May, with an extended period of ascospore ejection into mid-June from pseudothecia produced on sheep fescue and yellow foxtail (Rossi et al. 2005). The production, maturation, and ejection of *S. vesicarium* ascospores is influenced by the host and environmental conditions.

Asexual reproduction occurs via oval to ellipsoidal, pale to dark brown conidia (22–42 × 12–25 µm) with 3–5 transverse septa, 4–14 longitudinal septa, and a dark basal scar (Figure 1.1) (Simmons, 1969). Conidia are produced on yellow or brown conidiophores that are cylindrical and enlarged apically at the site of conidia production (Raghavendra Rao and Pavgi, 1975). Under warm (25 °C) and damp conditions, conidia and ascospores germinate on onion leaves, forming single or multiple germ-tubes (Simmons, 1969). The germ-tubes terminate in bulbous appressoria upon stomata or epidermal cells (Falloon et al., 1987; Aveling and Snyman, 1993;

Prados-Ligero et al., 2003). Appressoria that formed above epidermal cells successfully penetrated the host at a rate of 48–72% and those formed above stomata were 89–97% successful (Aveling and Snyman, 1993). The greatest success was observed after 24 h at 18–25 °C with a minimum leaf wetness period of 16 h (Aveling and Snyman, 1993).

Vegetable juice agars such as V8 medium are often used to induce sporulation of *Stemphylium* spp. and other pathogens (Simmons, 1969; Rissler and Millar, 1977; Chowdhury et al., 2015). V8 media contains a mixture of macro- and micronutrients including nitrogen, salts, and metals (especially copper) that supports sexual reproduction (Kent et al., 2008). In the human fungal pathogen *Cryptococcus neoformans* (San Felice) Vuill., copper in the V8 media enhanced mate recognition, cell fusion, filamentation, and up-regulated pheromone gene expression (Kent et al., 2008). Copper is an essential micronutrient used by enzymes for production of melanin (Eisenman and Casadevall, 2012). Copper deficiency can cause reduced pigmentation in cultures of many fungi when grown on potato dextrose agar (Griffith et al., 2007).

The genome of an isolate of *S. vesicarium* has recently been sequenced (Gazzetti et al., 2019) and this resource can now be used as a baseline for molecular confirmation of the species identity of new isolates. The internal transcribed spacer, glyceraldehyde-3-phosphate dehydrogenase, and translational elongation factor EF-1 alpha sequences were used to separate *S. vesicarium* (designated in the article as syn. *P. herbarum*) into a clade apart from several closely related *Stemphylium* species, but did not differentiate *P. herbarum* from *P. alfalfae*, *P. sedicola*, and *P. tomatonis* (Inderbitzin et al., 2009). However, new studies have suggested that these species be synonymized (Woudenberg et al., 2017). Additionally, *S. vesicarium* can be differentiated from *S. botryosum* based on the cytochrome *b* sequence (Graf et al., 2016).

1.3.4 Life cycle

Stemphylium vesicarium is dormant during the winter, surviving as pseudothecia or mycelia in diseased or asymptomatic leaves (Simmons, 1969). In spring, ascospores are ejected from pseudothecia at temperatures > 14 °C, and primary infections from conidia also occur (Misawa and Yasuoka, 2012). In the Holland Marsh, air-borne ascospores were trapped from April to July, but declined as the season progressed, which indicated that pseudothecia can overwinter in the region. However, pseudothecia were not found on onion leaf residue left in the field in the Holland Marsh in one year (Gossen et al., 2021), possibly because the leaf tissue was highly

degraded, so pseudothecia would have been difficult to identify, or pseudothecia are more common on other nearby hosts.

Conidia were captured in the Holland Marsh throughout the growing season, from May to September, with a peak in concentration around mid-June to mid-August (Gossen et al., 2021). Conidia of *S. vesicarium* germinated at temperatures as low as 4 °C and infected leaf tissues at 10 °C (Prados-Ligero et al., 2003). The release of air-borne ascospores and conidia showed a diurnal pattern in response to temperature, leaf wetness duration, and relative humidity (Suheri and Price, 2000b). In the Holland Marsh, the majority of spores were captured between 0600–1200 h (Gossen et al., 2021). Rainfall events within the past 24–72 h and long periods of leaf wetness were positively correlated with the concentration of air-borne *S. vesicarium* conidia concentrations (Gossen et al., 2021).

If the weather is warm (18–25 °C) and humid, with periods of leaf wetness > 16 h, SLB symptoms may occur (Suheri and Price, 2000b; Prados-Ligero et al., 2003). Early SLB symptoms on onion are characterized by small, yellow flecks, or streaks in the middle of the leaf (Saraswathi et al., 2017). These can develop into elongated, oval, or spindle-shaped spots, often reaching the leaf tips, with a characteristic pinkish margin (Raghavendra Rao and Pavgi, 1975). The centre of the spots turn grey, or dark olive brown when conidiophores and conidia begin developing (Figure 1.2) (Misawa and Yasuoka, 2012). Yellow mottled symptoms on leaves have also been reported on Welsh onion leaves (Raghavendra Rao and Pavgi, 1975). Also, a shorter wetting duration was required for infection and symptoms were more severe on wounded plants (Johnson and Lunden, 1986). The first symptoms develop in late June or early July in the Holland Marsh (Gossen et al., 2021). The spots can coalesce and the leaves may die-back from the tips (Misawa and Yasuoka, 2012). SLB is typically restricted to the leaves and inflorescences of onion (Misawa, 2008), and pseudothecia form at the end of the growing season on leaves and stalks (Raghavendra Rao and Pavgi, 1975; Basallote-Ureba et al., 1999).

Figure 1.2 Symptoms of Stemphylium leaf blight: A) dieback of the oldest leaves, B) oval-shaped lesions with dark areas of sporulation, leading to tip dieback, and C) chlorotic regions around lesion on scape, leading to collapse and termination of the flower.

This pathogen may also overwinter on alternative hosts (Raghavendra Rao and Pavgi, 1975) or on asymptomatic leaves of Welsh onion (Misawa and Yasuoka, 2012), and has been isolated from infested soil (Gaikwad et al., 2014) and onion seed (Aveling et al., 1993). Infected onion seed was suggested as a factor in the SLB epidemic in New York State in 1990 (Lorbeer, 1993). *Stemphylium vesicarium* can infect seed of parsley (0.25% incidence) (Koike et al., 2013), radish (25% incidence) (Belisario et al., 2008), and squash fruit (2% incidence) (Moumni et al., 2020) Likewise, *S. lycopersici* (Enjoji) Yamamoto has been isolated from blackened and rotted tobacco seeds (Kurose et al., 2014). Infected seed is a source of primary inoculum for seedling blight of carrot by *S. radicinum* (Murtaza et al., 1988) and Stemphylium blight of lentil caused by *S. botryosum* (Taylor et al., 2007). A very low incidence of *S. botryosum* (0.2%) was reported from onion seed in Turkey, but infection was limited to the seed coat, and did not lead to infested onion sets (Köycü and Özer, 1997). Further investigation is needed to determine the overwintering of this pathogen in southern Ontario, and if seed-borne inoculum is a potential source of primary inoculum (Figure 1.3).

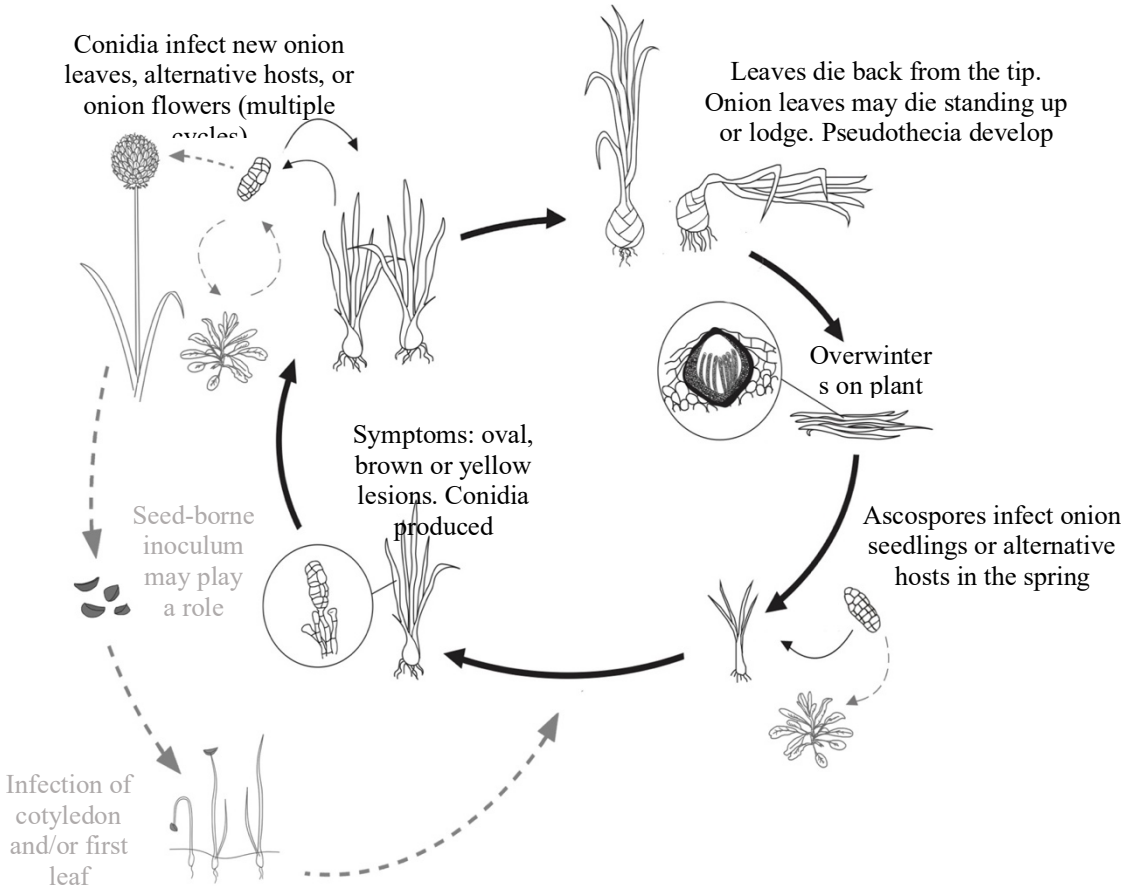

Figure 1.3 Possible life cycle of *Stemphylium vesicarium* on onion.

1.3.5 Host-specific toxins

Host specific toxins (HST) are compounds produced by plant pathogens that induce toxicity and promote disease only in the susceptible host species. HSTs have been extracted from isolates of *S. vesicarium* isolated from pear trees by refining the toxin from culture filtrates, but the chemical formulae and structures were not defined (Singh et al., 1999). Two compounds, SV-I and SV-II, induced vein necrosis on susceptible pear cultivars; likely by altering the plasma membrane, resulting in electrolyte leakage (Singh et al., 2000). These SV-toxins were host specific, and did not produce necrosis on resistant cultivars or non-host species such as tomatoes (Singh et al., 1999). Some SLB symptoms on onion and garlic may be due to a toxin released by the fungus (Basallote-Ureba et al., 1999).

Four other metabolites, stemphylin, stemphyloxin, stemphyperylenol, and stemphol, have been detected in extracts of *S. vesicarium* colonies using high-performance liquid chromatography and thin-layer chromatography (Andersen et al., 1995). Low doses of

stemphylin have been used to treat mouse leukemia cells, but a high concentration (40 μM) extracted from *S. botryosum* caused death of treated mice (Assante and Nasini, 1987). Stemphyperylenol obtained from *Alternaria cassia* Jurair & A. Khan induced host-specific necrosis on crabgrass (*Digitaria sanguinalis* [L.] Scop.) (Hradil et al., 1989) and exhibited antibacterial activity at a concentration of 3.12 μg mL^{-1} (Liu et al., 2010). Another compound, stemphyltoxin II, extracted from *Alternaria alternata* (Fr.) Keissl was mutagenic for a *Salmonella* species (Davis and Stack, 1991). There is one documented report of *S. vesicarium* producing patulin, a particularly toxic mycotoxin, in pear fruits (Laidou et al., 2001). Patulin is carcinogenic and toxic to plants and animals but its role in causing animal and human disease is unknown (Bullerman, 2003).

Overall, there is a paucity of literature on the animal toxicity of compounds produced by *S. vesicarium*, and toxin production in *Allium* spp. has not yet been confirmed. Detailed toxicity data are not available for these compounds, but a recent review developed in preparation for the current study concluded that *Stemphylium* spp. did not pose a risk to the health of humans or animals (Stricker et al., 2021a).

1.4 Factors affecting SLB severity

For disease to occur, the host plant must be susceptible, there must be a virulent pathogen present, and environmental conditions must be favourable for infection and symptom development (Agrios, 2005). *Stemphylium vesicarium* is a pathogen of crops around the world, which demonstrates that it has adapted to many ecological niches. For foliar pathogens, infection success is restricted by the duration of leaf wetness, humidity, and temperature (Magarey et al., 2005). Plant canopy density, the timing and amount of rainfall, and other environmental factors act in combination to affect infection success and disease development (Aveling, 1993; Basallote-Ureba et al., 1999; Mwakutuya, 2006; Misawa and Yasuoka, 2012).

1.4.1 Temperature

Plant pathogens have characteristic optimal temperatures for infection and growth that result in the most rapid disease development, but typically differ from the optimum for the host (Agrios, 2005). However, the optimal temperature can differ by the stage in pathogen's life cycle, the host infected, and location. The optimum temperature for mycelium growth of four *S. vesicarium* isolates on water agar was 25 °C (Bohlen-Janssen et al., 2018a), but infection by *Stemphylium* spp. on asparagus in New Zealand was higher at 14 °C than at 20 °C or 26 °C (Menzies et al.,

1991). In contrast, the optimal conditions for *S. vesicarium* infection of pear were > 24 h leaf wetness at 23 °C for fruits and 21 °C for leaves (Montesinos et al., 1995). Similarly, the percentage of successful infections of *S. vesicarium* into onion leaves was highest after 24 h of incubation at 25 °C, although the spores were able to germinate at temperatures as low as 4 °C (Suheri and Price, 2000b). The optimal temperature for germination of conidia was 23 °C for germination and 29 °C for germ tube growth (Bohlen-Janssen et al., 2018a). The optimal temperature for the development and maturation of pseudothecia on garlic was 5–10 °C (Prados-Ligero et al., 1998). The optimal temperatures for ascospores were 31 °C for germination and 30 °C for germ tube growth (Bohlen-Janssen et al., 2018b).

Spore production in garlic fields and air-borne ascospore abundance were linked to moderate winter temperatures (12–21 °C), and conidia were associated with warmer temperatures (15–32 °C) and spring rainfall events (Prados-Ligero et al., 2003). A correlation between the concentration of *S. vesicarium* conidia and temperatures of 15–25 °C was also reported in pear orchards (Rossi et al., 2005b). The concentration of air-borne *S. vesicarium* conidia on onion at the Holland Marsh was correlated with temperatures > 15 °C, and ascospore concentration decreased when average temperatures exceeded 15 °C (Gossen et al., 2021). However, the negative correlation between rising temperature and ascospore was more likely associated with ascospore depletion in early spring rather than a direct effect of temperature (Gossen et al., 2021).

1.4.2 Moisture

Rainfall and leaf wetness are key factors for the infection, development, and spread of most plant diseases caused by fungal pathogens (Agrios, 2005). In the field, the release of sexual and asexual spores of *S. vesicarium* coincides with precipitation events (Prados-Ligero et al., 2003). Increased leaf wetness duration was positively correlated with increased germination of *Stemphylium* spp. conidia, and clover (*Trifolium* spp.) grown in moist field conditions exhibited increased disease severity of Stemphylium leaf spot when leaf wetness exceeded 3 h (Bradley et al., 2003). In contrast to this short interval for infection in clover, *S. vesicarium* requires at least 8 h of leaf wetness at 10 °C for successful infection, and severity increases with temperature (10–25 °C) and leaf wetness duration (8–24 h) (Suheri and Price, 2000b). Also, severe foliage damage and yield loss of garlic caused by *S. vesicarium* in South Africa occurred only when leaf-wetness periods exceeded 24 h during warm, humid summers (Aveling and Naude, 1992). In

addition, infection by *S. vesicarium* can be irreversibly halted by a prolonged dry period (Llorente and Montesinos, 2002).

Leaf spot epidemics on garlic and onion are favoured by humid conditions followed by dry, warm weather (Basallote-Ureba et al., 1999). Daily wetness duration and mean air temperature variables have been used in disease forecasting models to predict when foliar fungicides should be applied to pear (Llorente et al., 2012), asparagus (Bohlen-Janssen et al., 2018a), and onion (Stricker et al., 2020).

Vapour-pressure deficit (VPD) has been linked to spore production and dispersal of plant pathogenic fungi. VPD a measure of the ability of air to hold moisture, and is calculated from relative humidity and air temperature, but affects evapotranspiration, dew formation, and leaf wetness (Deshpande et al., 1995). In garlic fields in Spain, the maximum production of *S. vesicarium* ascospores was associated with VPD ≤ 0.5 kPa, and severe symptoms of leaf spot occurred with VPD ≤ 0.7 kPa (Prados-Ligero et al., 2003). High VPD over the previous 4 days is associated with low air-borne *S. vesicarium* spores in asparagus fields (Granke and Hausbeck, 2010). A similar pattern has also been reported for other pathogens that produce air-borne spores, such as *Alternaria porri* (Everts and Lacy, 1990).

1.4.3 Other environmental factors

There is little information on the effects of environmental stress, such as drought or heat, on the severity of SLB on onion. However, anecdotal reports from the Holland Marsh suggest that SLB symptoms develop earlier and become more severe in onion crops under stress than on those under optimal growth conditions.

A recent report has suggested that bacterial stalk and leaf necrosis of onion caused by *Pantoea agglomerans* (Ewing and Fife) Gavini may be linked to damage caused by onion thrips (*Thrips tabaci* Lindeman) (Grode et al., 2019). A similar trend has been observed under controlled environments with *S. vesicarium* on onion, where a reduction in thrips damage led to over two-fold reduction in SLB symptoms (Leach et al., 2020). Additionally, use of insecticides to reduce damage by thrips on onions resulted in a 40–50% decrease in SLB severity under laboratory conditions and symptoms were reduced by 27% in the field (Leach et al., 2020). Field trials where the onion crop was not treated with fungicides or insecticides to suppress onion thrips also exhibited 27% higher SLB damage and 83% less green foliage at the end of the season than those treated with fungicides but not insecticides (Hoepting, 2017a).

Soil moisture is another factor that may affect drought response. It can be measured as gravimetric soil water content (GSWC), which is the mass of water per unit mass of dry soil, or as volumetric soil water content (VSWC), which is the percent by volume. To calculate VSWC, the site-specific bulk density of the soil is needed. Previous research conducted at the Holland Marsh using muck soil plotted the site-specific soil moisture desorption within the field and also determined the gravimetric water content at the permanent wilting point to be 125% GSWC (Kora, 2004). Muck soils have unique moisture profiles, and may develop a nearly water-repellent layer if the soil moisture content drops below a critical point (Hewelke et al., 2016).

Drought conditions may increase or decrease disease incidence and severity, depending on the particular pathosystem being assessed. For example, onion white rot (caused by *Sclerotium cepivorum*), wheat take-all (caused by *Gaeumannomyces graminis* var. *tritici* J. Walker), wheat crown rot (caused by *Fusarium* spp.), and black leg of canola (caused by *Leptosphaeria maculans* Sowerby P. Karst) are projected to decrease in severity over time in response to future drought conditions, whereas Sclerotinia rot of kiwifruit caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is expected to increase (Wakelin et al., 2018). All four of these pathogens are Ascomycetes fungi, as is *S. vesicarium*. The effect of drought conditions on SLB has not been investigated.

Plant health, which is linked to disease susceptibility, and sporulation of *Stemphylium* spp. are both influenced by the quality and quantity of light available. Sporulation of *S. vesicarium* followed a diurnal pattern (Suheri and Price, 2000a) and required exposure to ultraviolet light (Suheri and Price, 2000b). Similarly, *S. botryosum* produced conidiophores only when exposed to near-ultraviolet radiation (320–420 nm), but a subsequent exposure to darkness was required to produce conidia (Leach, 1967). In India, the development of Stemphylium blight of lentil was favoured by < 7.7 h of sunshine, which may be linked to leaf wetness parameters (Sinha and Singh, 1993). Light quality and quantity, temperature, moisture availability, latitude, and the interaction of these elements can influence plant disease severity.

1.5 Management of Stemphylium leaf blight

Fungicide efficacy trials have been conducted at the Muck Crops Research Station in the Holland Marsh for the past decade, but the last time that fungicides suppressed disease compared to the unsprayed control was 2013 (Tesfaendrias et al., 2014; Stricker et al., 2020). Efforts to use sanitation and biocontrol methods did not reduce SLB severity more than the level provided by

fungicides alone (Llorente et al., 2010b). This indicates that current approaches for management of SLB are not effective. Cultural management methods may be more effective than fungicide applications.

1.5.1 Cultural management methods

Cultural management methods refer to agronomic approaches that reduce pathogen levels or reduce the rate of disease development by altering aspects of the plant host. For example, crop rotation with other non-host vegetables or cereals is a recommended strategy for *S. vesicarium* because it allows time for inoculum to break down before the next susceptible crop is planted (Chand and Kumar, 2016).

Sanitation to remove sources of inoculum can also be effective. In European pear orchards, plant residue on the orchard floor, composed of fallen fruit, dead leaves, grasses, and weeds is an important inoculum source of ascospores and conidia of *S. vesicarium* throughout the growing season (Rossi et al., 2005a, 2008; Köhl et al., 2009). Removal of the residue, especially in combination with other management tactics, reduced the disease with similar efficacy of fungicides alone (Llorente et al., 2010b). Inoculum of *S. vesicarium* was also reduced in garlic and asparagus fields by removal or burial of crop debris at the end of the growing season (Johnson, 1990; Katoch and Kumar, 2017).

Another commonly used cultural method is to increase plant spacing, which decreases both humidity within the crop canopy and leaf wetness duration (Brewster, 2008). Also, orienting rows so that they follow the direction of the prevailing wind improved airflow and accelerated leaf drying (Brewster, 2008). Overhead irrigation, poor drainage, and watering late in the day can also favour foliar diseases (Agrios, 2005). Additionally, increased calcium in pear fruit, which can be achieved through the use of quince rootstocks, was correlated with tolerance to BSP (Maurizio et al., 2019).

Plant pathogens in the genus *Stemphylium* only sporulate after exposure to light in the ultraviolet (UV) range. For example, sporulation of *S. botryosum* and the development of Stemphylium leaf spot symptoms were prevented on Welsh onion in greenhouses constructed with UV-absorbing vinyl film that blocked radiation < 360 nm (Sasaki et al., 1985). In addition, exposure to UV radiation decreases insensitivity of some *S. vesicarium* isolates to mancozeb fungicide (Hussein et al., 2007), which also suggests that UV filter covers could be a useful management method under controlled conditions.

Cultural management can provide some reduction in disease. Detailed knowledge of the pathogen is required for IPM systems to be effective. The IPM system for SLB in southern Ontario must be improved to sustainability maintain, or intensify, onion production in the region.

1.5.2 Fungicides

Fungicides have been grouped by biochemical mechanism and target site within the target pathogen (mode of action), and assigned a code number by the Fungicide Resistance Action Committee (FRAC, 2017). Fungicides in the same FRAC group use the same biochemical mechanism to disrupt the disease of fungi.

The fungicide groups that are currently registered in Canada to manage SLB are phthalimides (group M04), chloronitrile fungicides (group M05), demethylation inhibitors (DMI, group 3), succinate-dehydrogenase inhibitors (SDHI, group 7), anilino-pyrimidine fungicides (group 9), and strobilurin fungicides (QoI, group 11) but only a few are registered for the management of SLB (Health Canada, 2020) (Table 1.4). To reduce the risk of the development of fungicide insensitivity, it is important not to use one mode of action exclusively, but to apply several modes of action products as a mixture, or rotate between products with different modes of action (Brent and Hollomon, 2007a). Fungicides containing active ingredients from FRAC group 7 (fluxapyroxad and fluopyram), and group 3 (difenoconazole) have exhibited the best SLB reduction in field trials in New York (Dr. F. Hay, Cornell University, *personal communication*, 2018). In Italian pear orchards, application of captan (group M04), captan mixed with tebuconazole (group 3), boscalid (group 7), fludioxonil (group 12), and fluazinam (group 29) have provided sufficient suppression of BSP (Brunelli et al., 2014).

Table 1.4 List of fungicides registered in Canada to manage *Stemphylium vesicarium* on onion (Health Canada, 2020).

FRAC group	Active ingredient	Product name	Manufacturer	Year registered
3	Difenoconazole	Quadris Top®	Syngenta	2012
		Miravis Duo		
7	Fluxapyroxad	Sercadis®	BASF	2015
	Fluopyram	Luna® Tranquility	Bayer	2012
	Benzovindiflupyr	Aprovia®	Syngenta	2015
	Pydiflumetofen	Miravis Duo		
9	Pyrimethanil	Luna® Tranquility	Bayer	2012
11	Azoxystrobin	Quadris Top®	Syngenta	2012
		Quadris® Flowable	Syngenta	2000

A recent study of *S. vesicarium* on spinach in Texas demonstrated that preventative application of several fungicides, such as chlorothalonil (group M05), mancozeb (group M03), flutriafol + azoxystrobin (group 3 + 11), penthiopyrad (group 7), or azoxystrobin (group 11), decreased disease severity in a controlled environment (Liu et al., 2020).

In an older study on treatments to eradicate *S. vesicarium* from onion seed, the fungicides benomyl (FRAC group 1), a carbendazim + flusilazole mixture (FRAC groups 1 & 3), procymidone (FRAC group 2), tebuconazole (FRAC group 3), thiram (FRAC group M03), and anilazine (FRAC group M08) were tested for their efficacy in reducing the pathogen both on seed and mycelium growth in culture. Mycelial growth was inhibited by all of the fungicides, however, procymidone did not reduce seed infection (Aveling et al., 1993).

1.5.3 Biocontrol agents

Biocontrol uses antagonistic organisms or their byproducts to target pathogens and decrease disease symptoms. Several biocontrol agents have been tested against *S. vesicarium*, such as the bacteria *Pseudomonas fluorescens* (Flügge) Migula and *Pantoea agglomerans*, which have been used in a greenhouse to prevent brown spot of pear (Montesinos et al., 1996). Fungi such as *Trichoderma* spp., have also been used to target the saprophytic survival of overwintering *S. vesicarium* in pear orchards (Rossi and Pattori, 2009; Llorente et al., 2010b). The application of *Trichoderma harzianum* Rifai reduced the production of *S. vesicarium* conidia in the laboratory and in the field (Rossi and Pattori, 2009). *Trichoderma asperellum* Samuels, Lieckf & Nirenberg reduced mycelial growth of *S. vesicarium* in dual culture plates, and inoculation of onions with *T. asperellum* followed by *S. vesicarium* decreased SLB severity by nearly 50%

under controlled environmental conditions (Zapata-Sarmiento et al., 2020). However, *Trichoderma* spp. did not always reduce disease caused by *S. vesicarium* when applied to pear trees in the field (Ponti et al., 1993). Endophytic bacteria, such as *Bacillus subtilis* (Ehrenberg) Cohn and *Bacillus cereus* Frankland & Frankland also reduced mycelial growth of *S. vesicarium* in dual culture, but have not been tested *in planta* (Al-Badri et al., 2020).

Biocontrol methods may also include the application of products with antimicrobial properties. For example, propolis is a resinous material produced by bees that has been used in alternative medicine for its antibacterial and antifungal properties. In a recent study, propolis extracts inhibited *in vitro* growth of *S. vesicarium* and decreased disease incidence on detached pears (Loebler et al., 2020). Antimicrobial peptides also reduced mycelial growth of *S. vesicarium* and infection on detached pear leaves (Puig et al., 2014). In another study, extracts from common rue (*Ruta graveolens* L.) inhibited growth of *S. vesicarium* by > 60% (Reyes-Vaquero et al., 2021). However, these extracts have not been tested in the field.

The consistency and efficacy of biocontrol agents alone may not be as high as synthetic pesticides, because their efficacy is influenced by temperature, pH, moisture, and competition with other microorganisms in the phyllosphere (Howell, 2003). However, when *Trichoderma* spp. were used in combination with leaf litter removal, the disease suppression was comparable to a fungicide alone (Llorente et al., 2010b). A biocontrol agent containing *Trichoderma atroviridae* was tested in one field season in the Holland Marsh, ON, but did not suppress SLB on onion in Ontario (Stricker et al., 2020). This is an ongoing investigation at the Muck Crops Research Station. Currently there are no biocontrol agents commercially registered for management of Stemphylium leaf blight of onion in Canada.

1.5.4 Genetic resistance

Another approach to reducing the impact of *S. vesicarium* is to develop resistant onion cultivars. Onion species and cultivars differ in response to *S. vesicarium* (Behera et al., 2013). Field trials at the Holland Marsh showed some variation in susceptibility among cultivars, although the results varied from season to season (Foster et al., 2019). The cultivar Braddock had relatively low SLB incidence but high numbers of lesions per infected leaf, Milestone had high numbers of lesions but average leaf dieback symptoms, and Highlander had low lesions per leaf but high leaf dieback. Overall, no cultivar consistently exhibited strong resistance to SLB (Tayviah, 2017). Results from other locations are similar (Hoepting, 2020a). An onion cultivar developed in

southern India (RO-1) exhibited moderate resistance to SLB (Sharma and Sain, 2003). Another study in northern India reported three cultivars with moderate resistance (Behera et al., 2013). The bunching onion cultivar Pusa Soumya and the red onion cultivar Red Creole 2 also exhibited moderate resistance to SLB in India (Dangi et al., 2019).

Welsh onions (*Allium fistulosum*) are resistant or moderately-resistant to *S. vesicarium* (Pathak et al., 2001; McDonald and Vander Kooi, 2015). A breeding program in Taiwan crossed *A. fistulosum* and *A. cepa* in an effort to develop onion lines resistant to SLB (Pathak et al., 2001), but no resistant cultivar has been released. An interspecific hybrid of *A. cepa* and *A. fistulosum* was developed that carried recombinant chromosomes with resistance genes against *S. vesicarium* and was partially resistant in a field trial (Kudryavtseva et al., 2019). More research is needed to develop a marketable onion cultivar that is resistant to SLB.

1.5.5 Activated resistance

Plants can respond to biotic and abiotic stresses through the expression of active defence mechanisms. When appropriately stimulated, plants enter a state of enhanced responsiveness and capacity for response termed 'induced resistance'. Disease symptoms can be reduced if these mechanisms are stimulated before the pathogen is present (Choudhary et al., 2007). There are two main forms or pathways for induced resistance in plants: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Walters and Fountaine, 2009).

A resistance activator product is a substance that, when applied to a plant, activates resistance responses that decrease damage caused by abiotic stresses or pathogen infection (Walters and Fountaine, 2009). Resistance activator products may have an acute toxicity to plant hosts, pathogens, or non-target organisms, but this is often lower than that of synthetic pesticides (Thakur and Sohal, 2013). Plant activators that have been used for disease management in crops include acibenzolar-S-methyl, 2, 6- dichloroisonicotinic acid, β-aminobutyric acid, probenazole, salicylic acid, riboflavin, potassium phosphonate, and methyl jasmonate (Sreeja, 2014).

There is limited literature on activated resistance in onion crops. In one study, foliar applications of salicylic acid reduced SLB of onion by up to 40% (Abo-Elyousr et al., 2009). The application of acibenzolar-S-methyl, a structural analog of salicylic acid, also provided some reduction of Xanthomonas leaf blight of onion caused by the bacteria *Xanthomonas axonopodis* pv. *allii* Kadota (Lang et al., 2007). In a growth chamber study, acibenzolar-S-methyl reduced

populations of *X. axonopodis* pv. *allii* on onion leaves as effectively as copper hydroxide-mancozeb pesticide (Gent and Schwartz, 2005).

Civitas (Intelligro, manufactured by Petro-Canada Lubricants, Inc., a Suncor Energy Business, Mississauga, ON, Canada) is a food-grade mineral oil product that is often mixed with Harmonizer™ Pigment Dispersion (polychlorinated copper phthalocyanine pigment), which has been registered for use in the United States and Canada for turfgrass diseases, as well as some insects (http://www.civitasturf.com). Civitas has an ISR mode of action (Cortes-Barco et al., 2010), while the mode of action of Harmonizer may involve both ISR and SAR components (T. Hsiang, University of Guelph, *personal communication*, 2017). The current research project included an early drench application of Civitas soon after emergence, to assess if this product can provide early-season protection from SLB.

1.5.6 Forecasting models

Growers in Ontario have used a calendar-based method weekly or bi-weekly, to time application of fungicides for SLB management. This method does not depend on weather conditions or knowledge of the biology of the pathogen, and can result in more applications than necessary (Llorente et al., 2012). Unnecessary applications are not economical for the grower and can increase the risk of fungicide insensitivity developing in the pathogen population (Alberoni et al., 2010b). More recently the IPM program has been providing indications of disease risk and spray timing based on the research presented in this thesis and previous research summarized in Stricker et al. (2021).

Forecasting models use environmental factors to predict conditions that are conducive to disease development and may recommend when growers should apply pesticides. When used correctly, forecasting models can provide the same or better disease suppression as calendar-based methods, often with fewer fungicide applications. For instance, FAST, a forecast model developed for *Alternaria solani* Sorauer on tomatoes, achieved comparable disease suppression for brown spot of pear (BSP) as the 7-day calendar spray with 28% fewer applications (Montesinos and Vilardell, 1992). Another forecasting model, BSPcast, was designed for *S. vesicarium* in pear orchards (Montesinos et al., 1995). Validation of the model confirmed that the BSPcast-guided schedules used 20–50% fewer sprays and provided the same degree of disease suppression as a weekly application of thiram (Llorente et al., 2010b). The PAMcast model (*Pleospora allii* maturation forecast) was designed to include the environmental

conditions conducive to the development of the sexual phase of *S. vesicarium* (Llorente and Montesinos, 2004). PAMcast has been used to determine the date to begin the BSPcast forecasting system (Llorente et al., 2006).

The TOMcast model was developed based on the FAST model to manage foliar diseases of tomato in Ontario (Pitblado, 1992). Disease severity values (DSVs) were calculated based on leaf wetness duration and the average temperature during the wet period and sprays were recommended when the sum of daily values reached a set threshold (Montesinos and Vilardell, 1992). Ideally a disease forecasting model should reduce the number of sprays without compromising onion yield and quality. In a recent study, fungicides applied to onion according to TOMcast maintained comparable SLB incidence and severity compared to a calendar spray treatment under high disease pressure (Stricker et al., 2020). In years when the disease pressure was low, the SLB severity was not different than the unsprayed control. However, there was suspicion at this time that the *S. vesicarium* population was largely insensitive to most of the active ingredients applied, which would explain why there was little or no reduction in SLB severity or increase in crop yield in that study (Stricker et al., 2020).

TOMcast has also been successfully used for management of purple spot of asparagus (Meyer and Hausbeck, 2000). On some cultivars of asparagus, TOMcast can even improve the management of Stemphylium leaf spot without increasing the number of fungicide applications (Foster and McDonald, 2018). In 2012 and 2013, TOMcast reduced the number of fungicide applications on asparagus by 1 or 2 sprays, but sometimes recommended more sprays than the calendar-based method depending on the threshold selected (Foster, 2018). To our knowledge, a forecasting model specifically for *S. vesicarium* on onion has not yet been developed.

1.6 Fungicide insensitivity

The application of fungicides in agricultural systems is an essential part of crop management to maintain healthy crops and to safeguard crop yield and quality. Frequent use of a single fungicide can result in insensitivity in pathogen populations and a loss of disease suppression over time (Lucas et al., 2015). Fungicide insensitivity builds up in a fungal population treated with fungicides, beginning with the survival of rare mutants that are not killed by the fungicide (Brent and Hollomon, 2007a). If the mutation is not associated with a major fitness cost, the mutants survive and reproduce, which results in a population containing more insensitive isolates

in the next generation (Lucas et al., 2015). Repeated fungicide treatments can result in a rapid shift in the fungal population, until eventually the whole population consists of completely insensitive individuals.

The ability to reproduce sexually often increases the population diversity, can limit the accumulation of deleterious alleles, and increases the potential for adaptation to changes in the environment (Ene and Bennett, 2014). The 'Red Queen Hypothesis' states that host-pathogen relationships lead to co-adaptation cycles, where sexual outcrossing of the host promotes further adaptation by the pathogen as both host and pathogen try to stay ahead in an 'arms race' (Van Valen, 1973). This has been confirmed by a study on roundworm (*Caenorhabditis elegans* Maupas) and a bacterial parasite (*Serratia marcescens* Bizio), where populations of the parasite that were unable to sexually reproduce were driven to extinction and those capable of outcrossing survived (Morran et al., 2011). Sexual reproduction may increase the risk of a pathogen population developing insensitivity to fungicides, although not always (Brent and Hollomon, 2007b). This highlights the importance of confirming whether sexual reproduction of *S. vesicarium* is occurring in or near the onion fields in Ontario.

There are two main types of fungicide insensitivity: qualitative and quantitative. Qualitative insensitivity is typically conferred by a single-point mutation in the fungicide target gene, which leads to complete insensitivity (Lucas et al., 2015). As a result, individuals in the population will be either fully sensitive or fully insensitive. Insensitivity to quinone outside inhibitor (QoI) fungicides due to the G143A mutation in the cytochrome *b* complex is an example of qualitative insensitivity (Lucas et al., 2015). Another example would be point mutations in the *Bcmdl$_1$* and *Bcpos5* genes, which resulted in anilinopyrimidine insensitivity in *Botrytis cinerea* Pers. ex Fr. (Mosbach et al., 2017). Quantitative insensitivity occurs when mechanisms are available to export or otherwise detoxify the fungicide, resulting in varying levels of insensitivity (Deising et al., 2008). For example, mutations in various locations within the ß-tubulin gene have been linked to benzimidazole (FRAC group 1) insensitivity in several plant pathogens. Moderately insensitive strains of *Gibberella zeae* (Schwein.) Petch exhibited single-point mutations, and a highly insensitive isolate had two mutations (Chen et al., 2009). Rapid increases in the frequency of extreme phenotypes of quantitative insensitivity are the outcome of strong selection pressure, where individuals that are able to survive have passed on

their genes, and repeated exposure to the fungicide has shifted the population towards increasing insensitivity.

Four key mechanisms by which fungicide insensitivity is acquired are alteration of the target protein, overexpression of the target, degradation of the fungicide by metabolic enzymes, and efflux pumps that remove the fungicide from within the cell or away from the fungicide action site (Lucas et al., 2015). Additionally, fungicides may be converted into nontoxic compounds through biotransformation and conjugation (Kim et al., 2004). Fungicides with a single site of action are more at risk for the development of insensitivity (Brent and Hollomon, 2007a). The sensitivity to a fungicide is typically measured by calculating the fungicide concentration that causes 50% inhibition (EC_{50}) of conidial germination or mycelial growth compared to the nonamended control treatment (Gupta, 2016).

Countermeasures to prevent the development of fungicide insensitivity include reducing the dose or number of fungicide applications, and mixing or alternating with fungicides that have different modes of action (Lucas et al., 2015). However, the use of reduced fungicide doses to slow fungicide insensitivity development is controversial; some studies report that low doses increased this phenomenon (Ayer et al., 2020), and others report that it did not impact fungicide insensitivity development (Pijls and Shaw, 1997). Monitoring for fungicide insensitivity is an important tool that augments resistance management strategies such as adjusting application frequency, using cultural management methods, or mixing with non-cross-resistant products to ensure the continued effectiveness of the product. It is especially important considering the high cost of research, development, and registration of new pesticides.

The development of fungicide insensitivity has been linked to multiple fungicide applications in a growing season and three major risk factors: high genetic diversity of the pathogen, short generation time, and high potential for reproduction and dispersal of propagules (Brent and Hollomon, 2007a). Pathogens with polycyclic life cycles that produce windborne propagules will have a higher risk for developing fungicide insensitivity, especially in cropping systems with large monoculture fields (Gossen et al., 2014).

1.6.1 Fungicide insensitivity in *Stemphylium vesicarium*

Researchers have reported *S. vesicarium* populations that are insensitive to fungicides for the past two decades. Populations on onion are at high risk of insensitivity because onion is grown

on a large-scale and routinely receives multiple fungicide applications during a growing season, and *S. vesicarium* possesses all three of the risk factors for fungicide insensitivity: short asexual reproductive generations, production of air-borne spores and potential for sexual reproduction each year.

Brown spot of pear caused by *S. vesicarium* has caused economic losses since the early 1980s (Ponti et al., 1982), which is likely why most publications on the fungicide insensitivity in *S. vesicarium* are focused on that disease. Several fungicides have previously provided effective SLB or BSP suppression, but the efficacy varies by product and crop, and has decreased over time. One fungicide group that is registered for use on onion in Canada is the quinone outside inhibitor group (QoI; FRAC group 11, also known as strobilurins). QoIs disable the production of energy (adenosine triphosphate) in fungal pathogens by blocking the transfer of electrons at the outer quinone site of the cytochrome *bc1* complex in the electron transport chain (Bartlett et al., 2002). This can have a profound effect on conidial germination as well as mycelial growth (Jin et al., 2009). Insensitivity to group 11 fungicides has been documented in pear orchards in Italy since 2006 (Collina et al., 2007).

The alternative respiration system, the use of efflux transporters, or mutations that may change the structure of proteins within the cytochrome complex could all result in QoI-insensitivity (Fernández-Ortuño et al., 2008). Qualitative insensitivity to this group of fungicides is commonly conferred by a single nucleotide polymorphism which results in an amino acid substitution of glycine with alanine at position 143 (G143A) on cytochrome *b* (Bartlett et al., 2002). Two other mutations have also been identified: phenylalanine to leucine at position 129 (F129L) and glycine to arginine at position 137 (G137R) (Gisi et al., 2002). QoI fungicides have been available since 1996, and insensitive fungi were reported 4 years later (Heaney et al., 2000).

A QoI-insensitive isolate of *S. vesicarium* was first detected in an Italian pear orchard, and the G143A mutation was confirmed (Collina et al., 2007). This mutation was also detected in azoxystrobin-insensitive strains of *S. vesicarium* in New York state (Hay et al., 2019). In addition, cross-insensitivity between azoxystrobin and pyraclostrobin (another QoI) has been documented (Hay et al., 2019). QoI insensitivity due to G143A has been documented in several other plant pathogenic fungi, including *Botrytis cinerea*, *Didymella rabiei* (Kovatsch.) Arx, *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, and *Cercospora beticola* Sacc. (Kim et al., 2003; Banno et al., 2009; Bolton et al., 2013; Owati et al., 2017). The F129L mutation site has been

documented in *Alternaria solani*, a relative of *S. vesicarium* (Rosenzweig et al., 2008). In addition, the frequency of the F129L substitutions within the *A. solani* population was dependent on management practices (Rosenzweig et al., 2008).

Dicarboximide fungicides (FRAC group 2) interfere with of the osmotic signal transduction pathway, which consists of histidine kinase (HK) and mitogen-activated protein (MAP) kinase cascades (Yamaguchi and Fujimura, 2005). Mutations in the HK gene *SvHK1* conferred degrees of sensitivity to dicarboximide fungicides in *S. vesicarium* isolates from pear orchards in Italy (Alberoni et al., 2010b). Isolates that are sensitive to procymidone and iprodione but moderately insensitive to vinclozolin exhibited the F267L mutation, while those highly insensitive to procymidone but moderately insensitive to the other dicarboximide exhibited the L290S mutation, and isolates highly insensitive to all dicarboximides exhibited T765R or Q777R mutations in the *SvHK1* gene (Alberoni et al., 2010b). This is an example of quantitative insensitivity. Dicarboximide-insensitive strains of *Alternaria brassicicola* (Schwein.) Wiltshire may also have mutations in *AbNIK1*, a HK gene (Avenot et al., 2005). Other researchers have reported other HK gene mutations that result in insensitivity to dicarboximide fungicides in *Alternaria alternata*, *Sclerotinia sclerotiorum*, and *B. cinerea,* among others (Dry et al., 2004; Fillinger et al., 2012; Duan et al., 2013). In a recent study, *S. vesicarium* isolates collected in New York state were sensitive to iprodione ($EC_{50} < 0.5$ µg a.i. mL^{-1}) (Hay et al., 2019). Iprodione is not registered for use on onion in Canada.

Phenylpyrrole fungicides (PP; FRAC group 12) are derived from an antifungal compound produced by *Pseudomonas* spp. (Gehmann et al., 1990). The mode of action of this group appears to differ for each active ingredient. One study has shown that exposure to fenpiclonil results in the accumulation of the lipophilic cation tetraphenyl-phosphonium bromide, which modifies the membrane potential of the mitochondria (Jespers et al., 1993). A subsequent study documented the intracellular accumulation of glycerol and mannitol, which suggested that the mode of action may be inhibition of the phosphorylation of glucose (Jespers and De Waard, 1995). The proposed mode of action for fludioxonil is that binding to the class III hybrid histidine kinase (HHK) mimics osmotic stress by activating Os-2/Hog1 MAP kinases (reviewed in Kilani and Fillinger, 2016). Only a few mutations for insensitivity have been documented and mapped to the class III HHK genes in *Alternaria* spp. (Iacomi-Vasilescu et al., 2004) and *B. cinerea* (Ren et al., 2016). In New York, *S. vesicarium* isolates were highly sensitive to

fludioxonil (Hay et al., 2019), but fludioxonil-insensitive isolates of *S. eturmiunum* have been isolated from garlic in China (Chen et al., 2020).

It has been proposed that anilinopyrimidine fungicides (AP; FRAC group 9) inhibit the biosynthesis of methionine, and the proposed target is cystathionine β-lyase or cystathionine γ-synthase (Jeschke et al., 2019). However, this was recently disputed, because pyrimethanil affects an unknown gene that simultaneously controls methionine, cystine, and cysteine biosynthesis in *S. sclerotiorum* (Hou et al., 2018). Another recent study identified nine genes for nuclear-encoded proteins targeted to the mitochondria that contain insensitivity-conferring mutations; *Bcmdl1*^{E407K} and *Bcpos5*^{L412F} mutations accounted for 76% of AP insensitive isolates in *Botrytis cinerea* on grape (Mosbach et al., 2017). Several isolates of *S. vesicarium* collected in New York state were insensitive (EC_{50} > 10 µg a.i. mL^{-1}) to the AP fungicides cyprodinil and pyrimethanil (11 and 15 insensitive isolates out of 46, respectively) (Hay et al., 2019). It is unclear if this type of insensitivity is qualitative or quantitative.

Succinate dehydrogenase inhibitor fungicides (SDHI; FRAC group 7) , which target the succinate dehydrogenase complex in the mitochondrial respiration chain (Keon et al., 1991), have been in use since the late 1960s. SDHIs inhibit spore germination, germ-tube elongation, mycelial growth, and sporulation by blocking electron transport in the mitochondrial respiratory chain through inhibition of succinate dehydrogenase (complex II) (Lunn, 2011). Succinate dehydrogenase is an enzyme necessary for aerobic respiration and is constructed of four nuclear encoded protein subunits: sdhA, sdhB, sdhC, and sdhD (Hägerhäll, 1997). SDHI fungicides bind to the ubiquinone binding site that is formed by the interface of the sdhB, sdhC and sdhD subunits (Avenot and Michailides, 2010). Mutations that result in SDHI insensitivity have been documented in all three of these subunit genes (Sun et al., 2016). In addition, each mutation confers differing degrees of insensitivity to individual active ingredients in the SDHI group (Scalliet et al., 2012). Isolates may possess one to eight different sdh substitution mutations, resulting in a range of sensitivity phenotypes (low, moderate, or high insensitivity to the active ingredient) (Pearce et al., 2019). In a recent study, most of the *S. vesicarium* isolates collected in New York were sensitive to the SDHI fungicides fluopyram and fluxapyroxad, but over half were insensitive to boscalid, with an EC_{50} > 10 µg a.i. mL^{-1} (Hay et al., 2019). Insensitivity to SDHIs is considered to be quantitative insensitivity.

Some genetic mutations can increase sensitivity to SDHI fungicides; the greater sensitivity to fluopyram and boscalid of some isolates of *Fusarium tucumaniae* O'Donnell & Aoki was attributed to the mutation G277R in sdhB (Sang et al., 2018). The sdhB-H277Y mutation in *Alternaria alternata* and sdhB-H267T in *Clarireedia jacksonii* Beirn, B.B. Clarke, C. Salgado & J.A. Crouch both resulted in sensitivity to fluopyram but also conferred decreased sensitivity to boscalid (Avenot and Michailides, 2010; Popko et al., 2018). Some isolates of *A. alternata* with the H277Y/R mutation in sdhB exhibited insensitivity to boscalid but were sensitive to fluopyram, while isolates with the H134R mutation in sdhC or H133R in sdhD were insensitive to both active ingredients (Avenot et al., 2014, 2019). Of 118 *A. solani* isolates with the D123E mutation in sdhD, 80% exhibited insensitivity to boscalid and 50% exhibited slight insensitivity to fluopyram (EC_{50} = 0.2–3 µg a.i. mL^{-1}) (Bauske et al., 2018). Additionally, isolates of *Botrytis cinerea* that were insensitive to boscalid were sensitive to fluopyram (EC_{50} = 0.01–1.96 µg a.i. mL^{-1}), which was associated with the sdhB-N230I mutation (Fernández-Ortuño et al., 2017). Fluopyram binds in a different manner to the cavity within the sdhB protein than other SDHI active ingredients, which may explain the few reported cases of cross-insensitivity with other group 7 fungicides (Fraaije et al., 2012).

Field trials in New York in 2017 demonstrated that fungicides containing fluopyram reduced SLB severity by 69% compared to the unsprayed control (Hoepting, 2018a). Additionally, *in vitro* studies in New York demonstrated a high level (> 90%) of sensitivity to fluopyram in the *S. vesicarium* population in New York (Hay et al., 2019). Over half of the isolates in New York were insensitive to another SDHI fungicide, boscalid (Hay et al., 2019). However, it is important to note that due to the different binding capacity, the results from boscalid cannot extrapolated to other fungicides in group 7 such as fluopyram, fluxapyroxad, and benzovindiflupyr.

Demethylation inhibitor fungicides (DMI; FRAC group 3) destabilise cell membranes by inhibiting C14 demethylation during sterol formation (Ziogas and Malandrakis, 2015). DMIs specifically prevent the formation of membrane sterols by targeting sterol 14α-demethylase cytochrome P450 (Joseph-Horne and Hollomon, 1997). Insensitivity to DMI fungicides is often quantitative and can be conferred through many different mechanisms, including modification of the target site CYP51 gene, overexpression of CYP51, reduced accumulation of DMIs in fungal cells, or the production of paralogues (gene copies within the genome which may be slightly

different) of the target gene (Ziogas and Malandrakis, 2015). Other point mutations (Mair et al., 2016), overexpression of the fungicide target gene (Ma et al., 2006), overexpression of transmembrane transport proteins (Price et al., 2015), or changes in sterol biosynthesis (Karaoglanidis et al., 2001) can also result in DMI-insensitivity.

Insensitivity to DMI fungicides has not been previously documented for *Stemphylium vesicarium*, but a recent study suggests that increased tolerance to DMIs in populations of *Alternaria alternata* in China may have increased. Isolates of *A. alternata* were collected from seven regions in China, and growth was inhibited by 0.12–0.2 μg a.i. mL^{-1} but the amount of inhibition differed by region (He et al., 2019). Reduced-sensitivity to the DMI fungicide difenoconazole has been reported for two other fungal pathogens, *Stemphylium solani* on cotton (Mehta and Oliveira, 1998) and *Venturia inaequalis* (Cooke) G. Winter in apple orchards (Mondino et al., 2015), which are both also in the order Pleosporales. However, of the 46 *S. vesicarium* isolates collected in New York, 98% were sensitive (EC_{50} < 0.5 μg a.i. mL^{-1}) to difenoconazole (Hay et al., 2019).

Insensitivity to multi-site fungicides, such as FRAC groups M03, M04, and M05, is rare. A mancozeb-insensitive (group M03) strain of *A. alternata* was reported in South Greece on tomatoes (Malandrakis et al., 2015), which may be the first and only report of insensitivity to this fungicide group. The mechanism of insensitivity has not been explained. Symptoms caused by *S. solani* on cotton in Brazil were reduced when treated with 2000 g a.i. ha^{-1} mancozeb (Mehta and Oliveira, 1998), but this treatment did not differ from the unsprayed control when the experiment was repeated. Also, recent field studies in New York using mancozeb did not suppress SLB (Hoepting, 2018a, 2019, 2020b; c). Fungicides containing mancozeb have not been registered for management of SLB in Canada. Insensitivity to fungicides in the FRAC group M05, such as chlorothalonil, has not yet been reported (FRAG-UK, 2016).

There has been no assessment of *in vitro* fungicide sensitivity of *S. vesicarium* in onion production systems in Canada. For several years, researchers at the Muck Crops Research Station in the Holland Marsh, ON, have been testing fungicides for the management of *S. vesicarium* on onions. From 2014–2019 field seasons, none of the commercial fungicides selected from various FRAC groups decreased the severity of SLB compared to the control (Stricker et al., 2020). To select fungicides that are effective in the field, the *S. vesicarium* population of southern Ontario should be screened for sensitivity to active ingredients of

commonly used fungicides, so the use of products for which high levels of insensitivity are present in the population can be avoided, or the use pattern modified.

1.7 Hypothesis and Objectives

The overall objective of this project was to improve the management of Stemphylium leaf blight of onion in Ontario. The specific objectives of the proposed work were as follows:

Pathogen identification:

1. Confirm the identify of *S. vesicarium* using molecular methods.

Sources of inoculum:

2. Assess alternative hosts in the Holland Marsh for susceptibility to *S. vesicarium.*
3. Determine the overwintering survival of *S. vesicarium* on onion in Ontario.
4. Assess infected seed as a potential source of primary inoculum for onions.

Fungicides:

5. Screen the pathogen population for *in vitro* insensitivity to common fungicides used on onion, especially azoxystrobin and fluopyram.
6. Assess existing disease forecasting models for SLB to identify the most effective ones.

Abiotic Factors:

7. Determine if plant stress by drought increases susceptibility to *S. vesicarium*.
8. Identify weather factors that affect the concentration of air-borne *S. vesicarium* spores.

The hypotheses of the proposed work are as follows:

1. The pathogen causing leaf blight symptoms in the Holland Marsh is *S. vesicarium.*
2. Common weed species in the Holland Marsh are hosts of *S. vesicarium* and act as reservoirs of inoculum.
3. *S. vesicarium* produces overwintering structures on onion residue.
4. Infection of onion seed by *S. vesicarium* can occur in the field during flowering and resulting seedlings are infected with *S. vesicarium*.
5. The *S. vesicarium* population in onion-growing regions of southern Ontario is insensitive to fungicides in the FRAC groups 11, 7, 3, and 9.

6. A forecasting model based on environmental conditions can be used to trigger fungicide applications to provide comparable suppression levels as a calendar-based spray schedule, with a lower number of applications.
7. Drought stress increases severity of SLB symptoms on onion.
8. Weather factors related to increased leaf wetness, humidity, and precipitation increase sporulation of *S. vesicarium*.

These objectives were chosen to address gaps in the existing research regarding SLB on onion in Ontario. The pathogen has previously only been identified based on morphology, but confirmation of the identity using molecular methods is desired because many species of *Stemphylium* are morphologically similar. Growers in the region report an increase in SLB severity over the past decade, which may be associated with plant stress, increased inoculum, or decreased fungicide efficacy. This research aimed to gain more information about the epidemiology of SLB and develop improved management strategies for onion growers in Ontario.

Chapter 2 Epidemiology and life cycle of *Stemphylium vesicarium*

The results from the gene sequencing of *S. vesicarium* have been published (Foster et al., 2019).

2.1 Introduction

Stemphylium vesicarium can infect and cause disease on a range of plant hosts. SLB of onion is currently managed through integrated pest management (IPM) strategies that include fungicide application and cultural management methods. Effective IPM requires knowledge of the pathogen's identity, life cycle, and alternative hosts. It is also important to correctly identify a pathogen to assess results from the available literature. Microscopy is often used to identify fungal spores, but identifying the spores of *Stemphylium* to species is difficult because of overlapping morphological characteristics within the genus and even among related species. The *Stemphylium* genus is somewhat morphologically similar to genera such as *Alternaria* and *Ulocladium*, which also produce multicellular conidia on proliferating conidiophores with apically-swollen conidiogenous cells (Simmons, 1967). Furthermore, symptoms caused by *Alternaria porri* on onion can be confused with SLB (Denis et al., 2010) by those who are not trained in disease identification. In the genus *Stemphylium*, spore size and colony morphology can be variable (Hassan et al., 2020), so identification based on the internal transcribed spacer (ITS) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene sequences has largely replaced morphology for species validation. Other gene regions that have been used to discriminate among *Stemphylium* spp. include the translation elongation factor 1α (*TEf-A*), cytochrome *b* (*cytB*), calmodulin, actin assembly-inducing protein, histidine kinase (HK) and the intergenic spacer between the vacuolar membrane ATPase catalytic subunit A gene and the vacuolar protein sorting-associated protein (vmaA-vpsA) (Câmara et al., 2002; Samac et al., 2014; Graf et al., 2016; Woudenberg et al., 2017; Das et al., 2019). The species *S. vesicarium*, *S. herbarum*, *S. alfalfae*, *S. tomatonis*, and *S. sedicola* were combined in a single clade within the genus because they could not be differentiated even using molecular methods (Inderbitzin et al., 2009; Wang et al., 2010; Woudenberg et al., 2017).

Once the identity of the pathogen has been established, the next step is to describe the disease cycle. The timing of fungicide applications can often be improved through an understanding of the biology of the target pathogen. Also, the efficacy of cultural management methods often depends on exploiting a vulnerable period in the disease cycle. Determining when

management practices should begin in a growing season can be an important component of IPM. For example, onion growers in Nova Scotia commonly use the first detection of *S. vesicarium* spores on onion or barley leaf tissue in the growing season as the action threshold to initiate the application of foliar fungicides to manage SLB (Dr. P. Hildebrand, Hildebrand Disease Management, *personal communication*). In Ontario, growers typically begin spraying when the onion crop has reached the 3–4 leaf growth stage, or when SLB symptoms have been reported in the area. A recent study demonstrated that the number of applications per season in Ontario could be substantially reduced using any one of several disease forecasting programs (Tayviah, 2017; Stricker et al., 2020).

Removal of plant residue, such as fallen fruit, dead leaves, and weeds, is used to manage brown spot of pear caused by *S. vesicarium* in pear orchards, since these residues may be a source of inoculum of *S. vesicarium* (Rossi et al., 2005a, 2008; Köhl et al., 2009; Llorente et al., 2010b). Removal of crop residue practice is also recommended in garlic and asparagus fields to manage diseases caused by *S. vesicarium* (Johnson, 1990; Katoch and Kumar, 2017). In Ontario, the current IPM recommendations for onions include rotation with non-host crops, destroying infected crops, application of registered fungicides, and minimizing damage caused by herbicide applications and onion thrips (OMAFRA, 2009).

Reduction of primary inoculum is one management tactic that can be incorporated into IPM strategies, so it is important to know where it originates. In theory, reducing initial inoculum for polycyclic diseases is not as important as reducing the rate of disease development. The relationship of initial development and disease development for SLB on onion in Ontario is not yet known. It is also not known how and where *S. vesicarium* overwinters in or near onion fields in Ontario. High levels of air-borne ascospores are present in early spring in or near onion fields in Ontario (Gossen et al., 2021). This indicated that pseudothecia, which are initiated in the fall to produce ascospores in spring, are certainly present near onion fields in late winter or early spring. One possible source of ascospores could be viable pseudothecia in overwintered onion leaf residue, as has been observed in warmer climates such as Spain, Italy, and India (Basallote-Ureba et al., 1999; Rossi and Pattori, 2009; Katoch and Kumar, 2017). However, a small study did not find pseudothecia after winter on onion residue in this region in one year of testing (Gossen et al., 2021). Another possibility is that the pathogen, which has a wide host range, overwinters on some other plant hosts in the region.

It is also possible that inoculum on the seed at planting contributes to early SLB development (Neergaard, 1977). The pathogen has been reported at high rates in seed of commercial onion (Aveling et al., 1993) and radish seed (Belisario et al., 2008), and has also been isolated from turfgrass seeds (Ban et al., 2021). Many seed companies take active measures to minimize this possibility, such as treating the seeds with a hot water bath prior to sale (Stokes Seed, *personal communication*). Therefore, there are several potential sources of primary inoculum of *S. vesicarium* in the Holland Marsh: onion leaf residues left in the field, local plant species that are alternative hosts, and seed-borne propagules of *S. vesicarium*.

The objectives of the study were i) to sequence diagnostic gene regions of isolates to validate the species identification (part of this work has been published, Foster et al., 2019), ii) to determine if *S. vesicarium* survives overwinter on onion leaves in the region; iii) to assess if local weed species are alternative hosts for *S. vesicarium*, and iv) to determine if seed-borne *S. vesicarium* is a potential source of primary inoculum in southern Ontario.

2.2 Materials and methods

2.2.1 Sample preparation and growth media

Isolates of *S. vesicarium* were obtained from symptomatic onion leaves. The leaves were surface sterilized as follows: samples were dipped in 70% ethanol for 30 sec, then in 5% commercial bleach (0.5% sodium hypochlorite v/v) for 1 min, followed by a rinse with sterile water for 2 min. Leaf pieces were cultured on V8-agar media (200 mL V8 juice [Campbell Soup Co., Etobicoke, ON], 3 g calcium carbonate [Sigma-Aldrich, Oakville, ON], 20 g agar [Fisher Scientific, Mississauga, ON], and 800 mL deionized water) in plastic Petri dishes (90 mm dia ×15 mm, Fisher Scientific, Fair Lawn, NJ) sealed with Parafilm (Bemis Company, Inc., WI). This medium was also used for routine culture of *S. vesicarium.* When culturing fungi from onion, leek, or asparagus samples collected from the field, the V8 media was amended with antibiotics (250 mg L^{-1} ampicillin [Sigma-Aldrich, Oakville, ON], 150 mg L^{-1} streptomycin [Sigma-Aldrich, Oakville, ON]). If conidia were required for identification or inoculation, the colonies were incubated under UV light (15 watt F15T8/BLB, Westinghouse Lamps, McNulty, PA) (12 h on / 12 h off) at room temperature for 3–5 days to stimulate production of conidia.

2.2.2 Pathogen identification

Morphological characteristics

Isolates were obtained from commercial onion fields in Ontario, at the Holland Marsh (44.0415°N, 79.6001°W), Grand Bend (43.241226°N, 81.816714°W), and Keswick (44.223634°N, 79.417749°W), and from asparagus fields from five counties in southern Ontario. Two historical isolates were purchased from the Canadian Collection of Fungal Cultures (Ottawa Research and Development Centre, Agriculture and Agri-Food Canada), and three isolates were contributed by Dr. P.D. Hildebrand from commercial onion fields in Kings County, NS. Five isolates were cultured from onion seed collected from flowers that had been naturally inoculated at the Muck Crops Research Station in the Holland Marsh (Table 2.1). Symptomatic tissue was collected, surface sterilized, and plated onto V8 agar amended with antibiotics. Fungal colonies that matched the desired phenotype were purified by hyphal-tip culture.

Table 2.1 *Stemphylium vesicarium* isolates, collection years, host plants, and collection sites.

Isolate	Year	Host Plant	Location collected
225106	1995	oat	Crop District 9B, western SK
225105	1995	oat	Crop District 8A, eastern SK
OA17[1]	2012	asparagus	Fairground, Norfolk, ON
OA20	2012	asparagus	Fairground, Norfolk, ON
OA23	2012	asparagus	Calton, Elgin, ON
OA46	2013	asparagus	Gilbertville, Norfolk, ON
OA47	2014	asparagus	Florence, Chatham-Kent, ON
OA48	2014	asparagus	Harrow, Essex, ON
NA51	2014	asparagus	Canning, Kings, NS
NA61	2014	asparagus	Canning, Kings, NS
OA65	2014	asparagus	Harrow, Essex, ON
OA66	2015	asparagus	Corinth, Elgin, ON
OO25	2013	onion	Holland Marsh, Simcoe, ON
OO26	2013	onion	Holland Marsh, Simcoe, ON
OO27	2013	onion	Holland Marsh, Simcoe, ON
OO31	2013	onion	Holland Marsh, Simcoe, ON
NO35	2013	onion	Avonport, Kings, NS
NO36	2013	onion	Avonport, Kings, NS
OO69	2016	onion	Holland Marsh, York, ON
OO1	2019	onion seed	Holland Marsh, York, ON
OO2	2019	onion seed	Holland Marsh, York, ON
OO3	2019	onion seed	Holland Marsh, York, ON
OO4	2019	onion seed	Holland Marsh, York, ON
OO5	2019	onion seed	Holland Marsh, York, ON

[1]Naming convention: first letter N = Nova Scotia, O = Ontario, second letter, A = asparagus was the host, O = onion was the host.

Isolates were identified based on conidial morphology. Conidia of *S. vesicarium* are brown, oval or ellipsoidal, 22–42 μm in length and 12–25 μm wide, with 3–5 transverse and 4–10 longitudinal septa (Simmons, 1969). The conidia were collected by gently scraping the colony surface with a sterile scalpel and viewed with a compound microscope at 125 × magnification to verify spore morphology. Photos of the conidia were taken using a light microscope (Nikon Eclipse 50i) and size was assessed using NIS-Elements BR 4.60 software. The isolates were maintained in long-term storage on V8 agar slants and water plus V8 agar cubes at 4 °C.

DNA extraction, PCR amplification and identification

The DNA of 17 *S. vesicarium* isolates collected from onion or asparagus in 2012–2016, 2 isolates from oat in 1995, and 5 isolates collected from onion seed in 2019 (Table 2.1) was extracted from mycelia as follows (Foster et al., 2019). The isolates were cultured on V8 agar for

3 days at room temperature (22 °C) and darkness, after which four 5-mm-dia. plugs were taken from the edge of an actively growing colony and transferred into a flask containing 100 mL potato dextrose broth media (Difco, Becton Dickinson, and Co., Sparks, MO). The inoculated flasks were incubated at room temperature on an orbital shaker at 90 rpm, and mycelial mats were collected 14 days later by filtering through sterile cheesecloth. The mats were transferred to sterile micro-centrifuge tubes containing one tungsten carbide bead and flash frozen in liquid nitrogen. DNA was extracted using a DNeasy Plant Kit (Qiagen, Toronto, ON) according to the manufacturer's protocol and stored at -80 °C.

Species-specific primers were selected based on previous literature (Table 2.2) to amplify regions of the ITS, *gapdh*, *TEf-A*, *cytB*, and vmA-vspA genes of *S. vesicarium*, based on GenBank accessions. In a previous study, the ITS, *gapdh*, and *TEf-A* sequences were used to separate *S. vesicarium* (syn. *P. herbarum*) from other closely related *Stemphylium* spp. (Inderbitzin et al., 2009). The *cytB* sequence (Graf et al., 2016) was included to differentiate *S. vesicarium* from *S. botryosum*, since the two pathogens have similar host ranges and distributions. VmaA-vpsA has been used for evolutionary studies of *Stemphylium* species (Inderbitzin et al., 2009; Graf et al., 2016).

Table 2.2 Description and sequence of the primers used to confirm the identification of *Stemphylium vesicarium* isolates collected in Ontario.

Gene region	Primer name	Primer sequence	Source
Internal transcribed spacer (ITS)	ITS1 ITS4	5′-TCCGTAGGTGAACCTGCGG-3′ 5′-TCCTCCGCTTATTGATATGC-3′	(White et al., 1990)
Glyceraldehyde 3-phosphate dehydro-genase (*gapdh*)	GPD1 GPD2	5′-CAACGGCTTCGGTCGCATTG-3′ 5′-GCCAAGCAGTTGGTTGTGC-3′	(Berbee et al., 1999)
Translational elongation factor EF-1 alpha (*TEf-A*)	EF446f EF1473r	5′-TCACTTGATCTACAAGTGCGGTGG-3′ 5′-CGATCTTGTAGACATCCTGGAGG-3′	(Inderbitzin et al., 2005)
Cytochrome *b* region (*cytB*)	KES1999 KES2000	5′- GACCGTCGGCCATATAAAGGGTCG-3′ 5′-AACCGTCTCCGTCTATCAATCCT GCT-3′	(Graf et al., 2016)
Intergenic spacer between vacuolar membrane ATPase catalytic subunit A gene and vpsA (vmaA–vpsA)	VATP2949f VATP3238r	5′-TCGATCAGTTACAGCAAGTAC-3′ 5′-GCCTTCTGCGCTTCGTCGTGG-3′	(Inderbitzin et al., 2005)

The PCR reaction was performed in a total volume of 50 µL containing 1x PCR buffer (50 mM Tris-HCl, pH 8.5); 2.0 mM $MgSO_4$; 0.2 mM dNTP; 0.2 µM of each primer separately; 0.04 U Taq DNA polymerase (Biobasic, Scarborough, ON); and 4 µL DNA template. Amplifications were performed in a Mastercycler pro384 thermal cycler (Eppendorf Canada, Mississauga, ON). The PCR program consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 7 min.

A sample of each PCR product was resolved using horizontal gel electrophoresis in 1% agarose gels in 0.5 × tris-borate-ethylenediaminetetraacetic acid buffers at 100 V cm^{-1} for 75 min. Gels were stained with ethidium bromide (0.5 µg mL^{-1}), digitally visualized, and photographed using a Gel Doc XR+ Imaging System (BioRad, Hercules, CA) (Figure 2.1).

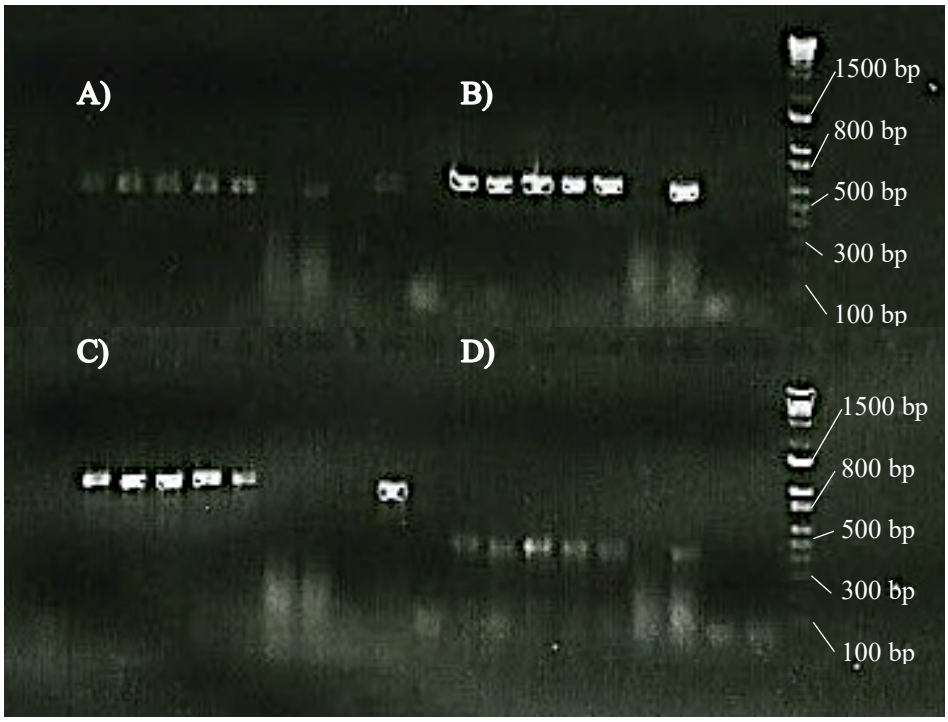

Figure 2.1 Gel electrophoresis of seven *Stemphylium vesicarium* isolates, one *Plasmodiophora brassicae* isolate and one *Colletotrichum fioriniae* isolate (in that order) using primers to amplify the: A) internal transcribed spacer; B) glyceraldehyde 3-phosphate dehydrogenase; C) translation elongation factor EF-1 alpha; and D) cytochrome *b* regions. Ladders on the right indicate regions of 100 bp–10 Kb.

The PCR products exhibiting a single band of the expected size were purified by removing the excess dNTPs, primers, and reagents using RapidTip 'clean-up' pipette tips (Diffinity Genomics, West Henrietta, NY) and the Mag-Bind SeqDTR kit (Omega Bio-Tek, Norcross, GA). Sanger sequencing was completed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and 3500 Series Data Collection Software 2 (Applied Biosystems, Foster City, CA). The forward and reverse sequences were assembled into contigs using the sequence assembly software CAP3 (http://doua.prabi.fr/software/cap3) using default settings. The resulting contigs were compared to *S. vesicarium* gene sequences in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLASTN 2.8.0+ (Zhang et al. 2000; Morguilis et al. 2008). The contigs of eight isolates (OA20, OO27, OO31, NO36, OA46, OA48, NA51, NO35) were additionally submitted to the online database (GenBank accession numbers MH628098–MH628128).

2.2.3 Sexual reproduction and overwintering

Yellow bulb onion cultivar LaSalle (Stokes Seeds, Thorold ON) was seeded in a growth room set at 22 °C and 17 h photoperiod and grown for 4 months. Mature leaves were inoculated with an isolate of *S. vesicarium* (Onion1) that had recently been cultured from onion leaf tissue collected at the Holland Marsh, ON. Plugs were cut from the edge of actively growing *S. vesicarium* colonies on V8 agar with a 5-mm cork-borer and affixed, culture-side down, to onion leaves with parafilm. After lesions had formed, the parafilm was removed and the leaves were allowed to naturally desiccate. Once dry, the leaves were cut into 6–10 cm sections surrounding the lesions. These leaf sections were stored in the dark at room temperature for 1–5 weeks, until enough samples were collected to complete the experiment.

The infected leaf sections were randomly allotted to one of four treatments: dry-buried, wet-buried, dry-surface, or wet-surface. Dry leaves simulated those stuck to farm equipment, in storage bins, or blown into barns and other sheltered areas. Additionally, the surface-sterilization process would eliminate the possibility that the pathogen was only surviving on the external surface of the leaf. In the dry treatment, the infected leaf was placed into a sealed plastic tube (Falcon 50 mL conical centrifuge tubes, polyethylene, Fisher Scientific #14-432-22) inside a fabric bag (polyester, 200 mm × 200 mm). For the wet treatment, the infected leaf was placed loosely into the same fabric bag as the sealed plastic tube. The fabric used to make the bags was chosen to allow exchange of gases and moisture but was fine enough to retain fragments of leaf tissue. These fabric bags were either buried (buried treatment) or fastened to the surface of the soil (surface treatment) using two metal wire stake flags (63.5 mm × 88.9 mm polyvinyl flag with a 533 mm wire stem, Empire, Mukwonago, WI) twined together (Figure 2.2). For the buried treatment, the bags were placed in the bottom of a large plastic pot (30.5 cm dia. ×13 cm depth, Panterra Bowl Clay, The HC Companies, Middlefield, OH), which was then filled to the brim with local muck soil (organic matter ≈ 69%, pH ≈ 6.1) and buried at the Muck Crops Research Station (MCRS) so that the top was flush with the surface of the ground.

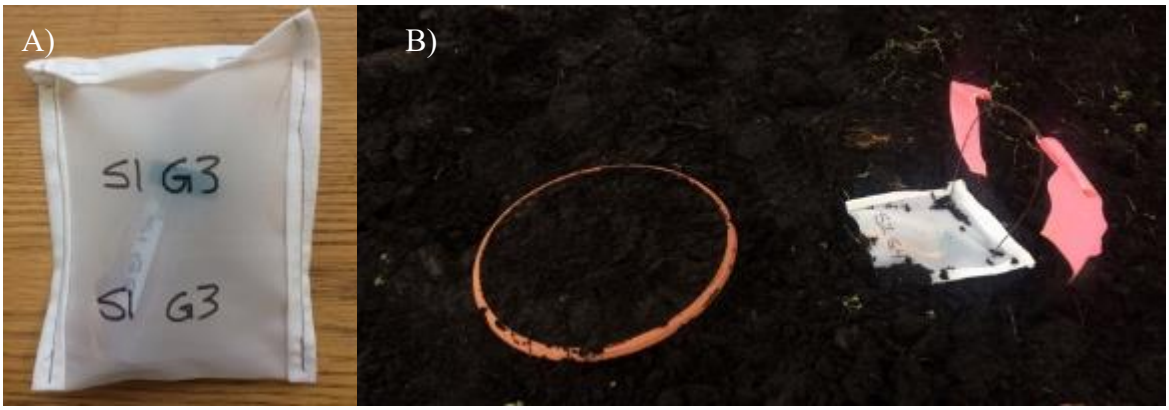

Figure 2.2 Sample preparation and deployment in a study to assess survival of *Stemphylium vesicarium* on onion leaves: A) fabric bag containing one onion leaf infected with *S. vesicarium* and a Falcon tube containing a second inoculated leaf, and B) one bag buried in a plastic pot (left) and one bag pinned to the soil surface (right).

On 8 November 2018 the buried/surface treatments were arranged in the field in a completely randomized design with five samples dates arranged as blocks, where each sampling date would be destructively harvested (Appendix 2.1). Each buried or surface treatment consisted of a fabric bag which contained a 'wet' leaf and a 'dry' leaf. There were five replicates for each treatment. The sampling dates were initially planned for December, January, February, March, and April. However, the January and February samples were frozen solid in the soil, and so could not be collected. The actual collection dates were 20 December 2018, 15 March 2019, 11 April 2019, 1 May 2019, and 30 May 2019.

After collection, the fabric bags were rinsed with sterilized water and the leaf sections were carefully removed and viewed under a dissecting microscope to check for the presence of pseudothecia. If present, several pseudothecia were collected using a sterile scalpel, placed onto a glass slide with a drop of water, covered with a plastic cover slip, firmly squeezed to crush the pseudothecia, and viewed under a microscope to check for ascospores. If present, the ascospores were collected with a 100 µL pipette, transferred to a fresh onion leaf from the growth room, and incubated at room temperature on a moist paper towel within a Petri dish sealed with Parafilm. After 24 h, a thin coating of clear nail polish was applied over the inoculation site, allowed to dry, then carefully removed with forceps. The nail polish peel was transferred to a glass slide, strained with 1–2 drops of lactophenol cotton blue, and viewed under a microscope to determine if the ascospores had germinated (Leandro et al., 2002).

The symptomatic areas of the overwintered onion leaf tissues were cut into 10 pieces (approximately 2 mm × 2 mm). Half of these were plated directly, and the other half were surface sterilized. The leaf pieces, five per plate, were placed onto antibiotic V8 media. Morphological characteristics such as colony color and spore shape were recorded.

The viability of the mycelium in the overwintered leaf tissue not used in for plating was assessed by incubating the samples in sealed Petri dishes with a moist paper towel for 3–5 days after collection. The presence of *S. vesicarium* conidia was confirmed using a light microscope.

2.2.4 Weeds as alternative hosts

Common weed species from the Holland Marsh, ON were collected as small plantlets and grown in propagation trays (28 cm × 53 cm plastic tray insert, with 4 × 8 configuration, each pot 57 mm deep and 57 mm square at the rim) filled with soil-less mix (Sunshine Mix LA4, Sun Gro Horticulture Canada Ltd, Agawam, MA) (Figure 2.3). The growth room was set at ~22 °C, with 17 h of light. The weeds were redroot pigweed (*Amaranthus retroflexus* L.), marshcress (*Rorippa palustris* [L.] Besser), yellow nutsedge (*Cyperus esculentus* L), perennial sowthistle (*Sonchus arvensis* L.), bull thistle (*Cirsium vulgare* [Savi.] Ten.), and purslane (*Portulaca oleracea* L.). These species were chosen to represent the wide variety of plant families (Amaranthaceae, Brassicaceae, Cyperaceae, Asteraceae, and Portulacaceae) commonly found in the area.

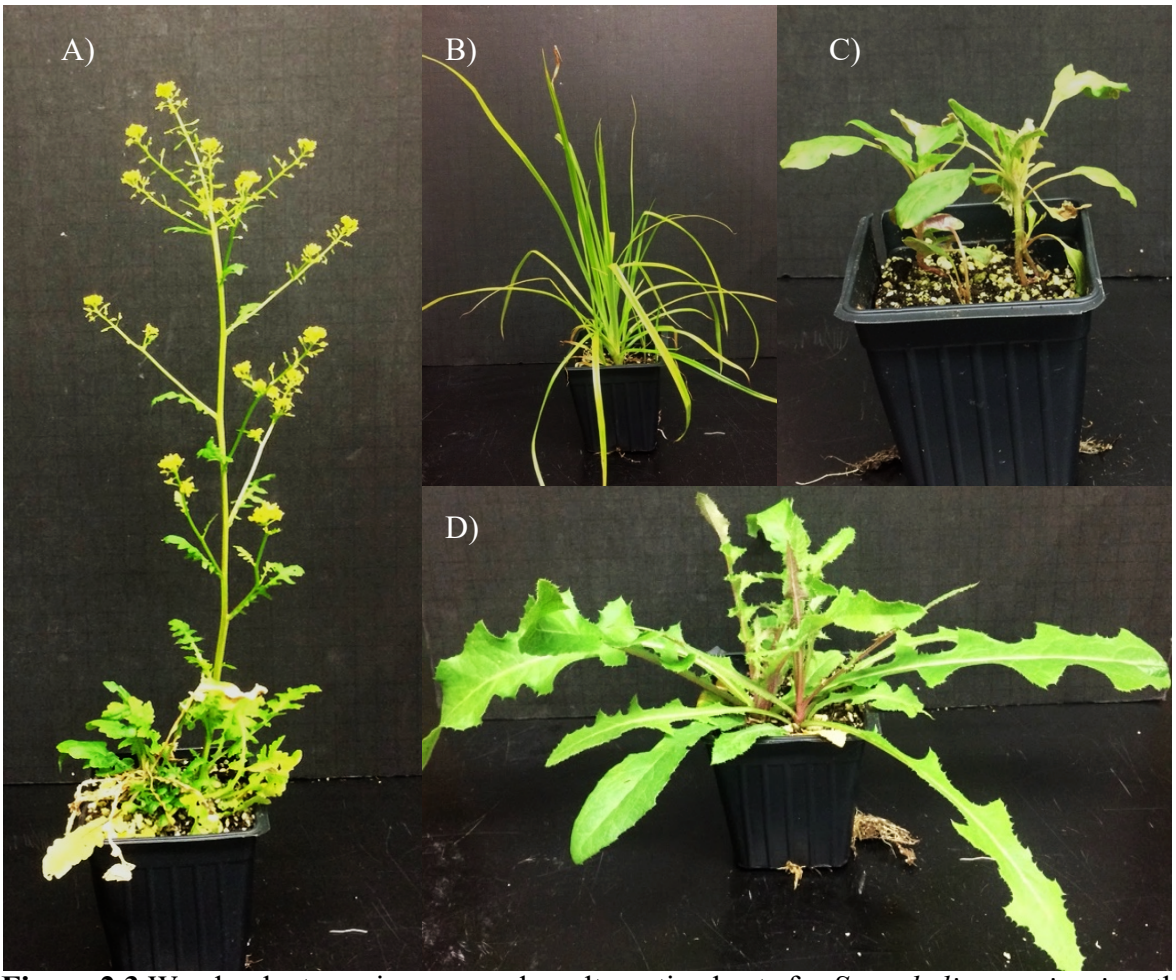

Figure 2.3 Weedy plant species assessed as alternative hosts for *Stemphylium vesicarium* that are common near the Holland Marsh in southern Ontario: A) marshcress, B) yellow nutsedge, C) redroot pigweed, and D) sow thistle.

The plants were allowed to acclimate to the growth room for 2–3 weeks, then sprayed with a conidial suspension of *S. vesicarium,* with a mock-inoculated treatment as a negative control and onion plants as a positive control. The inoculum consisted of a mixture of conidia from three recently collected, virulent isolates from the Holland Marsh (SS0006, Onion1, HP01), adjusted to 1×10^5 spores mL^{-1} plus one drop of Tween 20 (Sigma-Aldrich, Oakville, ON). A paperclip was affixed gently around the growing point of the plants to identify new leaves that grew after inoculation. Following inoculation, the plants were incubated for 3 days under high humidity using either clear plastic bags, or a larger humidity chamber in which the plants were sprayed with water for 30 s every 30 min, and then returned to the typical growth room settings. After ~18 days, leaf tissue was collected from the inoculated leaves.

For each species, four or five small sections (3 mm × 3 mm) of inoculated or mock-inoculated leaf tissue were surface sterilized and cultured on amended V8 agar as described above. The resulting colonies were identified based on spore morphology under a light microscope after 3–9 days of growth. The assessment of each weed species was repeated.

2.2.5 *Stemphylium vesicarium* as a seed-borne pathogen

Inoculation of seed

A commercial seed lot of cultivar LaSalle (Stokes Seed, ON) was soaked in water and strained to remove the pelleted seed coat. The seeds were surface sterilized as above, and half were inoculated with a suspension of *S. vesicarium* conidia (~3×10^4 spores mL^{-1} plus a drop of Tween 20, isolates FE02, FE03, FE04). The seeds were then allowed to dry in an unsealed plastic Petri dish at room temperature for 3 days. Ten seeds from each treatment were plated onto V8 media amended with antibiotic and incubated for 3 to 4 days under 12 h UV light / 12 h darkness. The resulting colonies were identified based on spore morphology. Of the remaining dried seed, 36 were planted in a 48-cell seeding tray filled with soil-less mix (Sunshine Mix LA4, Sun Gro Horticulture Canada Ltd, Agawam, MA). The pots were incubated in growth chambers set at ~24.5 °C, with 17 h of light for approximately 2 months. The mature onion plants were assessed for SLB symptoms, specifically leaf chlorosis, lesions, and tip dieback.

Inoculation of flowers – Growth room

Year-old onion bulbs of cultivar LaSalle were stored at 4 °C for 4 to 6 months, planted into 3.8-L pots filled with soil-less mix (Sunshine Mix LA4) and grown in growth chambers set at 24.5 °C, with 17 h of light (n = 16). Sixteen plants in total were grown until flowers were produced at ~3 months. When the florets began to open, they were manually pollinated by collecting several anthers from each plant, crushing the anthers to release the pollen, and using a paintbrush to carefully place pollen onto the stigmas of the florets. The flowers of eight plants were sprayed with a conidia suspension collected from three isolates of *S. vesicarium* (~3×10^4 spores mL^{-1} plus a small drop of Tween 20, isolates FE02, FE04, FE05), with the other eight serving as a non-inoculated control.

Once the inflorescences set seed at 4–6 weeks after flowering, the seeds were collected using forceps and air-dried in an unsealed Petri dish at room temperature. Up to 20 seeds (where available) were collected from each plant and stored at 4 °C until assessed. Ten of these seeds were plated directly onto amended V8 agar. The remaining seeds were surface sterilized, plated

onto the V8 agar and incubated under UV lights at room temperature for 2 weeks, and the colonies were assessed.

Inoculation of flowers – Field trial

Bulbs of onion cultivar LaSalle were stored over the winter in a cool storage room (dark, ~10 °C) for approximately 38 weeks. One hundred and twenty bulbs were planted at a site with high organic soil (organic matter ≈ 71%, pH ≈ 5.7) at the Muck Crops Research Station, King, Ontario. These plants were allowed to flower and pollinate naturally. Only 39 flowers were produced because many bulbs rotted after planting. The flowers were not inoculated, so any infection likely resulted from inoculum released from nearby onion fields. Eight flowers were collected early (23 July–10 August 2019) because mechanical or insect damage to the flower scape had terminated the flower. At the end of the growing season (30 August 2018), the remaining scapes were cut and 31 flowers were collected in individual paper bags. The flowers were stored at room temperature for 6 weeks to let the seeds air-dry.

As in the growth room study, up to 60 seeds (if available) per flower were plated onto V8 media + antibiotics on 6 November 2019 (surface-sterilized or not sterilized, 10 seeds per plate, with three replications), and fungal colonies were identified based on spore morphology 7 days later. Some colonies also produced immature pseudothecia in culture. Mycelium was collected from five representative colonies and pathogen identity was confirmed via DNA sequencing.

Seed-to-seedling transmission

Naturally-inoculated seed from the field trial was grown in a controlled environment to assess if *S. vesicarium* would be transmitted to seedlings. Approximately 35 seeds per flower (if available) were seeded into propagation trays (28 cm × 53 cm plastic tray insert, with 8 × 12 configuration, each cell 57 mm ×38 mm × 54 mm deep) filled with soil-less mix (Sunshine Mix LA4) in a growth room set at ~22 °C with 17 h of light. When the seedlings reached the first true leaf stage, the cotyledon and the first leaf were collected using disinfected forceps and scissors. A 10-mm section of the tip and the middle of the leaf / cotyledon were cut using a sterile scalpel, plated onto V8 media amended with antibiotics, and incubated at room temperature with 12 h UV light / 12 h darkness. The plates were assessed 6 days later for *S. vesicarium* colonies.

Seed Heat Treatment

Onion seeds collected from flowers naturally inoculated with *S. vesicarium* were divided into Falcon 50 mL conical centrifuge tubes, with 60 seeds per tube and three replicate tubes per treatment. Shortly before heat treatment, autoclaved deionized water was added to each tube (~5 mL) to cover the seeds. The tubes were then immersed in a water bath (40, 50, or 60 °C) for 10, 20, 30, or 40 min. The control was soaked in sterile water at room temperature (~21 °C) for 40 min. The water was removed using a sterilized metal strainer. The seeds were then placed onto sterile filter paper in a plastic Petri dish with the lid cracked open in a laminar flow hood for 2 h to dry. Seed from each treatment was plated onto amended V8 media with five seeds per plate on five plates (25 seeds total), with three replicates separated over time, incubated under UV light 12 h on / off for 5 to 7 days, and assessed for *S. vesicarium* colonies (as described previously) and germination.

Twenty-five seeds per treatment per replication were planted in a growth room following the protocol previously described. When the first leaf emerged, the cotyledon and the first leaf were collected using sterilized scissors and forceps. Two 10-mm pieces were cut from each leaf, one from the top and another from the middle of the sample, and these pieces were surface sterilized and plated as described previously. Seedling emergence and the number of fungal colonies produced were recorded for all fungal species and for *S. vesicarium*.

2.2.6 Data analysis

Statistical analyses were conducted using SAS v.9.4 (SAS Institute Inc., Cary, NC). The numerical values of the proportion of tissues producing pseudothecia were presented without statistical analysis. The overwintering survival response over time was plotted for location, sterilization, and moisture factor separately to show effects of each treatment.

The frequency of infection of weed species by *S. vesicarium* was assessed in two repetitions of the host range study. There was no repetition × species interaction, so the data were pooled to make the best possible assessment of the suitability of each species as an alternative host for *S. vesicarium* when compared to the onion host. After sorting the data by sterilization treatment, a Dunnett's test was used to compare the recovery from the weed species to recovery from onion. Additionally, a Student's T-test was used to assess the effect of sterilization on each plant species.

For the laboratory and growth room assessments of *S. vesicarium* as a seed-borne pathogen (the seed inoculation study and the growth room flower inoculation), the numerical observations are presented without statistical analysis because the main result was whether or not infection could occur, which was clear without statistical analyses.

For the field trial to assess inoculation of flowers, PROC GLIMMIX with a beta distribution was used to determine if main effects (early harvest or end of season, surface sterilized or not) had a significant effect on the percentage of seeds infected with *S. vesicarium.* A beta distribution is a continuous distribution bounded by 0 and 1 that is often used for proportion, frequency, or percentage data (Bowley, 2015). The scape that the seed was harvested from was treated as a replicate (random factor). For the seed heat treatment study, the response variables (germination, emergence, and fungal colonies produced) were compared to the control using Dunnett's test at $P < 0.05$.

2.3 Results

2.3.1 Pathogen confirmation

Conidia collected from fresh tissue or culture plates (Figure 2.4) matched the descriptions of conidial morphology of Simmons (1969).

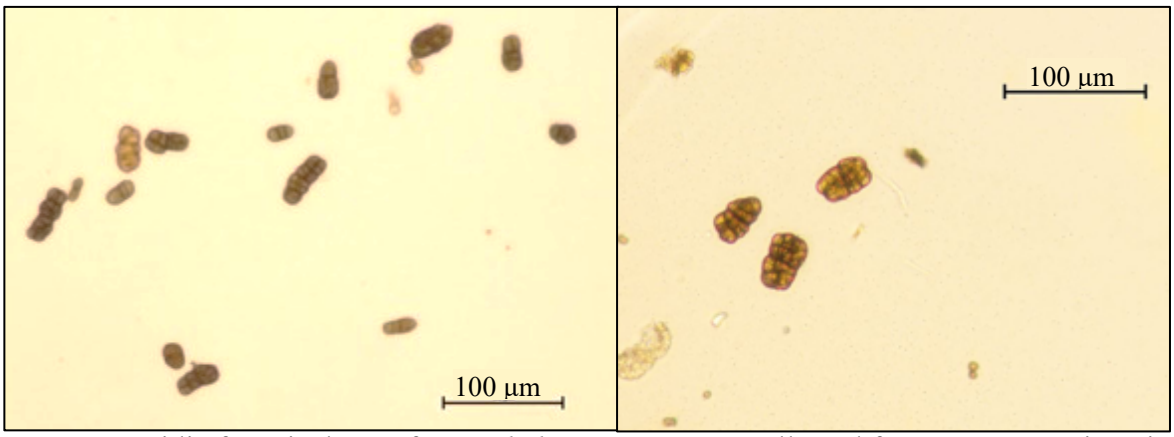

Figure 2.4 Conidia from isolates of *Stemphylium vesicarium* collected from symptomatic onion plants in Ontario.

Molecular analysis based on species-specific primers (GenBank accession numbers MH628098–MH628128) and DNA sequencing of 24 isolates confirmed that each isolate was *S. vesicarium.* For example, the alignment of retrieved sequences from the NCBI database from

the ITS, *gapdh*, *TEf-A*, and *cytB* regions of the fungal isolates OA20, OO27, OO31, NO36, OA46, OA48, and NA51 exhibited 98–100% homology over 83–99% query cover with *S. vesicarium* (GenBank accession numbers MG065799.1, MG020760.1, and KF993418.1 for the ITS region, and DQ000654.1, JF331624.1, and KJ934233.1 for the *gapdh*, *TEf-A*, and *cytB* regions, respectively). When the DNA sequences of the isolates were aligned using ClustalW (https://www.genome.jp/tools-bin/clustalw), the pairwise alignment scores of the created contigs were 96.6–100% for each gene sequence. The ITS, *TEf-A*, and *cytB* regions of NO35 also exhibited 99–100% homology with 83–98% query cover. The only issue was that the *gapdh* region of NO35 was not completely sequenced due to experimental error. The ITS, *TEf-A*, and *gapdh* regions (GenBank accession numbers AY329212.1, AY324708.1, and AY317016.1, respectively) aligned with 99–100% homology over 73–88% query cover to EGS37-067, which is an ex-type strain deposited by Simmons (1967) and later sequenced (Inderbitzin et al., 2005). Thus, representative isolates from Nova Scotia and Ontario, and from both asparagus and onions were all confirmed as *S. vesicarium*. The five isolates collected from onion seed naturally inoculated in the Holland Marsh in 2019 were also confirmed to be *S. vesicarium*, with 88–100% homology and 80–100 % query coverage using the ITS, *TEf-A*, *cytB* and *gapdh* sequences.

2.3.2 Over-winter survival

Colonies of *S. vesicarium* on V8 agar were grey to brownish grey with dark olive green concentric rings of sporulating tissue that formed under UV light (Arzanlou et al., 2012). Conidia produced were oval to oblong, brown, with 1–6 transverse septa and 2–6 longitudinal septa (Simmons, 1969; Raghavendra Rao and Pavgi, 1975). The sample size was relatively small (5 onion leaves per treatment per sampling date) and the experiment was only conducted in one year, so more assessments might show a difference. The main factors (burial, sterilization, wet or dry) were graphed to visualize the relationships over time. Survival of *S. vesicarium* decreased over time to as low as 3% survival (Figure 2.5). The dry / wet and buried / surface survival showed similar patterns; the survival of *S. vesicarium* in the wet or buried samples decreased steeply from 20 Dec to 11 April and remained <10% for the rest of the sampling period. The survival for dry or surface samples was nearly linear for the first three sampling dates and decreased after 11 April. For the main effect of surface sterilization, the relationship over time for surface-sterilized and non-sterilized samples exhibited a nearly linear decrease over the sampling period.

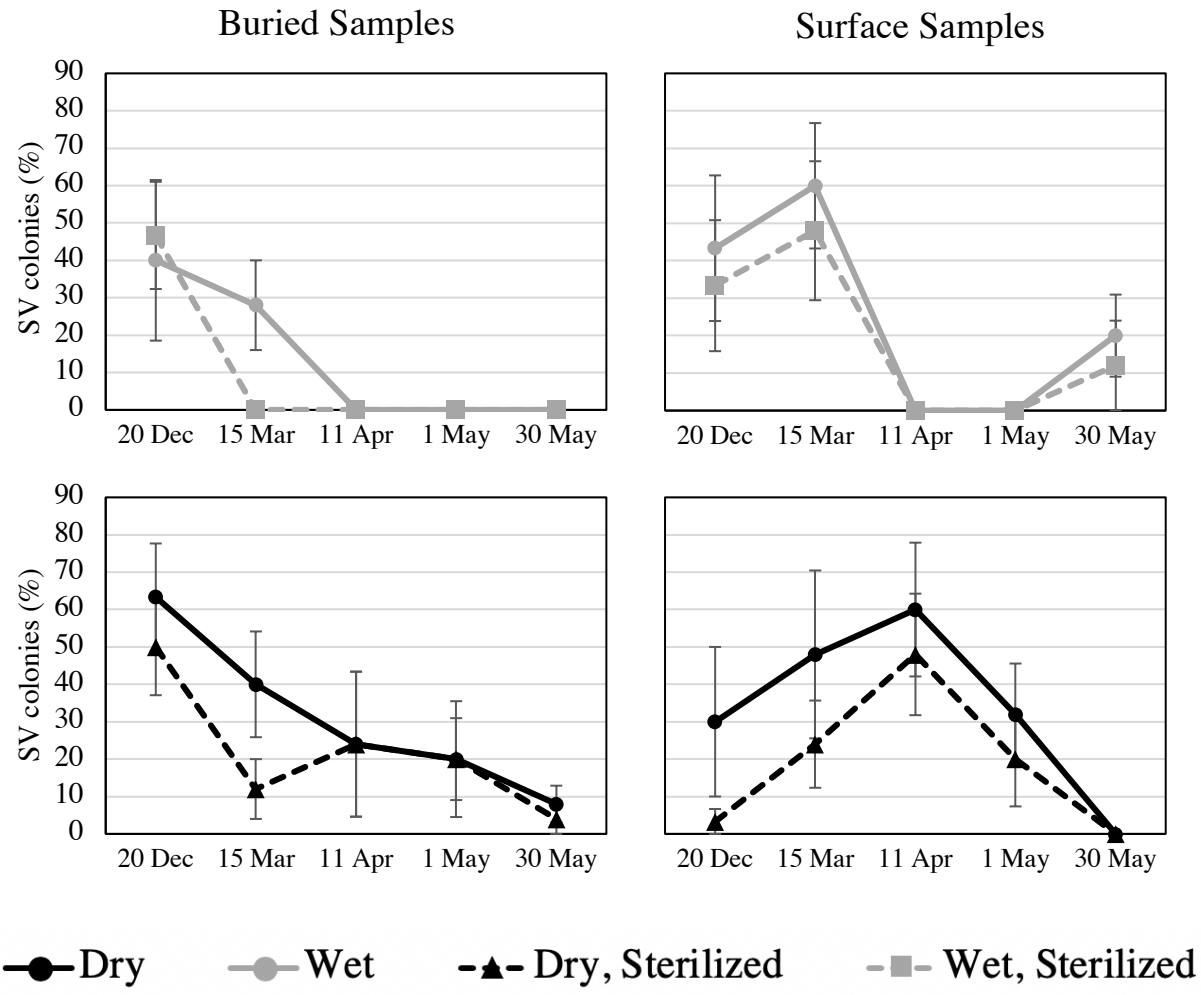

Figure 2.5 Percent of inoculated onion leaf samples overwintered at the Muck Crops Research station in the winter of 2018 on the soil surface or buried in soil, then collected at five sampling dates, that produced *Stemphylium vesicarium* colonies when cultured on V8 agar. Error bars represent standard error of five replicates.

Whether the overwintering leaf tissue was buried in soil or affixed to the soil surface did not have a major effect on pathogen survival; colonies of *S. vesicarium* were produced from both sets of samples, and the trend over time was not different between buried and surface samples. Overall, there was more survival on dry leaves relative to wet leaves.

There was no survival on buried wet leaves recovered on 11 April or later, suggesting that burying onion debris may reduce overwintering inoculum (Figure 2.5). Similarly, there were no colonies from the surface wet samples on 11 April and 1May. There was some survival on the surface wet leaves on 30 May. This may not be a reflection on the survival of the pathogen per se, and instead of the ability to compete against other saprophytic microbes when cultured on V8

agar. The wet samples were exposed to the environment and were highly degraded by the end of the winter. In culture, a wide array of non-target fungi and bacteria were produced, and they may have masked or outcompeted the *S. vesicarium* colonies. This may explain why no colonies were detected for wet samples on several sampling dates (Figure 2.5). The process of surface sterilization before plating the leaf tissue on antibiotic V8 agar decreased the number of colonies produced for every treatment.

It is important to note this was a small sample size (n = 5), and the experiment was only conducted once. This experiment reflected the ability to re-isolate *S. vesicarium* from samples which had overwintered in the field and is not necessarily reflect actual percent survival of the pathogen. In addition, the wet samples were highly degraded by the final sampling date, so pseudothecia or mycelial fragments may have broken free from the leaf tissue and been lost in the soil (Figure 2.6).

Figure 2.6 Onion leaves infected with *Stemphylium vesicarium* after 7 months of overwintering treatment A) at the soil surface, or B) buried 10 cm below the surface in a muck soil field. Five pairs of samples are pictured. In each pair, the leaf was either kept dry (Dry) in a sealed plastic container or exposed to the wet environment (Wet) within a polyester fabric bag.

2.3.3 Sexual reproduction

The number of pseudothecia produced on each sample was also assessed on samples collected from the overwintering study. The first sample was collected on 20 December 2018, 6 weeks

after the samples had been placed at the Muck Crops Research Station. The buried wet leaf tissue exhibited many pseudothecia-like structures. These structures did not contain ascospores, but later sampling dates contained viable ascospores (Figures 2.8 and 2.9).

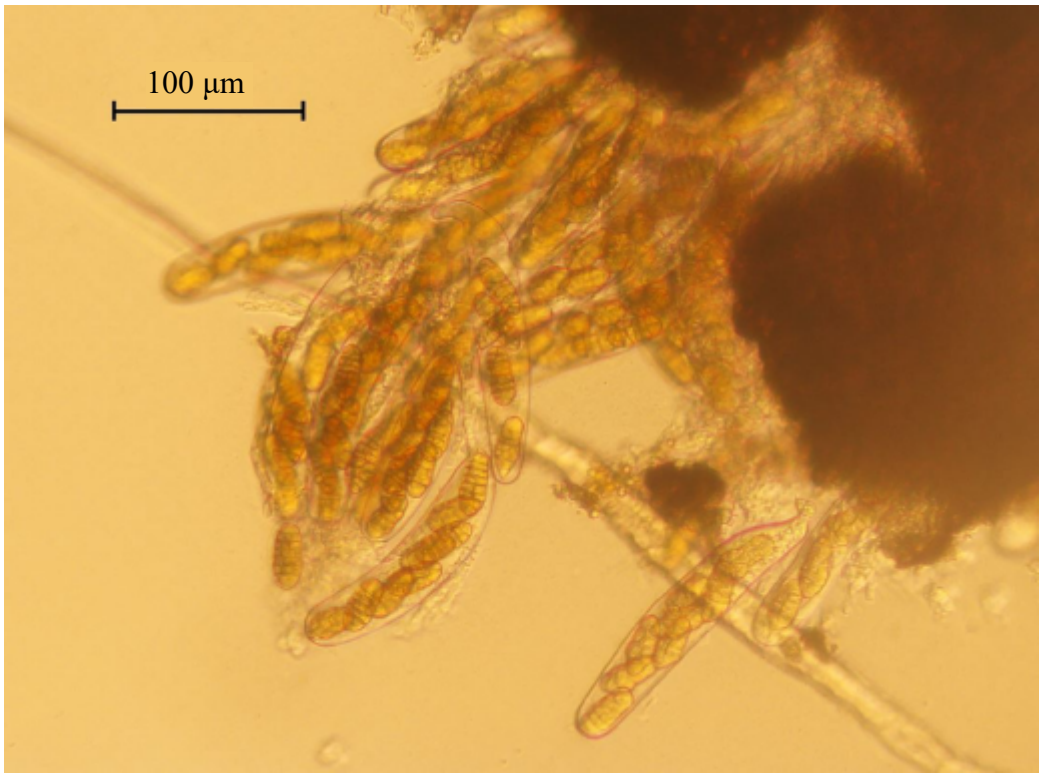

Figure 2.7 Asci containing ascospores protruding from a crushed pseudothecia of *Stemphylium vesicarium* collected from an overwintered onion leaf.

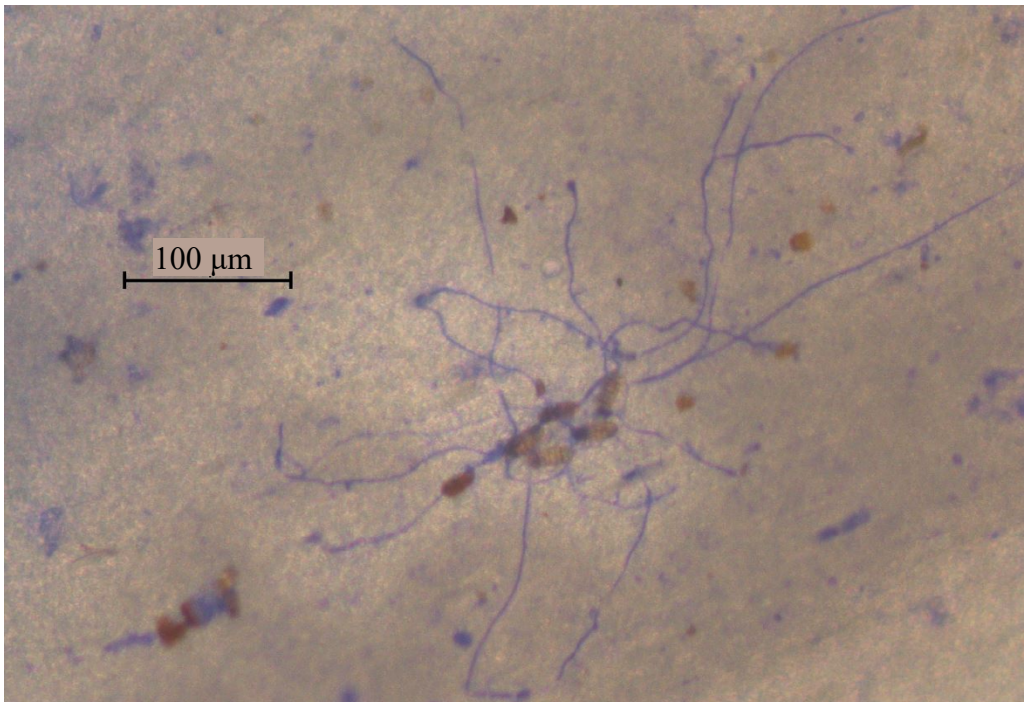

Figure 2.8 Germinated *Stemphylium vesicarium* ascospores and associated mycelium on a fresh onion leaf, visualised by peeling off the epidermis and staining the mycelium from germinated spores with lactophenol cotton blue.

In general, leaf tissue that had remained dry inside the plastic containers produced pseudothecia less frequently than samples in contact with moist soil (Figure 2.10). The previous experiment demonstrated that the dry leaf tissues could still produce *S. vesicarium* colonies when incubated on V8 media. When dry samples were incubated in humid Petri dish with paper towel, mycelium and conidia were produced from 100% of samples.

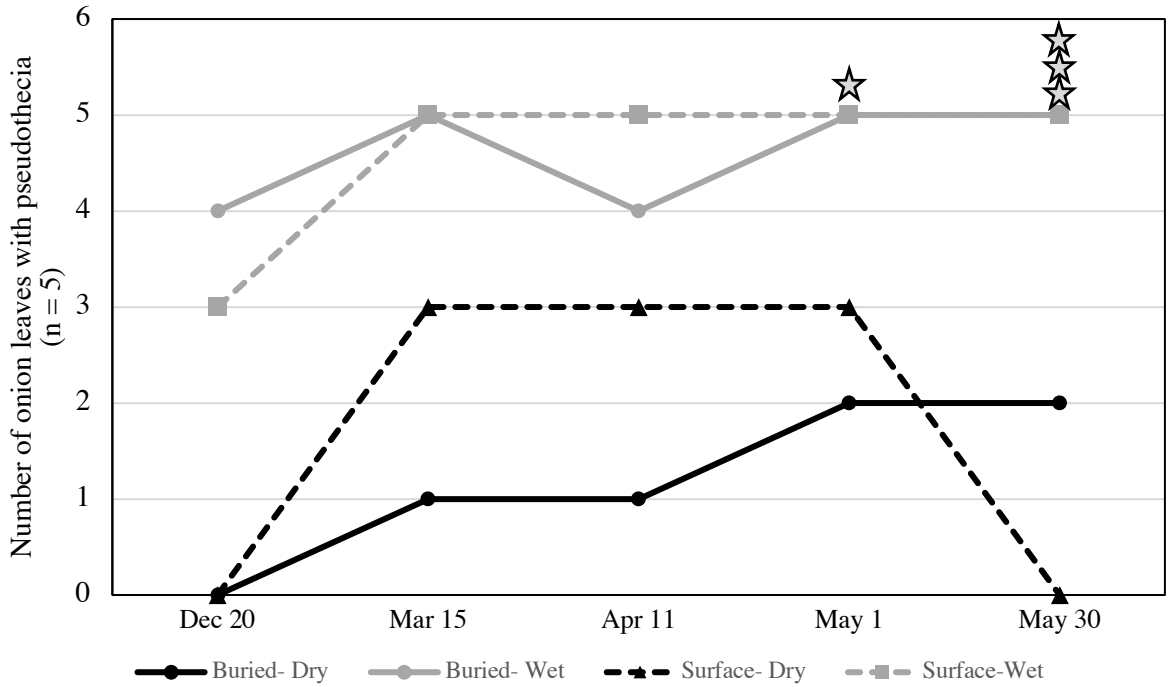

Figure 2.9 *Stemphylium vesicarium* pseudothecia produced on five onion leaves collected at five sampling dates from the soil surface or buried underground, which were kept dry in a tube or exposed to environmental moisture in a fabric bag. Stars above line indicate how many of the samples also produced ascospores in surface-wet treatment.

2.3.4 Weeds as alternative hosts

Redroot pigweed, yellow nutsedge, purslane, marshcress, sowthistle, and bull thistle were confirmed as asymptomatic alternative hosts when inoculated with *S. vesicarium* and incubated in a controlled environment. Occasionally, inoculated purslane presented with dark olive-green spots on inoculated leaves (Figure 2.12).

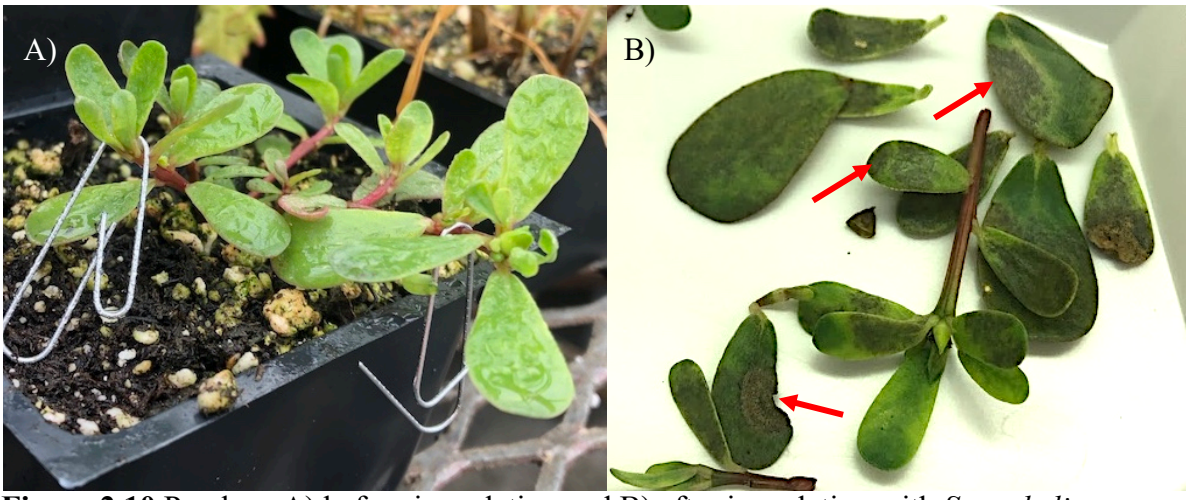

Figure 2.10 Purslane A) before inoculation and B) after inoculation with *Stemphylium vesicarium,* showing dark sunken circular or oval lesions (red arrows).

The interaction of sterilization × plant species and the main effect of plant species were not significant, but the main effect of surface sterilization was significant (Appendix 2.3). The simple effects were presented to show the effect of surface sterilization on each plant (Table 2.3). Recovery of *S. vesicarium* from nutsedge, pigweed, purslane, and bull thistle was similar to onion, but recovery from marshcress and sowthistle was lower (Table 2.3, Appendix 2.3). Examination of the interaction effects showed lower recovery from non-sterilized sowthistle and marshcress compared to onion following surface sterilization (Table 2.3). Surface sterilization generally decreased the number of samples producing *S. vesicarium* colonies, but this was only statistically significant for onion and there was increased recovery from marshcress (Table 2.3). There were no differences among species for pathogen recovery when the tissues were surface-sterilized (Appendix 2.3).

Table 2.3 Percentage of weed and onion leaf pieces producing *Stemphylium vesicarium* colonies after inoculation and incubation in a controlled environment for 10–20 days. (n = 8).

	Samples producing *S. vesicarium* colonies (%)			
Species	**Not sterilized**		**Sterilized**	
Onion	56	A[1]	13	B
Nutsedge	44		13	
Pigweed	23		8	
Purslane	22		19	
Bull thistle	19		16	
Sowthistle	*17		15	
Marshcress	*3	A	25	B
Main effect of sterilization	**26**	**A**	**16**	**B**

[1]Values followed by a different letter in a row were different at $P < 0.05$.
*Indicated values that differ from onion based on Dunnett's Test, $P < 0.05$).

This is the first report of *S. vesicarium* infecting redroot pigweed, yellow nutsedge, purslane, marshcress, and sowthistle (Table 2.4). However, *S. vesicarium* has a very wide host range and has previously been documented infecting species within the same genera as redroot pigweed, and sowthistle (Table 2.4) (Unamuno, 1941; Urries, 1957; Pennycook, 1989). Infection of bull thistle was previously reported in Russia (Babuschkina, 1995).

Table 2.4 First reports for Canada or worldwide of common weed species from the Holland Marsh, Ontario acting as hosts of *Stemphylium vesicarium*.

Species	**First report for Canada**	**Previous report**
Yellow nutsedge (*Cyperus esculentus*)	Yes	None
Redroot pigweed (*Amaranthus retroflexus*)	Yes	Genus only – New Zealand (Pennycook, 1989)
Purslane (*Portulaca oleracea*)	Yes	None
Bull thistle (*Cirsium vulgare*)	Yes	Russia (Babuschkina, 1995)
Sowthistle (*Sonchus arvensis*)	Yes	Genus only – Spain (Urries, 1957)
Marshcress (*Rorippa amphibia*)	Yes	None

2.3.5 *Stemphylium vesicarium* as a seed-borne pathogen

Inoculation of seed

In the small study of seed inoculated with a spore suspension of *S. vesicarium,* all of the inoculated seeds produced *S. vesicarium* colonies on V8 agar, and 1 of 10 seeds (10%) surface sterilized seeds produced colonies of *S. vesicarium*. No colonies grew from the non-inoculated commercial seed, as expected. When 36 inoculated seeds were planted, only six onion seedlings (17%) emerged from the surface-sterilized treatment, and 5 from the inoculated treatment. The low germination rate may be due to damage to the seed embryo during surface-sterilization. At maturity (10–12 leaf stage), none of the onion leaves exhibited symptoms of SLB. Note that this preliminary experiment had an extremely small sample size.

Inoculation of flowers – Growth room

Fourteen flowers from onion plants grown in a controlled environment were manually pollinated and half were inoculated with *S. vesicarium*. All seed was surface sterilized or rinsed with water and plated onto V8 media. In the inoculated surface sterilized treatment, 2 of 48 seeds (4.2%) from six flowers produced *S. vesicarium* colonies. Both seeds originated from the same flower. In the inoculated, non-sterilized treatment, 3 of 53 seeds (5.7%) produced *S. vesicarium* colonies. These infected seeds originated from two flowers. None of the seeds from the eight non-inoculated flowers produced *S. vesicarium* colonies, as expected.

Inoculation of flowers – Field trial

In the field study, eight flowers were harvested early due to mechanical or insect damage and 31 flowers were collected at the end of the season. The main effects (time harvested and surface sterilization) and their interaction on infestation with *S. vesicarium* were not significant so the data were pooled (Appendix 2.4). However, the main effects of time of harvest and sterilization on seed germination were significant, but infestation with *S. vesicarium* did not affect germination (Appendix 2.5). Germination was decreased by surface sterilization from 57% to 19% for early harvested seeds and from 54% to 17% for late harvested seeds. There was a small but significant difference between germination of early harvested seed (38%) and late harvested seed (34%). The interactions were generally not significant, except for a significant interaction between time harvested and infestation with *S. vesicarium* (Appendix 2.7). Infection of the seeds by *S. vesicarium* was 3% of those harvested early and 0.6% of those harvested at the end of the season (Table 2.5), which was lower than had been previously described in the literature.

Approximately one-third of the seeds germinated in the Petri dishes, and of these, approximately 1% of the germinated seeds also produced *S. vesicarium* colonies on the media.

Table 2.5 Observations from onion seeds naturally inoculated in the field, collected in July–August 2019 (Early) or on 30 August 2019 (End of season).

	Number / Percent observed	
Variable	**Early**	**End of season**
Number of flowers assessed	8	31
Number of seeds screened	300	1713
Number of colonies with *S. vesicarium* conidia	5 (1.7%)	5 (0.3%)
Number of colonies with *S. vesicarium* pseudothecia	7 (2.3%)	9 (0.5%)
Total # colonies with *S. vesicarium* structures	9 (3.0%)	10 (0.6%)
Number of seeds germinated in plate	115 (38.3 %)	614 (35.8%)
Germinated seeds with *S. vesicarium* structures	1 (0.9%)	7 (1.0%)

The percentage of colonies produced in the surface sterilization treatment was not statistically different from the non-sterilized treatment (Table 2.6). For the early-harvested seeds, the number of germinated seeds fell when surface-sterilized from 57% to 19% for the early harvested seeds, and from 54% to 18% for those harvested at the end of the season. This suggested that the surface sterilization was very damaging to the seeds. It also indicated the poor quality of the seeds generated (~ 50% germination) in this study, likely because the study relied on natural pollination between a limited number of flowers.

Table 2.6 The effect of seed harvested at two time points and subsequent surface sterilization on recovery of *Stemphylium vesicarium* colonies from onion seed that was naturally inoculated in the field during production at the Muck Crops Research Station in 2019.

Time harvested	**Surface sterilization**	**No. of seeds assessed**	**No. of seeds germinated**	**No. of seeds infected with *S. vesicarium* (%)**
Early	Sterilized	150	29	3 (2.0%)
	Not Sterilized	150	86	7 (4.7%)
End of Season	Sterilized	857	150	1 (0.1%)
	Not Sterilized	856	464	8 (1.1%)
	Total:	2013	729	19 (0.9%)

Seed-to-seedling transmission

There was no seed to seedling transmission of *S. vesicarium* from the early harvested seed. This null result may be due to the poor emergence (34%) and the small sample size (110). In seed harvested at the end of the season, only 1.2% transmitted *S. vesicarium* to seedlings (Table 2.8). This was consistent with the level of infection of seed assessed in the previous experiment.

Table 2.7 The effect of seed harvested at two time points on emergence and recovery of *Stemphylium vesicarium* colonies from the cotyledon and first true leaf of onions grown from seed that was naturally inoculated in the field during production at the Muck Crops Research Station in 2019.

Time harvested	Seeds planted	No. of seedlings	Emergence (%)	Seedlings infected with *S. vesicarium*	Transmission to seedling (%)
Early	110	40	33.5%	0	0%
End of season	945	504	53.3%	6	1.2%

Seed Heat Treatment

Naturally inoculated seeds collected from the field were exposed to 40, 50, or 60 °C for 10, 20, 30, or 40 min and assessed for infection. There was no interaction between time and duration of treatment. The main effect of temperature on seed infestation with fungi was significant, but not the duration of the heat treatment (Appendix 2.6). The opposite was found for seed germination, where the interaction of temperature and duration was significant (Appendix 2.7).In contrast, the interaction of temperature and duration was significant for seed germination (Appendix 2.7) and emergence of onion seedlings (Appendix 2.8).

Exposure of seed to 60 °C for 40 min reduced survival (17% germination, 0% emergence) compared to the control (57% germination, 88% emergence). Seed that had been exposed to heat treatments < 60 °C for 10 min germinated and emerged at similar rates as the control (Figure 2.12).

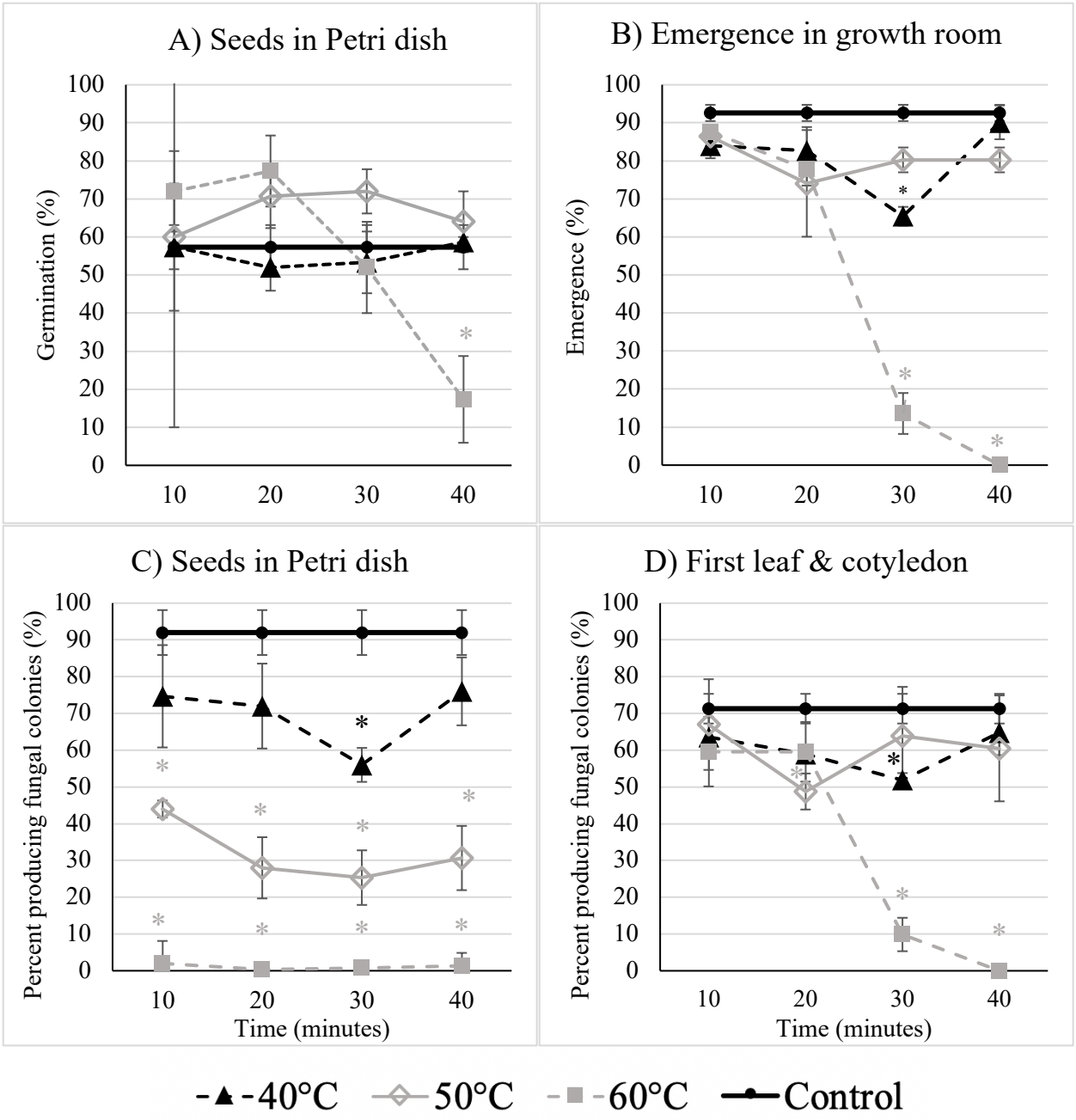

Figure 2.11 Effect of temperature and duration of heat treatment on A) onion seed germination, B) seedling emergence, C) total number of fungal colonies produced from seeds, and D) on the first leaf and cotyledon on V8 agar from onion seed grown in a controlled environment.
* Indicates significant difference from the control treatment, based on Dunnett's test at $P < 0.05$.

Only two *S. vesicarium* colonies were detected in the study, one from the 40 °C, 10-min treatment, and the other in the 40 °C, 40-min treatment (data not shown). Despite the low levels of *S. vesicarium* in the study, some conclusions can be drawn based on the colonies of other seed-borne fungi. When assessing seeds plated directly onto V8 media, the number of other

fungal colonies decreased as the temperature increased, but the duration of treatment did not affect the number of fungal colonies at 40 °C or 50 °C (Figure 2.13 C). The colonies produced from the cotyledon and first leaf showed that 60 °C reduced infection by fungi; the 30- and 40-min treatments reduced the number of fungal colonies from 71% to 3% and 0%, respectively. Interestingly, the number of fungal colonies produced by the cotyledon / leaf tissue was generally much lower (71%) than the direct seed assessment method (92%) (Figure 2.13 D), likely because the tissue collected from the growth room was surface sterilized after collection (Figure 2.13). A decrease in transmission from seed to cotyledon and first leaf is not surprising.

2.4 Discussion

The identity of the pathogen species causing SLB in the Holland Marsh, Ontario was initially identified as *S. vesicarium* based on conidia and ascospore morphology. The current study used molecular tests and confirmed the identification for the first time in the region, based on homology with accessions on GenBank (Foster et al. 2019), which has become the standard for identifications for this morphologically diverse group.

Three important aspects of the lifecycle of *S. vesicarium* were examined. The current study demonstrated that the pathogen could overwinter on infected onion leaf debris in Ontario, identified for the first time that common weed species in Ontario were alternative hosts, and confirmed that *S. vesicarium* could be seed-borne. The overwintering is consistent with results from New York State (Dr. S.J. Pethybridge, Cornell University, *personal communication*) and the seed-borne nature of the pathogen confirms an earlier report (Aveling et al., 1993). This study was the first to identify yellow nutsedge, redroot pigweed, purslane, sowthistle, and marshcress as alternative hosts of *S. vesicarium*.

Identifying the source of the primary inoculum could improve the efficacy of IPM strategies. *Stemphylium vesicarium* was recovered from overwintering onion leaf tissue at all of the five sampling dates from December to May. In early May, close to when the first onion transplants would be planted in the region, pseudothecia on the leaves contained mature, viable ascospores. It is important to note that the weather conditions over winter may have affected the timing of pseudothecial development in the overwintering study, especially since this study was only conducted in one year. Ascospores of *S. vesicarium* were previously reported in air samples collected at the Muck Crops Research Station in the Holland Marsh from late April to July

(Gossen et al., 2021). However, spore trapping does not assess if the ascospores were produced on onion or on other hosts.

No pseudothecia were found on overwintered symptomatic onion tissues in a previous study in the Holland Marsh, ON. In the previous experiment, the leaves were fresh when buried, so they degraded very quickly (Tayviah, 2017). In addition, it may have been more difficult to identify the symptomatic areas of the leaves after they had overwintered in the field for several weeks or months, since entire leaves and bulbs were used in the previous study. The current study improved upon the previous protocol by using dried leaf tissues, which degraded more slowly than fresh leaves. Also, dry leaf tissue simulated more closely what would occur naturally in the field. The samples in the current study were small sections of leaf surrounding a confirmed lesion, which narrowed the observation area after overwintering, as opposed to using the entire leaf. Finally, dry samples (contained in a sealed plastic tube) were included to represent onion leaves that may be stuck to equipment or had been blown into a sheltered area over winter.

The current study also assessed the viability of *S. vesicarium* after overwintering. The percentage of samples producing *S. vesicarium* colonies generally decreased over the winter period. This is likely due to competition by other microbes in the Petri dish when reviving the pathogen from field samples, or competition with other microbes in the soil. By comparison to opportunistic fungi such as *Penicillium* spp., *S. vesicarium* grows relatively slow in culture (S. Stricker, *personal observation*). The leaves in the buried, wet treatment were extremely degraded when collected on 1 May and 30 May, which may have also reduced culturing success. The percent survival may be an underestimation of the actual survival of the pathogen in the soil, since growth on V8 agar doesn't mimic field conditions exactly. The buried wet treatment did not produce viable colonies on 11 April–30 May samples, but pseudothecia were present. Ascospores were only present on samples which were wet and on the soil surface overwinter. This likely occurred be because daylight is required for the production of ascospores, or possibly because of greater degradation of the buried pseudothecia by microbial saprophytes. Further investigation is needed to confirm that burial of leaf litter decreases survival of *S. vesicarium*.

There was always some survival of the pathogen in treatments where the sample was dry or at the soil surface, even at the final sampling period, which occurred when most onion growers in the area would have onions planted. This confirms that onion leaf residue in the field (or adjacent fields) could act as a source of primary inoculum for onion transplants or seedlings.

An interesting finding was that even the dry samples, which had not produced pseudothecia at the time of collection, produced immature pseudothecia when incubated in a moist chamber. This indicated that all of the overwintered leaf samples had the potential to produce ascospores.

We conclude that *S. vesicarium* is able to survive overwinter in dried crop residue at the soil surface, but the resulting inoculum from dried tissue would likely be conidia and not ascospores when the tissue is rehydrated. The production of ascospores required prolonged moisture and time, with mature ascospores on wet surface samples collected on 1 May and on 30 May. It is possible that the buried wet samples had also produced pseudothecia with ascospores, but the leaf tissue was very degraded, so buried residue was unlikely to be a strong source of pseudothecia and ascospores.

Another possible source of primary inoculum was alternative hosts. The recovery of *S. vesicarium* from inoculated weeds in a controlled-environment setting ranged from 3–44%. When the leaf sections were surface sterilized, the recovery rate was reduced substantially for onion but did not result in a decrease for the weeds. Successful isolation of *S. vesicarium* from surface-sterilized leaves indicated that the pathogen had infected these plants and was not just surviving as conidia or as an epiphyte on the leaf surface. The survival on alternative hosts over winter was not tested, but the previous study showed that *S. vesicarium* can survive in dried residue at the soil surface. The results from this study showed that all of the weed species tested were asymptomatic hosts of *S. vesicarium,* with recovery after sterilization similar to onion. This may be due to the small sample size of the experiment. All of the local weed species tested were potential sources of inoculum and may also act as a living bridge for the pathogen to survive when onion plants are not available.

Weedy hosts may also serve a beneficial role, in the case of fungicide resistance management. The pathogen refuge hypothesis states that non-sprayed host plants offer a genetic refuge for susceptible alleles and so the emergence of fungicide insensitivity in a pathogen population will be slowed (Shaw, 2009). Conversely, these alternative hosts may exist as weeds within the onion field or in neighbouring crop fields that are treated with fungicides, thus further increasing the number of interactions between pathogen and fungicide, resulting in increased selection pressure that could shift the population towards fungicide insensitivity. Fungicide sensitivity is examined in detail in Chapter 4.

Over 500 species have been reported as hosts for *S. vesicarium*, but this is the first report of infection of redroot pigweed, yellow nutsedge, purslane, marshcress, bull thistle, and sowthistle in Canada. Therefore, the ditches and fallow areas near onion fields may act as sources of primary inoculum for SLB. As a result, removal of weeds from surrounding areas might reduce early-season infection. However, growers already make every effort to manage weeds within the onion crop and eliminating all weeds or possible alternative hosts near fields would be costly and potentially detrimental to the local ecosystem. One management recommendation would be that when onion fields are hand-weeded, that the weeds should be physically removed from the field because the plucked weeds could act as sources of inoculum for the pathogen. Fortunately, this is already a common practice as weeds can regrow if pulled and left on the soil. The wide host range of *S. vesicarium* also means that ornamental plants and grass species around dwellings in the Holland Marsh could be a source of inoculum, or plants in the natural areas around the Marsh. The main source of initial inoculum and the relative contributions of different plant species is not known.

Several researchers have suggested that *S. vesicarium* is a seed-borne pathogen, and *S. vesicarium* and other *Stemphylium* spp. have previously been isolated from seed of pea (*Pisum sativum*) (Teixeira, 2005), spinach (*Spinacia oleracea*) (Hernandez-Perez, 2005), squash (*Cucurbita maxima*, *C. moschata*) (Moumni et al., 2020), onion (Aveling et al., 1993), and other crops (Groves and Skolko, 1944). The current study confirmed that *S. vesicarium* can be seed-borne on onion. Onion plants grown from a commercial seed lot inoculated with *S. vesicarium* did not develop SLB symptoms, which indicated that surface infestation of seed did not result in seed-to-seedling transmission. It is possible that the study was too small to capture a small proportion of seed-to-seedling transmission.

The pathogen was isolated from 4–6% of surface-sterilized seed produced in inoculated flowers in a growth room study. This indicated that *S. vesicarium* could develop within the seed, not just on the surface, likely as the result of infection during seed formation. A field study confirmed that 19 of 2013 of seeds (0.9%) were infected with *S. vesicarium*. Seed-to-seedling transmission occurred in 1.2% of the onion seedlings grown from that seed. This infection rate is much lower than previously reported rates of 32% for seeds and 28% for onion seedlings (Aveling et al., 1993). This difference may possibly be due to a lower inoculum load in the current study than in the original study. Much commercial seed production takes place in regions

with low rainfall and low incidence of foliar diseases. Assessment of a larger number of infected seeds might change the proportion of naturally infected seeds or seed-to-seedling transmission, but the current study is consistent with earlier studies showing that this can occur.

Seedling infection from infected seed may result from infection of the tip of the cotyledon when the seed-coat remains attached to the tip of the cotyledon as the seedling emerges from the soil, as occurs with neck rot of onion caused by *Botrytis allii* Munn (Maude and Presly, 1977). There is also the possibility that the fungus grows systemically from the seed to the cotyledon and other leaves. The mechanisms of seed-to-seedling transmission have not been investigated for *S. vesicarium*. The heat treatment studies showed that heat treatments of 50 °C for 10 to 40 min reduced fungal infestation without affecting seed germination or seedling emergence.

This study investigated the etiology of SLB caused by *S. vesicarium* on onion, from primary inoculum to overwintering. It demonstrated that *S. vesicarium* overwintered in infected leaf debris to produce ascospores in early spring and confirmed that it can infest onion seed. In addition, it demonstrated that the local weed species are hosts of *S. vesicarium*, which is not surprising considering the vast and diverse number of previously described host species.

These conclusions support a number of existing management recommendations. Burial or removal of onion debris may reduce the initial inoculum, as has been previously recommended (WCPD, 2012). Infested seed could be a source of inoculum, but commercial seed is often treated with heat and /or fungicides, which probably reduce or eliminate the risk of seed as a source of inoculum. The role of common weeds as alternative hosts supports recommendations for keeping onion fields weed free to reduce competition. This study also provides support for the practice of removing weeds from the field when hand weeding.

Chapter 3 Scheduling fungicide applications for management of Stemphylium leaf blight of onion

The following results of the fungicide application timing field studies have been published (Stricker et al., 2020).

3.1 Introduction

Stemphylium leaf blight (SLB) was first reported on onion in the North America in Texas, USA in 1976 (Miller et al., 1978) and in New York state in 1985 (Shishkoff and Lorbeer, 1989). By 1990, SLB was present in almost all onion fields surveyed across New York state, resulting in severe foliar dieback in some fields (Lorbeer, 1993). SLB was first reported on onion in Ontario in 2008 (Paibomesai et al. 2012) and is a growing concern in both Ontario and New York.

SLB can lead to premature defoliation of the onion crop, resulting in loss of yield up to 90% (Lorbeer, 1993). Field trials in New York have reported yield losses of 28–38% (Hoepting, 2018a; c) and up to 74% premature plant mortality when disease pressure was high (Hoepting, 2017b). The pathogen, *S. vesicarium* also causes purple spot and Stemphylium leaf spot of asparagus and brown spot of pear (Lacy 1982; Hausbeck et al. 1999; Singh et al. 1999). Infection of asparagus or pear fruit results in an unmarketable produce due to cosmetic damage (Falloon et al., 1987; Llorente et al., 2010b).

None of the onion cultivars commercially grown in Ontario are resistant to *S. vesicarium* (Foster et al., 2019). Some cultivars exhibited more foliar lesions than others, whereas other cultivars were more susceptible to leaf dieback (Foster et al., 2019). This observation is supported by reports from a fungicide trial conducted in New York; some fungicide treatments resulted in high levels of leaf dieback with few SLB lesions, while others had less leaf dieback and high SLB lesion counts (Hoepting, 2020a). These results suggested that resistance to infection was independent of susceptibility to dieback.

Regular application of preventative or curative fungicides is an important tool for management of diseases caused by fungal pathogens (Llorente et al., 2012), especially where genetic resistance is not available. Onion growers often use a calendar-based method (e.g., weekly or bi-weekly) for scheduling fungicide applications to manage foliar diseases in the absence of disease forecasting information. Onion growers in the Holland Marsh, Ontario, typically begin fungicide applications for SLB when the disease is reported in the local area

(Tayviah, 2017). This method does not depend on weather conditions or knowledge of the biology of the pathogen, and can result in more applications than necessary (Llorente et al., 2012, Tayviah 2017). More recently, fungicides have been applied when disease forecasting using TOMcast indicated a high risk of disease. This change resulted from trials in the current study and a previous study (Tayviah, 2017). Applying more fungicide sprays than needed is not economical, and also increases the risk that fungicide insensitivity will develop in the pathogen population (Alberoni et al., 2010b). On the other hand, fewer applications than necessary could result in unacceptable levels of disease and loss of yield. Forecasting models use environmental factors to identify conditions that are conducive to disease development and recommend when growers should apply pesticides. A disease forecasting model should trigger fungicide applications that manage the disease without compromising crop yield or quality.

Effective forecasting models can provide the same level of disease suppression as calendar-based methods that result in a greater number of fungicide applications. Several forecasting models have been used for management of *S. vesicarium* on a range of crops, or of other foliar diseases of onion. FAST (Forecasting for *Alternaria solani* on Tomato), a forecast model developed for *A. solani* on tomatoes (*Solanum lycopersicum* L.), achieved comparable disease reduction for brown spot of pear (BSP) as the 7-day calendar spray, with 28% fewer applications (Montesinos and Vilardell, 1992). TOMcast (Tomato forecaster), a modification of FAST, was developed for management of Septoria leaf spot (*Septoria lycopersici* Speg.) and anthracnose (*Colletotrichum coccodes* [Wallr.] S. Hughes) on tomato in Ontario (Pitblado, 1992).

In the TOMcast model, disease severity values (DSVs) are calculated based on leaf wetness duration and the average temperature during the wet period, and fungicide sprays are recommended when cumulative DSVs meet or exceed the threshold value (Madden et al., 1978). When TOMcast with a DSV threshold of 15 was used for management of purple spot of asparagus, fungicide application was reduced by 60% (Meyer and Hausbeck, 2000). With some cultivars of asparagus, TOMcast improved the suppression of Stemphylium leaf spot without increasing the number of fungicide applications (Foster and McDonald, 2018). TOMcast also reduced fungicide sprays on onion in 2015 and 2016 while providing 35–44% SLB disease suppression (Stricker et al., 2020).

The BOTcast (Botrytis forecaster) model was developed for management of Botrytis leaf blight of onion caused by *Botrytis squamosa*. BOTcast uses similar weather parameters as TOMcast but a different combination of temperature and leaf wetness to estimate disease risk based on favourable conditions for infection (Sutton et al., 1986). A combination of daily inoculum value (0–2) and daily infection value (0–2) is used to calculate cumulative disease severity index (CDSI). Fungicide applications are recommended at one of two thresholds: medium risk at 21–30 CDSI, and high risk of disease at 31–40 CDSI. In previous trials at the Muck Crops Research Station (MCRS) in Ontario, BOTcast showed potential to reduce the number of spray applications for managing SLB on onion (McDonald and Vander Kooi, 2014). A third model, BSPcast (Brown spot of pear forecaster), was developed specifically for use with *S. vesicarium* in pear (*Pyrus* spp.) orchards in Spain (Montesinos et al., 1995). As with the previous models, this model integrated the effect of daily leaf wetness duration and the temperature during the wetness period to calculate a fungicide application recommendation. BSPcast-guided schedules for application of thiram used 20–50% fewer sprays to provide the same degree of disease suppression as weekly application on pear (Llorente et al., 2010b). Most recently, the forecasting model SIMSTEM (Simulation of *Stemphylium*) was developed for management of purple spot of asparagus in Germany (Bohlen-Janssen, 2018). SIMSTEM uses area-specific weather data to predict the beginning of an epidemic, the time of first treatment, and disease progress. The simulator will be available to growers on a website platform in the future (www.isip.de).

Fungicide seed treatments can be highly effective in reducing, or even eradicating, seed-borne fungal pathogens for many vegetable crops (Mancini and Romanazzi, 2014). Systemic fungicides such as penflufen and azoxystrobin may also protect the first few leaves of an onion seedling before the plants are big enough to be sprayed with a foliar fungicide. Studies presented in Chapter 2 demonstrated that *S. vesicarium* can be seed-borne, as suggested by Aveling et al. (1993), and ascospores are present in the field even before the crop emerges (Gossen et al. 2021), so seed treatments may offer early-season protection against SLB.

The objectives of the current project were i) to assess existing disease forecasting models for the management of SLB, and ii) to investigate seed treatments as alternative or additional management methods for SLB.

This research focused on yellow bulb onion grown in the Holland Marsh, Ontario on organic muck soil (50–75% organic matter, pH 5.5–7.2), which is where the majority of Ontario onions are produced. The fungicide timing study presented below has already been published (Stricker et al., 2020).

3.2 Materials and methods

3.2.1 Plant material

Commonly used cultivars of yellow cooking onion (cv. LaSalle in 2018, cv. Fortress in 2019; Stokes Seeds, Thorold, ON) were direct seeded in organic soil (organic matter ≈ 69, pH ≈ 6.1) at the Muck Crops Research Station (King, ON, 44.0406° N, 79.5977° W). Cultivar LaSalle was selected because it was susceptible to SLB in a previous study (Tayviah, 2017). In 2019, this cultivar was not available, so another susceptible cultivar, Fortress, was used.

The experimental design was a randomized complete block design with four replicates. One plot consisted of two adjacent beds, each 6 m × 1.75 m, seeded with four double rows 7.5 cm apart with 35 cm separation between pairs of rows. Beds were separated by 45 cm-wide wheel row furrows. There were four plots across the field and each set of four beds was separated by a 1.5 m-wide path (Appendix 3.1).

Onion plots were seeded on 9 May 2018 and 17 May 2019. Planting was delayed in 2019 due to high rainfall and poor drainage in the field. Seed was sown at 35 seeds m^{-1} using a Stanhay S870 Precision belt planter (Stanhay, Bourne, UK). The granular fungicide Dithane™ DG (75% mancozeb, 6.6 kg ha^{-1}, Dow Agrosciences, Calgary, AB) was applied in the furrow at seeding to manage onion smut caused by *Urocystis cepulae*. Insecticides, herbicides, and irrigation were applied as required according to OMAFRA production recommendations. The seed was pelleted, and the pellet contained insecticide (0.18 g clothianidin + 0.6 g imidacloprid per 1,000 seeds, Sepresto 75 WS, CropScience, NC) for management of onion maggot (*Delia antiqua* Meigen). Herbicides were applied for weed management when necessary, and plots were also weeded by hand.

In 2019, barley was manually sown after onion was seeded. Interplanting with barley is a common cultural practice to reduce soil erosion by wind when planting carrot or onion crops in muck soil (Zandstra and Warncke, 1993). Herbicide (125 g ha^{-1} fluazifop-P-butyl and s-isomer;

Venture L, Syngenta, Guelph ON) was applied to kill the barley when the barley leaves reached 15 cm and the onions reached the 2–4 leaf stage.

3.2.2 Fungicide spray programs

A tractor-mounted sprayer fitted with spray nozzles (D-3 hollow cone, TeeJet) delivered 500 L solution ha^{-1} at 620 kPa. In 2018, the foliar sprays consisted of azoxystrobin plus difenoconazole (Quadris Top, applied at 200 g ha^{-1} azoxystrobin, 125 g ha^{-1} difenoconazole; Syngenta, Guelph, ON) alternated with fluopyram plus pyrimethanil (Luna Tranquility, applied at 150 g ha^{-1} fluopyram, 450 g ha^{-1} pyrimethanil; Bayer Crop Science Inc., Calgary, AB). The foliar fungicide spray program in 2019 used benzovindiflupyr (Aprovia applied at 75 g a.i. ha^{-1}; Syngenta, Guelph, ON) alternated with chlorothalonil (Bravo Zn applied at 1.8 kg a.i. ha^{-1}; Syngenta, Guelph, ON) in an effort to use fungicides that might be more effective to reduce SLB, since no disease suppression was observed in 2018.

There were seven treatments and an unsprayed check (Table 3.1). The weekly calendar sprays were applied every 7–10 days where possible. There were also two fungicide seed treatments in the trials: EverGol Prime (2.5 g penflufen kg^{-1} seed; Bayer Crop Science, Guelph, ON) or FarMore F300 (0.075 g mefenoxam kg^{-1} seed, 0.0275 g fludioxonil kg^{-1} seed, 0.025 g azoxystrobin kg^{-1} seed; Syngenta, Guelph, ON). The seed treatments were applied commercially (Incotec, Salinas, CA) using industry-standard procedures. In 2019, the seed treatments were also tested without additional foliar fungicide applications. The action thresholds for these disease forecasting models were based on previous research (Tayviah, 2017; Bohlen-Janssen, 2018).

Table 3.1 Timing of fungicide application treatments for the fungicide timing field trials on Stemphylium leaf blight of onion at the Muck Crops Research Station, Ontario in 2018 and 2019. Values within brackets indicate the number of foliar fungicide applications each year.

Treatment	2018	2019
Control	Unsprayed (0)	Unsprayed (0)
Weekly, early	Weekly foliar starting at 2-leaf stage (7)	Weekly foliar starting at 2-leaf stage (7)
Weekly, late	Weekly foliar starting at 4-leaf stage (5)	n/a[1]
TOMcast 15	Every 15 DSVs (5)	Every 15 DSVs (6)
BSPcast (modified)	Every time threshold of 0.5 was reached (6)	Every time threshold of 0.5 was reached (5)
Mineral oil	Drench, weekly, 2-leaf stage (7)	n/a
Mefenoxam, fludioxonil & azoxystrobin seed treatment + spray	Treated seed, weekly foliar starting at 2-leaf stage (7)	Treated seed, weekly foliar starting at 2-leaf stage (7)
Penflufen seed trt + spray	Treated seed, weekly foliar starting at 2-leaf stage (7)	Treated seed, weekly foliar starting at 2-leaf stage (7)
Mefenoxam, fludioxonil & azoxystrobin seed trt only	n/a	Treated seed (0)
Penflufen seed trt only	n/a	Treated seed (0)

[1]n/a –not applicable, this treatment was not used.

In 2018, a treatment was added where a food-grade mineral oil product (Civitas™, 25.5 L ha^{-1} mineral oil; Petro-Canada Lubricants, Inc., Mississauga, ON) was applied as a drench at emergence, followed by weekly calendar sprays starting at the 2-leaf stage. Drench applications were applied to the base of the plants with a CO_2 backpack sprayer equipped with a single Syngenta 65-06 vegetable nozzle calibrated to deliver 1000 L ha^{-1} along the row. Products containing mineral oil have been registered for use against several foliar diseases of turfgrasses, and this product induced systemic resistance against three diseases of *Agrostis stolonifera* L. caused by Ascomycete fungi. (Cortes-Barco et al., 2010).

3.2.3 Disease forecasting models

The field experiment evaluated two forecasting models, TOMcast and a modified BSPcast in. comparison to weekly spray treatments that were similar to fungicide regimens commonly used by local growers. The 'early' weekly spray program began at the 2–3 leaf stage of onion growth, and the 'late' program began the 4–5 leaf stage (Table 3.2).

Table 3.2 Fungicide applications in the fungicide application timing field trials for management of Stemphylium leaf blight of onion at the Holland Marsh, ON.

	Fungicide application schedule by year (days after planting[1])	
Treatment	**2018**	**2019**
Weekly – early[2]	41 50 57 64 70 79 86	40 48 55 66 75 82 90
Weekly – late	57 64 70 79 86	
BSPcast	41 57 70 79 86	40 55 66 75 82 88
TOMcast (15)	47 57 64 70 79 86	40 48 55 66 75 82 90

[1]Date planted: 9 May 2018, 17 May 2019.
[2]Seed treatments in 2018 and 2019 received the same spray treatment as 'weekly early'.

The BSPcast model was designed to predict disease severity in pear grown in Mediterranean countries (Llorente et al., 2000). In the original model, disease severity was divided by 3.7942, which was chosen based on maximum daily disease severity predicted by the equation for weather data collected in Spain and Italy during the study period (1995–1997). The original BSPcast model over-estimated the infection risk, and was later slightly revised (Llorente et al., 2011). The maximum disease severity predicted using weather data collected at the Holland Marsh in 2017 and 2018 was 6.182, so this was used to modify the BSPcast model. The modified BSPcast was calculated as follows:

$$D = \frac{-1.70962 + 0.0289T + 0.04943W + 0.00868TW - 0.002362W^2 - 0.000238T^2W}{6.182}$$

where D = disease risk, T = average hourly temperature (°C) during leaf wetness, and W = duration of leaf wetness ≥50%. Weather data were collected hourly using an Onset® automatic weather station (Onset Corporation, Bourne, MA) and leaf wetness sensors (S-LWAM003, Onset Corp.). The disease risk was calculated daily at 12:00 PM based on the data collected in the previous 24 h. The 3-day cumulative value was calculated, and a threshold of D = 0.5 was used to recommend spray treatments in the BSPcast treatment.

The TOMcast model was developed to predict the severity of disease caused by different Ascomycete pathogens on tomatoes in Ontario, Canada (Pitblado, 1992). TOMcast used leaf wetness duration and average temperature during wetness periods to calculate a daily severity value (DSV) (Table 3.3) (Montesinos and Vilardell, 1992). A threshold of 25 was chosen to trigger the first spray application, and subsequent applications were applied when the model reached a threshold of 15, based on previous research conducted at the MCRS (Tayviah, 2017).

Table 3.3 Mean temperature and leaf wetness duration values used in calculating disease severity values (DSV) for the TOMCAST model (Madden et al., 1978).

Mean temp	**Leaf wetness duration (h) required to produce DSV:**				
(°C)	**0**	**1**	**2**	**3**	**4**
13–17	0–6	7–15	16–20	21+	
18–20	0–3	4–8	9–15	16–22	23+
21–25	0–2	3–5	6–12	13–20	21+
26–29	0–3	4–8	9–15	16–22	23+

3.2.4 Disease and yield assessment

SLB symptoms were assessed every second week after the onions had reached the 10-leaf stage. For each replicate, SLB incidence (presence of upright leaves with dieback or lesions) and severity of 20 randomly selected plants in the middle rows of each plot was assessed. A scale of 0 to 4 was used to assess severity on each of the three oldest leaves per plant and assign them into rating classes, where: 0 = no chlorosis, 1 = 1–10% chlorosis, 2 = 11–25% chlorosis, 3 = 26–50% chlorosis, and 4 > 51% chlorotic area. These data were used to calculate a disease severity index (DSI), which is a value that ranges from 0 to 100 (Chester, 1950). The DSI was calculated as:

$$\text{DSI} = \frac{\sum [(\text{class value})\ (\#\ \text{leaves in each class})]}{(\text{total}\ \#\ \text{leaves assessed})\ (\#\ \text{classes} - 1)} \times 100$$

The area under the disease progress curve (AUDPC) was calculated over the entire growing season using the biweekly DSI values to compare SLB severity for each treatment. Since the rating timing differed among years, the AUDPC was divided by the number of days between first and last rating (2018 = 39, 2019 = 34) to generate the standardized AUDPC (sAUDPC), which is a unit-less value that can range from 0 to infinity (Vidhyasekaran, 2004).

Shortly before lodging, a final destructive disease assessment was conducted. Twenty onion plants were harvested from the inside rows of each plot, and every leaf was removed. The leaves were sorted based on the same classes as described previously and DSI was calculated as described above. In late September to early October, the yield and marketable yield were assessed by harvesting two 2.3-m-long sections of row from the middle rows of each plot. The plants were topped using an agriculture-grade onion topping machine, bagged, weighed, and graded based on diameter to determine marketable yield, which was converted to tonnes ha^{-1}. The onions were sorted into four classes: cull (< 32 mm dia.), small (32–45 mm), medium (45–76 mm), and jumbo (> 76 mm). Onions in the medium and jumbo classes were deemed marketable, and the percent of marketable onions (by weight) was calculated for each treatment.

3.2.5 Data analysis

A mixed model analysis of variance was used to assess the disease and yield data (PROC GLIMMIX) in SAS University Edition (SAS Institute Inc., Cary, NC). DSI and marketable yield values, which were expressed as a percent, were divided by 100 for analysis using a beta distribution. Variance was partitioned into random (block or replicate) and fixed (treatment) effects. Means were separated using Tukey's Honest Significant Difference at $P \leq 0.05$. Pearson's correlation (PROC CORR, $P \leq 0.05$) was used to test for a linear relationship between yield and SLB severity each year. In the pooled analysis across years, the treatments occurring in only one year were removed prior to analysis.

3.3 Results

3.3.1 2018 Field season

Disease pressure and SLB severity was high in 2018, starting early in the growing season. The weekly schedules resulted in five (4-leaf stage) to seven (2-leaf stage) foliar applications of fungicide. Weekly foliar fungicide applications and the mineral oil drench did not reduce blight incidence or severity compared to the untreated control (Table 3.4). The forecasting models reduced the number of fungicide applications compared to the weekly schedule starting at the 2-leaf growth stage, with five applications recommended by TOMcast and six applications by BSPcast. However, neither of the forecasting models reduced SLB severity compared to the unsprayed control based on in-field disease incidence and severity. In contrast, fungicide seed treatments in combination with weekly foliar sprays reduced SLB incidence by 22–30%, DSI by

42–51%, and DSI only for penflufen plus foliar sprays by 34% in the destructive disease assessment relative to the control (Table 3.4).

Table 3.4 Effect of fungicide applications on Stemphylium leaf blight incidence (%) and severity (disease severity index, DSI, and standardized area under disease progress curve, sAUDPC[1]) on onion at the Muck Crops Research Station, 2018.

	Applications	14 Aug		27 Aug	
Treatment	**(#)**	**Incidence**	**DSI**	**DSI**	**sAUDPC**
Control	0	98 a[1]	57 a	88 a	21 a
Weekly spray (2-leaf)	7	93 ab	51 a	77 a	17 b
BSPcast	6	90 ab	45 ab	72 ab	16 b
Weekly spray (4-leaf)	5	86 ab	46 ab	79 a	16 b
Mineral oil + weekly spray	7	89 ab	42 abc	76 a	14 bc
TOMcast	5	88 abc	40 abc	79 a	15 bc
Mefenoxam, fludioxonil & azoxystrobin seed trt + weekly	7	76 bc	33 bc	75 ab	13 c
Penflufen seed trt + weekly	7	69 b	28 c	58 b	8 d

[1] Means in a column followed by the same letter do not differ based on Tukey's test at $P = 0.05$.

Based on sAUDPC, an assessment of SLB severity over the whole season, all of the treatments reduced sAUDPC compared to the control in 2018, with the greatest decrease (62%) decrease for the penflufen plus foliar sprays treatment. Only the two seed treatments decreased incidence and severity at the last in-field disease assessment (14 August 2018), and only the penflufen seed treatment decreased DSI in the destructive assessment (27 August 2018).

There were no differences in yield among treatments. The penflufen seed treatment resulted in fewer, slightly larger bulbs (Table 3.5), but this was likely associated with low emergence in this treatment (Figure 3.1). There were no differences in SLB severity or yield between the weekly spray programs that began at 2 true leaves or 4 true leaves.

Table 3.5 Effect of fungicide application on yield and size distribution of onions at the Muck Crops Research, 2018.

Treatment	Yield (T ha^{-1})	Bulbs (m^{-1})	Bulb wt. (g)	Size distribution by weight (%)		
				Cull	Marketable	Jumbo
Control	41 ns[1]	21 a[2]	93 ab	11ns	84 ns	5 ns
Weekly spray (2-leaf)	35	17 ab	101 ab	10	79	12
BSPcast	37	18 ab	101 ab	11	78	12
Weekly spray (4-leaf)	33	20 a	84 b	13	81	6
Mineral oil + weekly spray	44	21 a	100 ab	10	86	4
TOMcast	47	20 a	109 ab	7	83	9
Mefenoxam, fludioxonil & azoxystrobin seed trt + weekly spray	37	18 ab	100 ab	9	86	5
Penflufen seed trt + weekly	35	12 b	126 a	5	76	20

[1] ns –Not significant.
[2] Means in column followed by the same letter do not differ based on Tukey's test at $P = 0.05$.

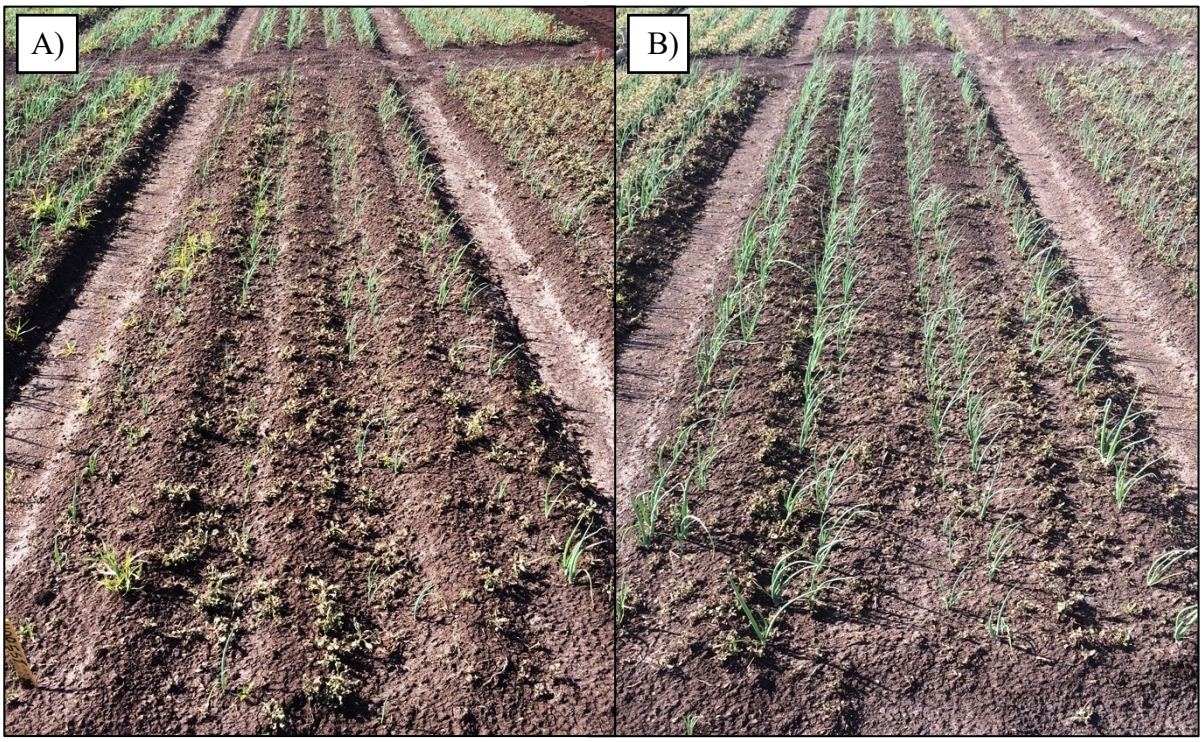

Figure 3.1 A) Poor emergence in penflufen-treated seed compared to B) the control treatment on onion in a field trial at the Muck Crops Research Station in 2018.

3.3.2 2019 Field season

Disease pressure was relatively low in 2019. Foliar fungicide applications made weekly or timed using forecasting models did not reduce SLB incidence or severity relative to the untreated control. The weekly schedule resulted in seven foliar fungicide applications. The forecasting models reduced fungicide applications, with six applications recommended by TOMcast and five applications by BSPcast. The penflufen fungicide seed treatment in combination with weekly foliar sprays reduced incidence by 27% and severity by 43% compared to the control in the in-field assessment on 15 Aug (Table 3.6). There were no differences for SLB severity when assessed using the destructive sampling method or sAUDPC. When severity was assessed at the end of the season with the destructive assessment, the weekly spray starting at the 2-leaf stage exhibited the lowest DSI (but this was not statistically significant), and the penflufen seed treatment exhibited the highest DSI. This may be because the penflufen seed treatment without foliar sprays had fewer leaves (555 leaves assessed in total) compared to the weekly spray treatment (670 leaves), which may have skewed this data assessment. The penflufen seed treatment plus foliar sprays had 631 green leaves and the unsprayed control had 586, which may indicate that the foliar fungicide sprays contributed to plant growth or health. There were no differences in yield among treatments (Table 3.7).

Table 3.6 Effect of fungicide applications on Stemphylium leaf blight incidence (%) and severity (disease severity index, DSI and standardized area under the disease progress curve, sAUDPC) on onion at the Muck Crops Research Station, 2019.

	Applications	15 Aug		22 Aug	
Treatment	**(#)**	**Incidence**	**DSI**	**DSI**	**sAUDPC**
Control	0	96 ns[1]	37 a[2]	23 ns	21 ns
Mefenoxam & fludioxonil & azoxystrobin seed trt (no spray)	0	84	34 a	19	15
Mefenoxam & fludioxonil & azoxystrobin seed trt + weekly	7	88	30 ab	18	15
BSPcast	5	85	28 ab	16	15
Penflufen seed trt (no spray)	0	84	29 ab	26	18
Weekly spray(2-leaf)	7	81	27 ab	14	14
TOMcast	6	80	29 ab	19	14
Penflufen seed trt + weekly spray	7	70	21 b	17	12

[1] ns –Not significant at $P = 0.05$ based on Tukey's HSD test.
[2] Means in column followed by the same letter do not differ based on Tukey's test at $P = 0.05$.

Table 3.7 Effect of fungicide application on yield and size distribution of onions at the Muck Crops Research Station, 2019.

	Yield	Bulbs	Bulb wt.	Size distribution by weight (%)		
Treatment	**(T ha^{-1})**	**(m^{-1})**	**(g)**	**Cull**	**Marketable**	**Jumbo**
BSPcast	70 ns[1]	24 ns	133 ns	2 ns	94 ns	5 ns
Mefenoxam, fludioxonil & azoxystrobin seed trt only	72	21	149	1	89	10
Penflufen seed trt + weekly spray	72	25	125	3	89	8
Weekly spray (2-leaf)	73	21	149	1	85	15
Mefenoxam, fludioxonil & azoxystrobin seed trt + weekly	74	21	158	1	80	18
Control (no spray)	74	22	149	1	87	13
TOMcast	75	22	148	1	88	11
Penflufen seed trt (no spray)	75	27	128	3	87	9

[1] ns –Not significant based on Tukey's HSD test at $P = 0.05$.

3.3.3 Pooled years

The SLB severity in the weekly fungicide spray programs in 2018 and 2019 did not differ from the unsprayed control. This lack of response may be linked to a lack of efficacy of the fungicides assessed, as discussed in Chapter 4. There was a weak positive correlation between SLB severity

and yield in 2018, but no correlation in 2019. There was a negative correlation between SLB severity and marketable yield in 2019, but no correlation in 2018 (Table 3.8).

Table 3.8 Correlation (r) between Stemphylium leaf blight severity and yield (total and marketable) for 2 years of field experiments on onion.

	Correlation with SLB severity			
	Yield (T ha^{-1})		**Marketable yield (%)**	
Year	**r**	***P* value**	**r**	***P* value**
2018	-0.14	0.43	**-0.41**	**0.02**
2019	**0.41**	**0.02**	-0.06	0.74

Bold values are significant based on Pearson's correlation at $P < 0.05$.

When the treatments where pooled, and treatments used in only one year removed, the interaction of treatment and years was significant for SLB severity (Appendix 3.6). Disease severity was generally lower in 2019, and the proportion of the decrease from 2018 to 2019 was not equal among treatments. For SLB incidence, there was no interaction, the main effect of treatment was significant, and the effects of treatment pooled over the two years were examined (Figure 3.2, Appendix 3.6). Both of the fungicide seed treatments reduced SLB incidence compared to the unsprayed control (Figure 3.2). Incidence in the weekly spray and BSPcast treatments were not different from the unsprayed control. The TOMcast treatment exhibited 13% lower SLB incidence than the unsprayed control. The seed treatments in combination with foliar fungicide application reduced SLB incidence relative to the control by 15–27%.

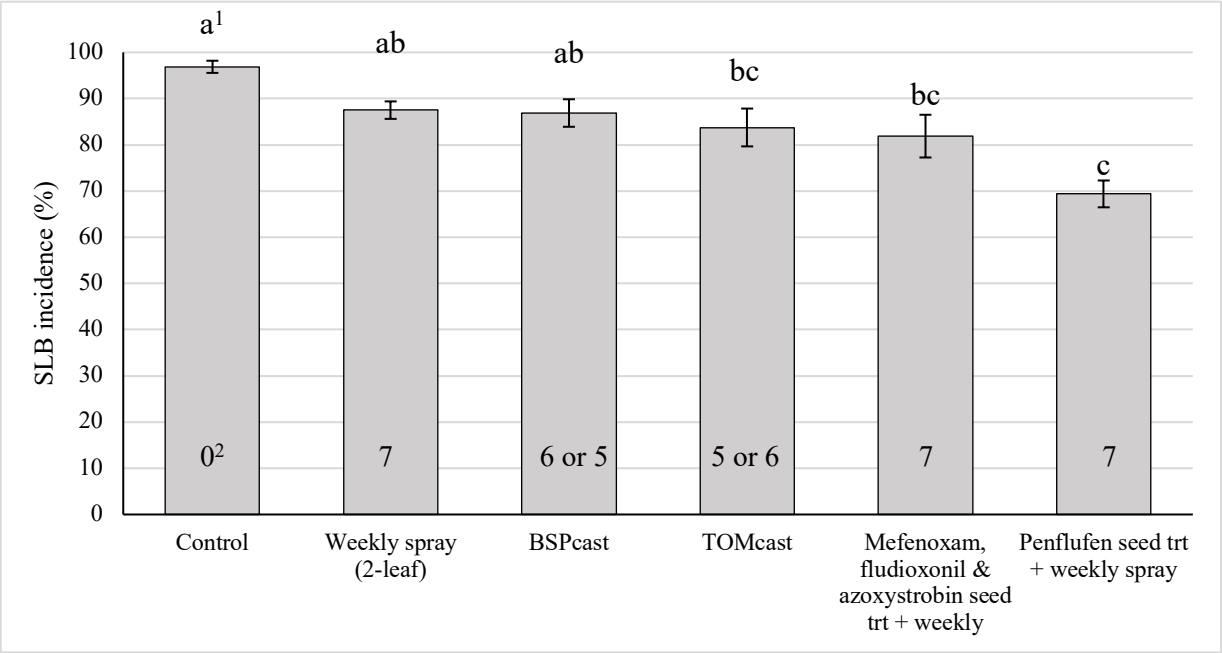

Figure 3.2 Effect of seed treatments and foliar fungicides on mean Stemphylium leaf blight incidence in field trials at the Holland Marsh, ON data from 2018 and 2019 pooled. Values at the base of each bar indicate the number of foliar fungicide applications. Bars topped with the same letters do not differ based on Tukey Test at $P < 0.05$, and the capped lines represent standard error.

To directly compare AUDPC over years, each estimate was normalised by dividing the total AUDPC by the number of days between the first and the last SLB assessments each year. The sAUDPC values for the untreated control were the same for 2018 and 2019 (21 for both years) and values for the other treatments were similar (±1–4).

Although disease severity was lower in 2019 (Figure 3.3.), symptoms were observed on nearly every plant in the plots, which was indicated by the high SLB incidence values. The symptoms included long chlorotic streaks down one side of the onion leaf, leaf tip dieback, oval to spindle-shaped lesions that were often sunken and sometimes exhibited target-like rings of sporulating conidia (Figure 3.4). The symptoms were most frequently seen on the oldest, outermost leaves and were not observed on the newest leaves, indicating that older leaves are more susceptible to infection than young leaves, or had been exposed to inoculum for a longer time. In the rare case of hail damage, the damaged areas were soon colonized by *S. vesicarium*. In New York, a certain symptom of colonization by *S. vesicarium* is referred to as 'dirty tips', where necrotic tips affected by dieback become dark brown or black, as a result of sporulation of

S. vesicarium (C. Hoepting, Cornell University, *personal communication*). This was rarely, but occasionally observed in the field in the current trials.

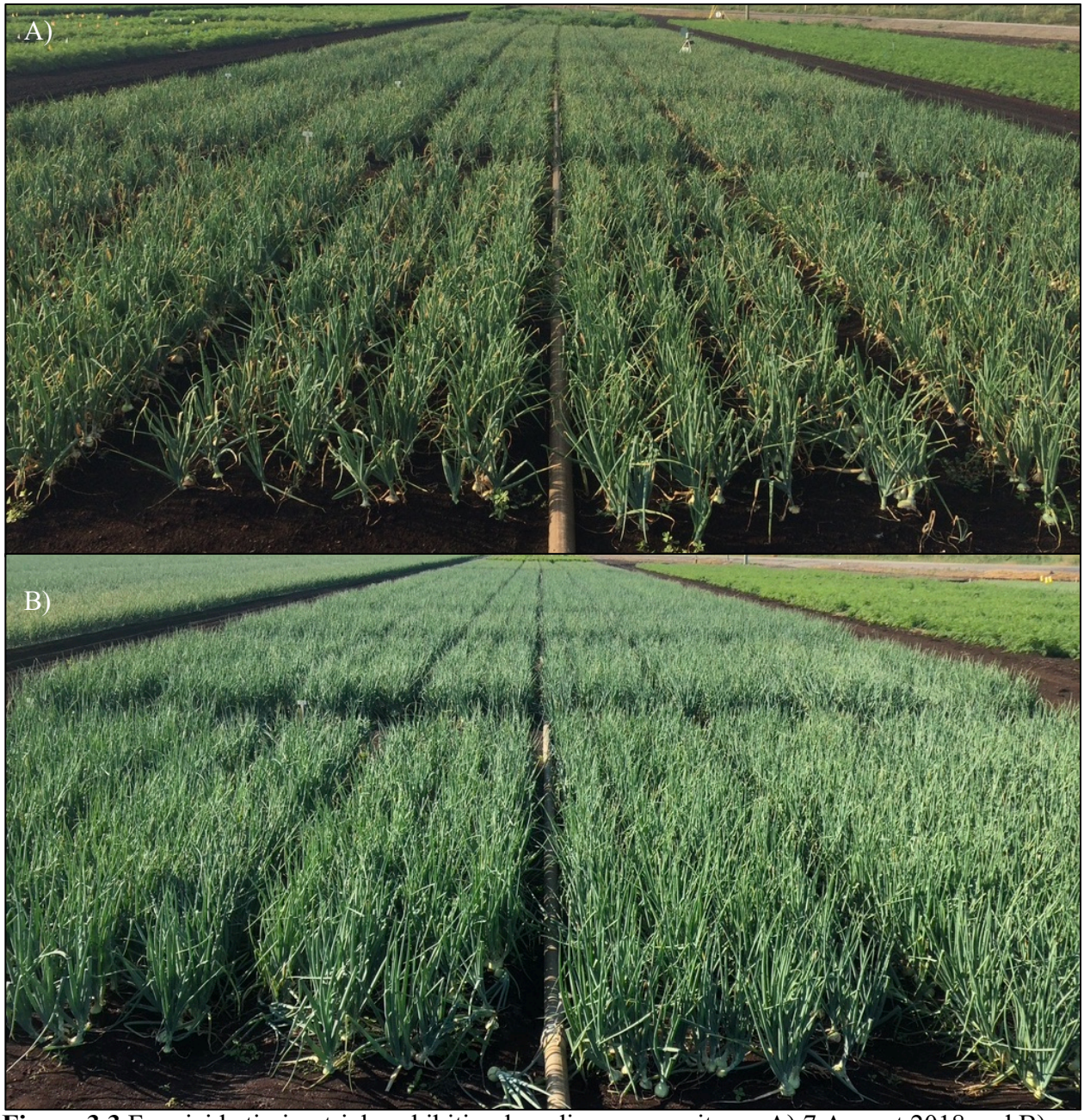

Figure 3.3 Fungicide timing trials exhibiting low disease severity on A) 7 August 2018 and B) 15 August 2019 at the Muck Crops Research Station.

Figure 3.4 Stemphylium leaf blight symptoms in the Holland Marsh, ON: A) Chlorotic streaks along the entire edge of oldest leaf with a lesion near the base, B) tip dieback with a lesion in the midst of the chlorotic region, C) leaf dieback and twisting with a brown region of sporulation, D) chlorotic streak on one edge of leaf with sunken spindle-shaped lesions, E) diffuse chlorosis of entire leaf with streaks of brown sporulating areas, F) tip dieback with oval-shaped lesion, and G) tip dieback and darkening ('dirty tips') with oval-shaped lesion with concentric rings of sporulation.

The approximate cost per hectare for each fungicide program was calculated based on the price of each fungicide purchased by the MCRS staff from the local commercial retailer and the rate at which it was applied (Table 3.9). The cost for each product was estimated to be: Luna Tranquility \$138 ha^{-1}, Quadris Top \$86 ha^{-1}, Bravo Zn \$81 ha^{-1}, Aprovia \$97 ha^{-1}, and Civitas \$548 ha^{-1}. Note that the application and labour cost was not factored into this analysis. The additional cost of various seed treatments adds approximately \$0.25–0.50 per 1000 seed to the price of seed, which would amount to an increase of \$200–400 ha^{-1}. Therefore, the estimated cost of the seed treatments was \$300 ha^{-1}. Currently penflufen is applied to seed to protect against onion smut, so it would be applied even if there was no risk of SLB. By reducing the fungicide applications by 1 or 2 sprays, the forecasting models saved \$86–224 ha^{-1} in 2018 and \$97–178 ha^{-1} in 2019, with no reduction in yield.

Table 3.9 Approximate cost of fungicide application programs used to manage SLB in the Holland Marsh, ON in 2018 and 2019.

	Cost of fungicide treatment[1] per hectare (\$ ha^{-1})	
Treatment	**2018**	**2019**
Control	**\$0**	**\$0**
Weekly, early	4×QT + 3×LT = **\$758**	4×A + 3×BZ = **\$631**
Weekly, late	3×QT + 2×LT = **\$534**	nd
TOMcast 15	3×QT + 2×LT = **\$534**	3×A + 3×BZ = **\$534**
BSPcast (modified)	3×QT + 3×LT = **\$672**	3×A + 2×BZ = **\$453**
Mineral oil	CV + 4×QT + 3×LT = **\$1306**	nd
Mefenoxam, fludioxonil & azoxystrobin seed treatment + spray	FM + 4×QT + 3×LT = **\$1058**	FM + 4×A + 3×BZ = **\$931**
Penflufen seed trt + spray	EP + 4×QT + 3×LT = **\$1058**	EP + 4×A + 3×BZ = **\$931**
Mefenoxam, fludioxonil & azoxystrobin seed trt only	nd	**\$300**
Penflufen seed trt only	nd	**\$300**

[1]Acronymns are as follows: QT–Quadris Top, LT-Luna Tranquility, CV-Civitas, FM–FarMore F300, EP-EverGol Prime, A–Aprovia, BZ-Bravo Zinc, nd – not done.

3.4 Discussion

Stemphylium leaf blight can result in severe defoliation of onion, and concern has been mounting over this disease in Ontario in the past decade. Currently, SLB is managed with repeated applications of foliar fungicides. In this study, two forecasting models were assessed over 2 years compared to a weekly (7–10 day interval) spray program. Both forecasting models reduced the number of fungicide applications while providing equivalent disease suppression. This resulted in a cost savings of \$86–224 ha^{-1}, not including application costs. Repeated applications of a fungicide also increase the risk of fungicide insensitivity developing in the pathogen population. Reducing the number of applications by using disease forecasting models is an important management tactic that could extend the useful life of fungicides in the field, in addition to being more economical for growers. However, none of the fungicide spray programs resulted in an increase in yield in comparison to the untreated check and there is the possibility that no fungicide sprays should have been applied during these years.

The mineral oil drench treatment did not decrease SLB severity compared to the unsprayed control. Additionally, the application of this product increased the cost of the fungicide treatment by \$548 ha^{-1}, which is much more costly than any other fungicide products on the market. This treatment was not repeated in 2019 because of the lack of disease suppression and the cost of the product, which would be a barrier for growers.

Fungicide seed treatments reduced the severity of SLB when combined with foliar fungicide applications. Treatment with penflufen consistently reduced SLB severity during the growing season when used in conjunction with weekly foliar sprays. This indicated that infection may be occurring early in the season, as soon as onion leaf tissue was present. Penflufen is a new active ingredient in the SDHI group (FRAC group 7) that was registered for use on onion in Ontario in 2019. The *in vitro* sensitivity of *S. vesicarium* to penflufen has not yet been assessed. There is a possibility that the seed treatment provided early-season protection with this systemic fungicide, or that the seed treatment led to healthier seedlings that were more resistant to infection. The other seed treatment, FarMore F300, contained azoxystrobin that has been proven to be ineffective because of fungicide insensitivity (Stricker et al., 2020). It also included mefenoxam, a FRAC group 4 fungicide which is not normally applied to onion seed in Ontario and is not expected to have any activity against an Ascomycete fungus such as *S. vesicarium*.

It is interesting to note that the final destructive assessment did not detect any differences among the treatments in 2019. The destructive assessment included all of the leaves of each plant, whereas the earlier severity ratings each season only include the oldest three leaves. As a result, the final (destructive) assessment may represent a more accurate assessment of the impact of SLB on the whole plant. Although DSI values were lower in 2019 compared to 2018, the sAUDPC in 2019 was equal to 2018. The 2019 season was cooler in May, resulting in a lower disease severity at the first rating date, but high temperatures in July may have contributed to the rapid increase in severity.

The poor emergence in the seeds treated with penflufen (FRAC group 7) in 2018 was an anomaly; the same product has been used before and since, but this was the only time this effect was observed. There is a possibility that, since the seed was older, the germination of the seed was lower overall and extended exposure of the embryo within the seed to the penflufen seed treatment may have resulted in toxicity. A decrease in emergence has previously been documented for wheat seeds treated with benomyl (FRAC group 1) or carboxin (FRAC group 7) and stored for > 30 days (Khaleeq and Klatt, 1986). Phytotoxicity of fluopyram has also been reported in soybean seedlings, resulting in a 'halo effect' symptom on cotyledons, but the plants were able to survive and yield was not impacted (Kandel et al., 2018).

SLB severity in 2019 was low to moderate, which was likely associated with below-average seasonal rainfall. However, the disease forecasting programs still recommended several fungicide applications. The DSI for the unsprayed control at the end of the season was only 33, but TOMcast recommended six applications in 2019. In a previous study on disease forecasting models conducted at the same location, TOMcast recommended five applications in 2016, when final severity was only 28 DSI (Stricker et al., 2020). This level of severity arguably did not merit the number of fungicide applications recommended. There clearly is room for improvement in SLB forecasting, especially when disease risk is low.

The relationship between SLB severity and yield was inconsistent among years, which may be due to confounding factors such as weather, cultivar used, and differences in seeding date. Weather likely had the greatest effect. For example, high temperature stress could reduce yield but not directly affect SLB severity. Overall, only a small proportion of the variation in yield was explained by variance in SLB severity. The trials conducted at the MCRS may be too small to capture small changes in yield, even though differences in yield were identified in

similar trials in New York State (Hoepting, 2017c, 2018a). Yield was much higher in 2019 than 2018, but this may be due to cultivar differences, since LaSalle was used in 2018 and Fortress in 2019. In disease forecasting trials conducted at the same location in 2015 and 2016, the relationship between yield and SLB severity was not significant (Stricker et al., 2020). The poor emergence in the penflufen seed treatment in 2018 may also have confounded the yield data and the relationship between SLB severity and yield.

Even if defoliation caused by SLB does not affect yield directly, especially if it occurs later in the growing season, it can reduce the efficacy of sprout inhibitors. The sprout inhibitor, maleic hydrazide, should be applied to onions that have at least 5–8 green leaves shortly before lodging (Ilić et al., 2011; Arysta LifeScience Canada, 2020). Onions with moderate to severe SLB might not have enough green leaves at the end of the season to take up the sprout inhibitor. If sprout inhibitor is not applied or is not taken up by the crop, the harvested bulbs will have a shorter storage life and shelf life. This can require the crop to be sold earlier, often at a time of lower prices, leading to lower returns. However, SLB severity was not high enough in the unsprayed control to prevent the application of maleic hydrazide in either year of this study.

Previous studies of disease forecasting models were conducted at the MCRS in 2015 and 2016. Versions of TOMcast and BOTcast were used to time the application of Luna Tranquility (Stricker et al., 2020). In 2015, treatments scheduled based on either of these disease forecasting models decreased leaf dieback (AUDPC) relative to the unsprayed control. In 2016, however, there was lower SLB severity overall and no differences among treatments, which may be linked to fungicide insensitivity. Similar to this study, SLB severity for the two forecasting models was the same as the weekly spray programs with fewer fungicide applications. Also, there were no differences in yield among the treatments in 2015 or 2016, but this may be linked to relatively low SLB severity (15–47 sAUDPC for leaf dieback) (Tayviah, 2017).

Based on the current fungicide timing trials and previous studies at the Holland Marsh (Stricker et al., 2020; Gossen et al., 2021), one might conclude that applications of foliar fungicide to suppress SLB on onion were not warranted, since there was little or no reduction in yield or quality of the treatments compared to the untreated control. However, recent trials conducted in New York State provided a different picture of SLB, even though the onion production systems in the two regions are quite similar. In 2016 and 2017, fluopyram plus pyrimethanil (Luna Tranquility) alone or azoxystrobin plus difenoconazole (Quadris Top) alone

reduced SLB severity compared to the untreated control, resulting in an increase in marketable yield of 20–63% (Hoepting, 2017c, 2018a). Severity of SLB in New York State was moderate in the untreated control (2.2–3.2 on a scale of 0–6) but premature plant mortality was very high by mid to late September. Based on these studies, growers were advised to begin weekly spray programs near the end of July, or around the time 2.5-cm-dia bulbs had formed (Hoepting, 2017c, 2018c). Fungicides containing azoxystrobin or pyraclostrobin (FRAC group 11) were originally effective against SLB in New York, but had become ineffective by 2015 (Hoepting, 2016). Similarly, the active ingredient boscalid (FRAC group 7) declined in efficacy by 2017 (Hoepting, 2016, 2017c, 2018c). Recent trials have identified reduced performance of fungicides containing active ingredients in FRAC groups 2, 7, and 9 compared to previous years (Hoepting, 2017c, 2018a, 2019, 2020b; a).

It is difficult to directly compare the studies in New York and Ontario because the timing of initial infection and the rate of SLB development could have been very different in the two regions, and severity values may have been influenced by the different methods used for SLB assessment. However, the start date for recommended fungicide applications in New York is substantially later than indicated by the current study, where seed treatment to minimize early season infection was essential for SLB suppression, even though application was initiated at the 2- or 4-leaf stage (often mid-late June). Also, the fungicides identified as highly effective in New York were no longer effective during the same years as studies conducted at the Holland Marsh (Stricker et al., 2020).

It has been suggested that a portion of SLB symptoms on onion and garlic may be due to a host-specific toxin produced by the pathogen (Basallote-Ureba et al., 1999). Previous studies have shown that secondary metabolites produced by *S. vesicarium* caused leaf dieback, reduced yield, and may increase susceptibility to other diseases (Singh et al., 1999). A few lesions of *S. vesicarium* on a plant early in the growing season may result in severe defoliation due to the production of host-specific toxins. Subsequent fungicide sprays would not control the earlier infections and thus be less effective in reducing the damage.

The current study indicated that foliar sprays in combination with seed treatments may be the best method to manage SLB. However, field trials conducted in the past 6 years in Ontario, culminating in the current study, have not identified a fungicide that provides effective SLB suppression (Stricker et al., 2020). Further research should focus on a) development of a

forecasting model that is also effective under low disease pressure, b) assessment of the efficacy of new fungicides and of biological controls, and c) continued assessment of the sensitivity of *S. vesicarium* to recommended fungicides to alert growers to changes in efficacy.

Chapter 4 Fungicide Sensitivity of *Stemphylium vesicarium* isolates

The results of the *in vitro* fungicide sensitivity assessments for azoxystrobin, pyrimethanil, and difenoconazole have been published (Stricker et al., 2020).

4.1 Introduction

Fungicides are an important tool for integrated pest management (IPM) systems in agriculture. However, fungicide insensitivity, also known as fungicide resistance, may develop in a pathogen population in response to selection pressure from frequent fungicide applications (Shaw, 2009). Several fungicides with different modes of action are registered in Canada for management of SLB on onion, but growers in the Holland Marsh are frustrated by a perceived lack of efficacy. In New York State, several of the foliar fungicides registered for management of SLB on onion no longer provide economic levels of SLB suppression, mostly due to fungicide insensitivity (Pethybridge et al., 2016).

The Fungicide Resistance Action Committee (FRAC) has classified the active ingredients in synthetic fungicides based on their mode of action, which is the biochemical mechanism by which they affect target pathogens. FRAC has also characterized the inherent risk of pathogen populations developing insensitivity to each mode of action grouping (Brent and Hollomon, 2007b). For example, FRAC group 11, also known as the QoI group or strobilurins, has a high risk for developing insensitivity (Gisi et al., 2002). Mutations that result in insensitivity to one active ingredient in the QoI group often confer partial or complete insensitivity to all of the active ingredients in the group (known as cross-insensitivity) (Gisi et al., 2002). For instance, sensitivity of *Botrytis cinerea* (Markoglou et al., 2006) to the strobilurin fungicide pyraclostrobin resulted in reduced sensitivity to other strobilurins, including azoxystrobin, fluoxastrobin, trifloxystrobin, and picoxystrobin (Markoglou et al., 2006).

Field trials on fungicide efficacy for the management of SLB on onion have been conducted by Dr. M.R. McDonald at the Muck Crops Research Station at the Holland Marsh, ON since 2011, but for the past 6 years (2014–2019) none of the fungicides assessed reduced SLB severity (Table 4.1, Stricker et al., 2020). In other studies, high concentrations of mancozeb, azoxystrobin, propiconazole, and propineb inhibited *in vitro* growth of *S. vesicarium* (Mishra and Gupta, 2012) but had little or no impact on the incidence of SLB in the field (Hoepting and Pethybridge, 2016). Several studies have reported that populations of *S. vesicarium* are

insensitive to specific fungicides (Alberoni et al., 2005, 2010b; Hay et al., 2019). This is not unexpected; *S. vesicarium* is at high risk for developing fungicide insensitivity because there can be multiple generations per season, there is abundant production of air-borne spores through sexual and asexual reproduction (Gossen et al., 2021), and management of SLB involves multiple fungicide applications per growing season (Misawa and Yasuoka, 2012). The fungicides currently registered for management of SLB in Canada are listed in Chapter 1, Table 1.4. The fungicides are in FRAC groups 3, 7, 9, and 11.

Table 4.1 Effect of fungicides on Stemphylium leaf blight (disease severity index) of at the Holland Marsh, ON, from 2011–2019 (Stricker et al., 2020).

			Disease severity index								
Fungicide	**Active ingredient(s)**	**FRAC Group**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Unsprayed			67a[1]	32a	30a	56 ns[2]	96 ns	28 ns	46 ns	54 ns	33 ns
Bravo®	Chlorothalonil	M05	38b	23ab							
Manzate®	Mancozeb	M03	37b	20bc	16b						
Switch®	Cyprodinil + Fludioxonil	9 + 12	37b	23ab	20ab	66	95				
Inspire®	Difenoconazole	3	25bc	17bc	20ab	55	88				
Pristine®	Boscalid + Pyraclostrobin	7 + 11	34bc	20bc	18ab	55	93	30	43	60	31
Luna Tranquility®	Fluopyram + Pyrimethanil	7 + 9	21bc	13c	13b	47	88	31	28	52	35
Fontelis®	Penthiopyrad	7	20c	19bc	19ab	59	95	28	49	61	
Quadris Top®	Azoxystrobin + Difenoconazole	3 + 11		12c	12b	50	96	27	37	56	
Dithane™	Mancozeb	M03				61	89	35			
Sercadis®	Fluxapyroxad	7						36	47	59	27
Miravis® Bold	Pydiflumetofen	7						36	47		
Merivon®	Fluxapyroxad + Pyraclostrobin	7 + 11							46	56	
T-77®	*Trichoderma atroviride*	BM02								59	27
Aprovia® Top	Difenoconazole + Benzovindiflupyr	3,7									31

[1]Values within a column followed by the same letter do not differ based on Tukey's test at $P < 0.05$.

[2]ns –Values within the column do not differ.

This study focused on the four active ingredients from the FRAC groups present in Luna Tranquility and Quadris Top. Luna Tranquility (fluopyram + pyrimethanil, groups 9 and 7) and Quadris Top (azoxystrobin + difenoconazole, groups 11 and 3) have been registered for use on onion in Canada since 2012 (Health Canada, 2020a). The first QoI-insensitive isolate of *S. vesicarium* was detected in 2016 in Italy (Collina et al., 2007). QoI-insensitivity was also identified recently in New York State, where 67% of 105 isolates from onion fields were insensitive to 0.5 µg a.i. mL^{-1} and the G143A mutation was detected in 70% of this sample (Hay et al., 2019). Difenoconazole (FRAC group 3) is a locally systemic DMI fungicide with efficacy against a broad range of fungal pathogens (Fonseka and Gudmestad, 2016; Jurick et al., 2017; Ali et al., 2018). Over 95% of *S. vesicarium* isolates tested in New York State were sensitive to difenoconazole, despite the fact that Quadris Top and Inspire Super (cyprodinil & difenoconazole; Syngenta) have been applied on onion crops in New York for 10 years in mixed formulations (Hay et al., 2019). Dual insensitivity between difenoconazole and pyrimethanil was not detected in the 33 isolates tested against both active ingredients (Hay et al., 2019). The activity of anilinopyrimidine fungicides (AP; FRAC group 9) such as pyrimethanil is mostly through direct contact, but they have some systemic activity (Rosslenbroich and Stuebler, 2000). Several isolates of *S. vesicarium* collected in New York State were insensitive to the AP fungicides cyprodinil and pyrimethanil (Hay et al., 2019). Succinate-dehydrogenase inhibitor (SDHI; FRAC group 7) fungicides such as fluopyram exhibit contact and some systemic activity (Lunn, 2011). *In vitro* studies in New York demonstrated that > 90% *S. vesicarium* isolates were sensitive to fluopyram (Hay et al., 2019).

Mutations conferring fungicide insensitivity may also have pleiotropic effects that can result in adverse effects and a fitness cost (Anderson, 2005). Fitness parameters are a method for determining the relative ability of a pathogen to survive, infect, and reproduce (Dekker, 1976). For example, azoxystrobin-insensitive isolates of *Ustilago maydis* exhibited decreased *in vitro* growth and pathogenicity on corn plants (Ziogas et al., 2002). Similarly, QoI-insensitive *Cercospora beticola* isolates exhibited a 30% reduction in mycelial growth, 18–54% reduction in pathogenicity on sugar beet, and half of the isolates exhibited reduced sporulation (Malandrakis et al., 2006). Isolates of *Stemphylium solani* that were insensitive to fludioxonil (FRAC group 12) exhibited decreased *in vitro* mycelial growth and sporulation, and reduced *in planta* aggressiveness on tomato (Wu et al., 2015). Fitness costs associated with mutations conferring

insensitivity to QoIs have also been observed in insensitive populations of *Plasmopara viticola* (Heaney et al., 2000) and *Magnaporthe grisea* (Avila-adame and Köller, 2003) but did not affect fitness parameters in *Erysiphe graminis* É.J, Narchal (Heaney et al., 2000), *Plasmopara viticola* (Corio-Costet et al., 2011), or dicarboximide-insensitive *S. vesicarium* isolates from pear (Alberoni et al., 2006, 2010b). In fact, QoI-insensitive isolates of *Alternaria alternata* were more aggressive than sensitive isolates on detached pistachio leaves. Fitness costs can also vary with environmental conditions such as temperature (Brown et al., 2006). The fitness of fungicide-insensitive isolates of *S. vesicarium* from onion have not been assessed. Future research may include screening the genome of *S. vesicarium* for RNA microsatellites related to pathogenicity or aggressiveness of isolates.

If use of a fungicide or mode-of-action group is discontinued, selection pressure can shift an insensitive pathogen population back towards to sensitivity if there is a fitness cost associated with the mutation conferring fungicide insensitivity (Andersson and Hughes, 2011). For example, successive sub-culturing of pyraclostrobin-insensitive *C. beticola* isolates on fungicide-free medium resulted in a shift towards sensitivity (Malandrakis et al., 2006). However, this is not always the case. Genes conferring insensitivity to the fungicide may persist in a population if the fitness costs are low or even fitness-increasing, if these genes are linked to other beneficial traits, or if subsequent mutations compensate for the fitness cost (Andersson and Hughes, 2011).

Fungicide sensitivity is often assessed using mycelial growth assays or germination of fungal spores (Russell, 2004). These methods compare the response of isolates on a fungicide-amended medium relative to non-amended medium. Initial studies generally assess response to a wide range of fungicide concentrations. However, a discriminatory dose can be used to reduce the time and effort needed to perform assessments on a large population. The decision to use a discriminatory dose was based on the anticipation of analyzing a large sample of isolates. However, the actual number of isolates was lower than expected due to research restrictions during the COVID-19 pandemic. A discriminatory dose is a specific concentration or dose that can be used to differentiate between sensitive and insensitive isolates, based on decreased growth or germination in previous trials (Russell, 2004).

When testing sensitivity to QoI fungicides, salicylhydroxamic acid (SHAM) is commonly added to media because it inhibits the alternate oxidase (AOX) pathway (Mizutani et al., 1996; Wood and Hollomon, 2003). It has been used when testing many fungi including *S. vesicarium*

(Hay et al., 2019), *S. solani* (Wu et al., 2015), and many others (Pereira et al., 1997; Ziogas et al., 2002; Ma et al., 2003). SHAM alone generally does not affect mycelial growth or conidial germination (Pereira et al., 1997; Wang et al., 2016), but can reduce mycelial growth of some fungal pathogens (Avila-Adame and Köller, 2002; Seyran et al., 2010; Kunova et al., 2013; MacLean et al., 2017). Also, a low dose (20 mg L^{-1}) enhanced the inhibitory effect of pyraclostrobin on *Clarireedia jacksonii* (Liang et al., 2015).

AOX inhibitors are generally not used in field trials because they are rapidly broken down in the environment and may inhibit plant respiration (Wood and Hollomon, 2003). One hypothesis states that AOX inhibitors are not needed for *in planta* studies because plant metabolites, such as flavonoids, also inhibit the alternative oxidase pathway (Tamura et al., 1999). However, respiration via AOX differs among species and the alternative oxidation pathway was active during infection of barley with *Mycosphaerella oryzae* (Catt.) Jacz. (Avila-Adame and Köller, 2002) and on wheat infected with *Mycosphaerella graminicola* (Fuckel) J. Schröt. (Miguez et al., 2004). Since the energy requirements changes during the infection period and saprophytic *in vitro* growth, the efficiency of the AOX pathway will depend on many factors (Wood and Hollomon, 2003). Therefore, whether or not to include SHAM in a fungicide sensitivity assay should be determined for each fungal pathogen. To date, assessments with and without SHAM have not been conducted for *S. vesicarium*.

Screening for insensitivity should provide information that would allow growers to avoid fungicides that are not effective and may explain the lack of SLB suppression by certain fungicides in the field. There have been no assessments of fungicide sensitivity of *S. vesicarium* in onion production in Canada. The objective of the study was to assess the *in vitro* sensitivity of *S. vesicarium* to the active ingredients of fungicides commonly used in Canada for management of SLB. The results of the *in vitro* fungicide sensitivity assessments for azoxystrobin, pyrimethanil, and difenoconazole conducted as part of this study have already been published (Stricker et al., 2020).

4.2 Materials and methods

4.2.1 Isolate collection

Symptomatic leaf tissue was collected from onion and leek crops grown in commercial and research fields in 2018 and 2019 from the Grand Bend, Holland Marsh, and Keswick areas of

Ontario. The leaves were incubated for 24–48 h to induce sporulation in a moist chamber. The moist chamber consisted of a white plastic propagation tray covered with a clear plastic humidity dome (279 mm × 543 mm, Mondi Products, Vancouver, BC) and lined with moist paper towels. The resulting colonies were incubated at room temperature for 3–4 days under UV light (Westinghouse Lamps, McNulty, PA), 12 h on / 12 h off, to stimulate production of conidia. The resulting conidia were collected by gently scraping the colony surface with a sterile scalpel. Conidia were transferred to Petri dishes containing 10 mL of V8-agar media amended with ampicillin and streptomycin (as in Chapter 2). In total, 122 isolates were collected from onion samples in Ontario: 1 from Keswick, 17 from Exeter, 60 from Grand Bend, and 44 from the Holland Marsh. An additional 10 isolates were collected from garlic in Exeter and 3 from leek in the Holland Marsh. All isolates were collected from a single lesion but could reflect a range of genetics since several conidia were collected from each lesion.

In addition, two isolates of *S. vesicarium* that had not been exposed to fungicides (baseline isolates) were purchased from the Canadian Collection of Fungal Cultures, Ottawa, ON. These were originally isolated from oat in Saskatchewan in 1995. Four isolates were also revived from a collection created by Dr. J. Foster; the isolates were collected from commercial asparagus and onion fields in southern Ontario and Nova Scotia between 2012 and 2016 (Foster, 2018).

The type of media used can be a confounding factor in fungicide sensitivity testing. For example, the EC_{50} values for *Alternaria alternata* exhibited a wider range on minimum media compared to potato dextrose agar (PDA) media. This may have been due to the fact that the fungus grew slower on MM, so inhibition was more difficult to measure (Vega and Dewdney, 2015). Likewise, *S. vesicarium* and other *Stemphylium* spp. have exhibited lower growth rates when grown on PDA compared to V8 agar (Chowdhury et al., 2015; de Souza et al., 2018); so inhibition may be more variable on PDA than it would be on V8 agar. Several other studies have used V8 media to culture the fungal isolates and then transferred the cultures to PDA or water agar media for fungicide sensitivity screening (Pasche et al., 2005; Dube et al., 2014; Hay et al., 2019). Some studies have used fungicide-amended V8 media (Geary et al., 2007; Cox et al., 2009) and others recommend using V8 media for mycelial growth assessments and water agar media for conidial germination assessments (Secor and Rivera, 2012). Media can impact the growth and development of fungi, so it is important to follow standardized protocols. In order to

compare our results with those recently published on *S. vesicarium* isolates collected in New York (Hay et al., 2019), fungicide-amended PDA was used for screening the fungicide active ingredients.

4.2.2 Determination of discriminatory concentrations

To establish the discriminatory concentration, a subset of 6–11 isolates was exposed to a range of fungicide concentrations for each fungicide. Technical-grade active ingredients of azoxystrobin, difenoconazole, and fluopyram (Sigma Aldrich, Missouri, United States) were dissolved separately in 100% acetone (Fisher Scientific) and technical-grade pyrimethanil (Sigma Aldrich, Missouri, United States) was dissolved in dimethyl sulfoxide (Čuš and Raspor, 2008) to create stock solutions. Serial dilution with sterile water was used to develop solutions that, when added to PDA media, produced the desired range of a.i. concentrations and a solvent concentration of 1% or lower, unless otherwise stated (Appendix 4.1 for complete recipes).

The final concentrations of azoxystrobin-amended media used for identification on a discriminatory dose were 0.05, 0.5, 5, 50, 100, and 200 µg a.i. mL^{-1}. PDA with 500 µg azoxystrobin mL^{-1} was also prepared, but the azoxystrobin precipitated out of the media into crystals within 24 h so this was not assessed. In addition, salicylhydroxamic acid (SHAM; Sigma Aldrich) dissolved in 100% methanol (Fisher Scientific) at a final concentration (v/v) of 100 µg mL^{-1} SHAM, and 0.3% total solvent (Wood and Hollomon, 2003) was added to the media to inhibit the alternative respiration pathway.

The final concentrations for pyrimethanil-amended media were 0.05, 0.5, 5, 50, 100, and 200 µg a.i. mL^{-1}, with a final acetone concentration of 0.2–0.9%. The final concentrations for difenoconazole-amended media were 0.1, 1, 10, 100, and 1000 µg a.i. mL^{-1}, with 1% acetone. Fluopyram was highly insoluble at high concentrations, resulting in final concentrations of 0.01, 0.1, 1, 10, and 100 µg a.i. mL^{-1}, with 10% acetone for the 100 µg a.i. mL^{-}PDA and 1% acetone for all other concentrations. The same concentration of solvent used in the amended media was added to the non-amended media. Acetone, when added to media at concentrations of 0.9–1.1%, can result in a slight inhibition of fungal respiration (~4.5%) (Rissler and Millar, 1977), but 0.2% acetone plus 0.1% methanol did not reduce mycelial growth of *M. grisea* (Avila-Adame and Köller, 2002).

The discriminatory concentrations chosen were 5 µg a.i. mL^{-1} for azoxystrobin, pyrimethanil, difenoconazole, and 10 µg a.i. mL^{-1} for fluopyram. This dosage is 10× higher than

the discriminatory concentration recommended for difenoconazole but equal to the recommended concentration for pyrimethanil, based on a study on *Venturia inaequalis* (Henriquez et al., 2011). The discriminatory concentration for azoxystrobin was 10× higher than that used for assessments of *S. vesicarium* isolates collected in Italian pear orchards (Alberoni et al., 2010a) but was consistent with the discriminatory concentration used in a recent survey of isolates collected from onion fields in New York State (Hay et al., 2019) and was ½ the concentration used to discriminate sensitive from insensitive isolates of *Plasmopara viticola* Berkeley & Curtis ex de Bary (Corio-Costet et al., 2011). The discriminatory dose for fluopyram of 10 µg a.i. mL^{-1} was similar to the 15 µg a.i. mL^{-1} dose used to assess *B. cinerea* in Spain (Fernández-Ortuño et al., 2017).

The isolates were separated into two groups: sensitive (when growth was less than 50% of growth on unamended media) or insensitive. When testing azoxystrobin, an additional group was added because preliminary experiments demonstrated that some isolates of *S. vesicarium* exhibited a high degree of insensitivity to azoxystrobin. This group, insensitive+, did not exhibit decreased growth or germination at a 100 µg a.i. mL^{-1}.

4.2.3 Sensitivity assessment – conidial germination

Germination of *S. vesicarium* conidia on fungicide-amended media relative to a fungicide-free control was used to assess sensitivity to the fungicides at the discriminatory concentrations described above. For assessment of each isolate × fungicide treatment, conidia were added to a micro-centrifuge tube containing 700 µL sterile deionized water. Mycelial fragments were filtered out using sterile cheesecloth. Approximately 50 µL of each conidial suspension (~1×10^4 spores mL^{-1}) was pipetted onto 15 mm × 15 mm sections of amended PDA on a glass slide, covered with a glass coverslip, and incubated in the dark at room temperature. Three replications per isolate were assessed for each fungicide concentration. After 24 h, the coverslips were lifted, the spores were stained with a drop of lactophenol cotton blue (Sigma-Aldrich), and germination of ~100 conidia was counted using a light microscope. Conidia were considered germinated if the germ tube was longer than the length of the conidium (Hay et al., 2019).

4.2.4 Sensitivity assessment – mycelial growth

The mycelial growth on fungicide-amended media was compared to growth on fungicide-free controls using the discriminatory concentrations described above. The PDA was poured into

square Petri dishes (08-757-11A Fisher Scientific, Ottawa, ON), allowed to set, and a sterile scalpel was used to make eight parallel slices in the medium. Four agar strips were removed and discarded leaving five 'lanes' of fungicide-amended media, each 5 mm apart, so that five isolates could be tested in the same Petri dish. The strip-agar method has been used previously to assess fungicide sensitivity of fungal populations (Edgington et al., 1973; Hsiang et al., 1997; Gourlie and Hsiang, 2017). Mycelial plugs (5-mm-dia.) were cut from the outer margins of actively growing cultures of *S. vesicarium* using a cork borer and placed onto the amended media with the mycelium facing down. The study was arranged in a randomized complete block design with three replicates, where each Petri dish was a replicate. The dishes were incubated at ~21 °C in darkness and the growth of each colony was marked after 48 h and 96 h for the first experiment and from 65–118 h for the second. Only mycelium in contact with the agar surface was considered as growth. Aerial hyphae extending beyond the colony edge, which is typical when fungi are grown on fungicide-amended media, were not included in the assessments (Shi and Hsiang, 2015).

4.2.5 Impact of SHAM

A small study to test the effect of SHAM on inhibition of mycelial growth was conducted using PDA amended with 0.05, 0.5, 5, or 50 µg a.i. mL^{-1}azoxystrobin, with or without 100 µg mL^{-1} SHAM dissolved in 100% methanol. Methanol was added to the fungicide-amended media without SHAM so that all treatments contained 0.3% total solvent (v/v). Six isolates were chosen to represent a wide geographic diversity and time range: 225016 from oat in Saskatchewan in 1995, KK01 from Keswick onion in 2018, OO69 from onion in the Holland Marsh in 2016, SS0006 from onion in the Holland Marsh in 2018, and FA05 and FE04 from onion fields in Grand Bend in 2019. The study was conducted in a randomized complete block design with three replicates. The study was repeated using three isolates (225106, SS0006 and KK01).

4.2.6 Data analysis

Statistical analyses were conducted using SAS University Edition (SAS Institute Inc., Cary, NC). In the assessment of the initial selected isolates, EC_{50} values (effective concentration to cause 50% inhibition) were determined for each isolate to each fungicide using probit analysis of inhibition expressed as a ratio (SAS University Edition, PROC PROBIT). Probit transformation is used to straighten the dose response curve and allows for a more accurate estimation of EC_{50}

values compared to untransformed data (Sokal and Rohlf, 1981). The PROC PROBIT function generates EC_{50} value and fiducial limits. A fiducial limit is similar to a confidence interval but is based on fiducial statistical theory, which considers unknown population parameters to be random variables, and fiducial limits are typically used for dose response curves (Irwin, 1943).

A mixed model, factorial analysis of variance in PROC GLIMMIX was used to assess conidial germination and mycelial growth values on the control media and a covariance test (PROC COVTEST) was used to determine if repeated experiments could be pooled. Inhibition by each fungicide active ingredient was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{(a - b)}{a} \times 100$$

Where a = mean conidial germination (%) or mycelial growth (cm) in the control (0 µg a.i. mL^{-1}) and b = conidial germination or mycelial growth in the fungicide treatment (5 µg a.i. mL^{-1}). The mean inhibition plus the standard error was calculated and isolates were sorted into two or three classes based on the reaction to the fungicide: Sensitive (> 50% inhibition at 5 µg a.i. mL^{-1} compared to the control), Insensitive (< 50% at 5 µg a.i. mL^{-1}), or Insensitive+ (< 50% at 100 µg a.i. mL^{-1}; this classification used for azoxystrobin only).

Mycelial growth was considered a proxy for fitness and fitness costs were assessed by comparing the mycelial growth on unamended media of isolates classified as sensitive or insensitive for each active ingredient. The hourly growth rate was calculated for each isolate on unamended media. Mycelial growth on agar is not the best characteristic to use since it is not necessarily an indicator of aggressiveness on a host or ability to reproduce, but has previously been used to estimate fitness parameters of other fungal pathogens (Chen et al., 2016; Fernández-Ortuño et al., 2017; Ren et al., 2017). The mycelial growth data were analyzed using a mixed model with isolate and replicate as random factors, and sensitivity classification (sensitive or insensitive) as a fixed factor. Means separation was completed using Tukey's honest significance test with $P = 0.05$.

4.3 Results

4.3.1 Azoxystrobin

In the small studies with and without the addition of SHAM, mycelial growth was decreased by the addition of SHAM at 0.05 µg a.i. mL^{-1} and 50 µg a.i. mL^{-1} for isolate 225106, but not at

intermediate concentrations. When the test was repeated with this isolate, there was no effect of SHAM. SHAM also reduced mycelial growth for isolate SS0006 at 5 µg a.i. mL^{-1}, but when the test was repeated, there was no effect (Table 4.2). Based on these results, SHAM was included in all subsequent assessments of reaction to strobilurin fungicides.

Table 4.2. Effect of SHAM at four concentrations of azoxystrobin on mycelial growth of *Stemphylium vesicarium* relative to unamended media.

	Mycelial growth (mm) of *S. vesicarium* when exposed to concentrations of azoxystrobin (µg a.i. mL^{-1})							
	0.05		**0.5**		**5**		**50**	
Isolate	**SHAM**	**Control**	**SHAM**	**Control**	**SHAM**	**Control**	**SHAM**	**Control**
225106[1]	**26**	**0***	60	32	43	19	**51**	**20***
225106 (repeat)[2]	15	26	30	39	65	52	81	67
KK01[1]	5	0	12	28	14	6	17	17
KK01 (repeat)[2]	0	8	26	3	8	25	21	34
SS0006[1]	18	4	10	9	5	13	23	15
SS0006 (repeat)[2]	0	23	3	17	**0**	**21***	5	52
FA05[1]	0	0	5	4	5	10	32	39
FE04[1]	5	21	2	15	5	23	28	44
OO69[1]	6	9	18	1	15	8	24	28

*Indicated a significant difference with addition of SHAM. All others are not significant, based on a t-test at $P < 0.05$.
[1]Growth measured for first experiment from 48–96 h.
[2]Growth measured for second experiment from 65–118 h.

The isolates initially assessed displayed a wide range in response to azoxystrobin, with the EC_{50} values were of 0.53–3.95×10^{8} µg a.i. mL^{-1} for conidial germination and 0.11–2.65 ×10^{52} µg a.i. mL^{-1} for mycelial growth (Table 4.3). Ten isolates had EC_{50} values well over 15 µg a.i. $mL^{-1}$and four other isolates had values were below 5 µg a.i. mL^{-1} for conidial germination. The baseline isolate isolated from oat in 1995 was sensitive to azoxystrobin with an EC_{50} value of 0.53 µg a.i. mL^{-1} for conidial germination and 0.11 µg a.i. mL^{-1} for mycelial growth. When tested a second time, this isolate exhibited a higher EC_{50} value for conidial germination (4.06 µg a.i. mL^{-1}) but it was still below the 5 µg a.i. mL^{-1} discriminatory concentration. The isolates selected for this initial assay were those available at the time of assessment since long-term storage of *S. vesicarium* was inconsistent.

In the main azoxystrobin assessment, the sensitivity of 52 isolates was tested in the mycelial growth assessment and 44 in the conidial germination assessment (including those previously tested in the initial assessment) (Appendix 4.2). This included 40 isolates from onion, 5 isolates from garlic, 3 isolates from leek, 3 isolates from asparagus, and 1 isolate from oat. Some isolates could not be tested for both conidial germination and mycelial growth because they did not produce conidia after revival from storage or produced atypical mycelial growth after revival from storage.

Table 4.3 Estimates of EC_{50} values to azoxystrobin calculated for inhibition of conidial germination and mycelial growth of isolates of *Stemphylium vesicarium* and sensitivity classification to azoxystrobin (S – sensitive, Insen – insensitive) of these isolates.

				EC_{50} value (µg a.i. mL^{-1})		
Isolate	**Host**	**Province**	**Year**	**Conidial germination**	**Mycelial growth**	**Sensitivity classification**
225106	Oat	SK	1995	0.53	0.11	S
225106 (repeat)				4.06	0.15	S
OA03	Asparagus	ON	2012	0.24	55.6	S / Insen
Onion	Onion	ON	2018	2.06	nd	S
FC04	Onion	ON	2018	nd	18.0	Insen
FA05	Onion	ON	2018	139	33.2	Insen
OO69	Onion	ON	2016	nd	55.2	Insen
HP01	Onion	ON	2018	282	6.93	Insen
FC07	Onion	ON	2018	2.30×10^{5}	9.84	Insen
Leek2	Leek	ON	2018	3.63×10^{4}	15.9	Insen
AE02	Onion	ON	2019	5.01×10^{28}	32.61	Insen
Leek3	Leek	ON	2018	290	77.91	Insen
SS0006	Onion	ON	2018	3.68	310	Insen
OV11	Onion	ON	2019	nd	1.57×10^{4}	Insen
FE04	Onion	ON	2018	260	1.40×10^{9}	Insen
EX01	Onion	ON	2018	nd	4.57×10^{10}	Insen
FA02	Onion	ON	2018	nd	2.65×10^{52}	Insen
Leek1	Leek	ON	2018	1.05×10^{4}	nd	Insen
KK01	Onion	ON	2018	1.65×10^{6}	nd	Insen
Onion2	Onion	ON	2018	3.95×10^{8}	nd	Insen

nd –Not done.
Where two classifications are presented, the first classification is for conidial germination and the second is for mycelial growth.

Of 40 isolates of *S. vesicarium* collected from onion fields Ontario in 2018 and 2019, 39 isolates (97.5%) were insensitive to azoxystrobin at 5 µg a.i. mL^{-1} (Appendix 4.2). The

remaining isolates were categorized as sensitive using the conidial germination assay but were insensitive based on the mycelial growth assay (100% insensitive). The three isolates collected from leek in the Holland Marsh in 2018 were also insensitive. Two isolates collected from asparagus in 2013–2014 were sensitive, and one isolate from asparagus in 2012 was insensitive based on the conidial germination assay but sensitive in the mycelial growth assay. All of the five isolates collected from garlic in 2019 were insensitive to 5 µg a.i. mL^{-1}. Overall, 47 of 51 isolates (98%) collected from various hosts in Ontario from 2012–2019 were insensitive to azoxystrobin, based on both assessments of conidial germination and mycelial growth. Of these, 20 were also insensitive to 100 µg a.i. mL^{-1}. The baseline isolate collected in 1995 (225106) was sensitive to azoxystrobin, one isolate from 2013 and one from 2014 were sensitive, but the isolate from 2016 was insensitive even to 100 µg a.i. mL^{-1}. These findings coincide with the time that fungicide efficacy in the field declined (Stricker et al., 2020).

4.3.2 Pyrimethanil

In the first repetition of the study of sensitivity to pyrimethanil using 0–200 µg a.i. mL^{-1}, the EC_{50} of the historical isolate was 9.70×10^{3} µg a.i. mL^{-1} for conidial germination and 13.47 µg a.i. mL^{-1} for mycelial growth. When the experiment was repeated using 0–500 µg a.i. mL^{-1}, the EC_{50} values were < 0.01 µg a.i. mL^{-1}. The change in EC_{50} occurred because first repetition was unable to create a dose-response curve since only the highest rate of a.i. (200 µg a.i. mL^{-1}) was able to inhibit conidial germination. By adding a higher concentration in the second experiment, the PROBIT model was able to estimate an EC_{50} value that was within the range of concentrations tested. The mean EC_{50} for the second repetition was 98.04 µg a.i. mL^{-1} for conidial germination and 5.15 µg a.i. mL^{-1} for mycelial growth. The conidial germination data were highly variable (Figure 4.1), as illustrated by differences among isolates 225106, OA03, and FE04 when they were assessed a second time (Table 4.4). The isolates selected for the EC_{50} assay were those actively growing in the collection at the time of assessment since long-term storage of *S. vesicarium* was inconsistent.

Table 4.4 EC_{50} values for inhibition of conidial germination and mycelial growth of *Stemphylium vesicarium* and fungicide sensitivity classification (S – sensitive, Insen – insensitive) based on exposure to selected rates of pyrimethanil on agar medium.

				EC_{50} value (µg a.i. mL^{-1})		
Isolate	**Host**	**Province**	**Year**	**Conidial germination**	**Mycelial growth**	**Sensitivity classification**
225106	Oat	SK	1995	9.70×10^{3}	13.5	Insen
225106 (repeat)				98.0	5.2	Insen
AE02	Onion	ON	2018	nd	0.54	S
Onion2	Onion	ON	2018	nd	0.97	S
FA01	Onion	ON	2018	3.40×10^{13}	1.30	Insen / S
FC07	Onion	ON	2018	31.8	5.20	Insen / S
Leek2	Leek	ON	2018	133	5.04	Insen
Leek1	Leek	ON	2018	1.84×10^{7}	10.6	Insen
Leek1 (repeat)				497	8.34×10^{5}	Insen
Leek3	Leek	ON	2018	1.46×10^{7}	12.9	Insen
FC04	Onion	ON	2018	10.6	16.1	Insen
OV11	Onion	ON	2019	6.80×10^{5}	20.6	Insen
OO69	Onion	ON	2016	924	37.5	Insen
FE04 –	Onion	ON	2018	1.12×10^{4}	44.0	Insen
FE04 (repeat)				1.18×10^{6}	444	Insen
HP01	Onion	ON	2018	6.37×10^{11}	177	Insen
OA03	Asparagus	ON	2012	2.02	309	Insen
OA03 (repeat)		ON		523	nd	Insen
SS0006	Onion	ON	2018	239	1.22×10^{3}	Insen
SS0006 (repeat)				5.76×10^{3}	nd	Insen
EX01	Onion	ON	2018	1.00×10^{4}	1.06×10^{4}	Insen
KK01	Onion	ON	2018	3.67	1.82×10^{14}	Insen

nd –Not done.

Where two classifications are presented, the first classification is for conidial germination and the second is for mycelial growth.

Figure 4.1. Variable germination of conidia of *Stemphylium vesicarium* isolate GB7_3 on media amended with 100 µg a.i. mL^{-1} pyrimethanil and stained with lactophenol cotton blue.

In the pyrimethanil assessment, the sensitivity of 51 isolates was tested in the mycelial growth assessment and 49 in the conidial germination assessment. This included 41 isolates from onion, 4 isolates from garlic, 3 isolates from leek, 2 isolates from oat, and 1 isolate from asparagus. Of 41 isolates collected from onion fields in Ontario in 2018 and 2019, 16 isolates (39%) were insensitive to the discriminatory concentration of pyrimethanil using both conidial and mycelial assessments. Of the isolates from onion, none of the isolates from Keswick (1), Exeter (6), and Grand Bend (16) were insensitive to pyrimethanil based on mycelial growth. Three of 18 isolates from the Holland Marsh (17%) were insensitive. In the conidial germination assay, the results were different: 33% insensitive for Exeter, 50% for Grand Bend, and 33% for the Holland Marsh.

All four isolates from garlic were insensitive in the conidial germination test, but two were sensitive in the mycelial growth assessment. The one isolate from asparagus was sensitive in the mycelial growth assessment but insensitive in the conidial germination assessment. Of the three isolates from leek, one was insensitive in both tests and the other two were insensitive in the conidial germination assessment. In total, 20 of 49 isolates (40.8%) were sensitive to the

discriminatory concentration of pyrimethanil using both assessments. The baseline isolates (225105 and 225106) collected from oat in 1995 were insensitive to pyrimethanil and one isolate from asparagus in 2012 and one isolate from onion in 2016 were also insensitive (Appendix 4.2). This suggested that this active ingredient had little to no efficacy for more than two decades and this active may never have been effective for management of *S. vesicarium*.

4.3.3 Difenoconazole

In the initial assessment of the response to difenoconazole, the EC_{50} values for mycelial growth ranged from 0.50 to 1.18 µg a.i. mL^{-1} (Table 4.5). The historical isolate collected in 1995 was sensitive to difenoconazole, with an EC_{50} value of 0.50 µg a.i. mL^{-1} for mycelial growth. The EC_{50} values for conidial germination ranged from 6.22×10^{7} to 1.53×10^{130} µg a.i. mL^{-1} difenoconazole (Table 4.5). The isolates selected for the EC_{50} assay were those actively growing in the collection at the time of assessment since long-term storage of *S. vesicarium* was inconsistent.

Table 4.5 EC_{50} values for inhibition of conidial germination and mycelial growth of *Stemphylium vesicarium* and fungicide sensitivity classification (S – sensitive, Insen – insensitive) based on exposure to selected rates of difenoconazole-amended agar medium.

				EC_{50} value (µg a.i. mL^{-1})		
Isolate	**Host**	**Province**	**Year**	**Conidial germination**	**Mycelial growth**	**Sensitivity classification**
225106	Oat	SK	1995	2.18×10^{9}	0.50	Insen / S
GB4_6	Onion	ON	2019	1.53×10^{130}	0.60	Insen / S
GED18	Garlic	ON	2019	4.17×10^{19}	0.70	Insen / S
EO9	Onion	ON	2019	1.11×10^{11}	0.89	Insen / S
MS01	Onion	ON	2018	7.03×10^{8}	0.99	Insen / S
GB6_9	Onion	ON	2019	2.25×10^{51}	0.44	Insen / S
KG11	Onion	ON	2019	6.22×10^{7}	0.52	Insen / S
GB7_8	Onion	ON	2019	4.72×10^{84}	0.56	Insen / S
EO11	Onion	ON	2019	1.06×10^{74}	1.06	Insen / S
GED6	Garlic	ON	2019	2.08×10^{24}	1.18	Insen / S

Where two classifications are presented, the first classification is for conidial germination and the second is for mycelial growth.

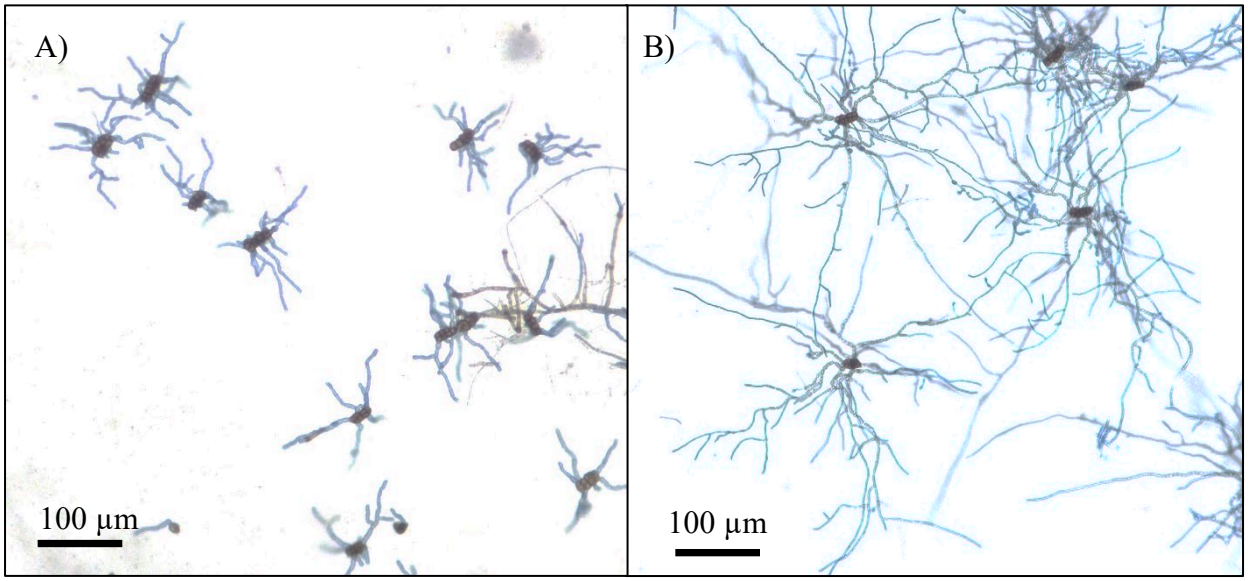

Figure 4.2 Germination of conidia of *Stemphylium vesicarium* isolate EO9 conidia stained with lactophenol cotton blue A) on media amended with 1000 µg a.i. mL^{-1} difenoconazole compared to B) the unamended control.

Of 106 *S. vesicarium* isolates collected from onion in 2018 and 2019, 105 isolates (99.0%) were sensitive at the discriminatory concentration in the mycelial growth assay (Appendix 4.2). Of the isolates from onion, none from Exeter (17) and the Holland Marsh (35) were insensitive to difenoconazole using the mycelial growth assessment, and only 1 of 54 from Grand Bend (2%) was insensitive. The baseline isolates (225105 and 225106) were sensitive to difenoconazole in the mycelial growth assessment. In addition, all 8 isolates collected from garlic were sensitive. However, all 10 isolates tested for conidial germination, including the baseline isolate, were insensitive to the discriminatory concentration of difenoconazole.

4.3.4 Fluopyram

In the initial assessment of response to fluopyram, the EC_{50} values for conidial germination ranged from 12.7 to 2.50×10^{125} µg a.i. mL^{-1} fluopyram, and 6.35×10^{-3} to 1.91×10^{8} µg a.i. mL^{-1} for mycelial growth (Table 4.6). The baseline isolate collected in 1995 was insensitive to fluopyram. The isolates selected for the EC_{50} assay were those actively growing in the collection at the time of assessment since long-term storage of *S. vesicarium* was inconsistent.

Table 4.6 EC_{50} values for inhibition of conidial germination and mycelial growth of *Stemphylium vesicarium* and fungicide sensitivity classification (S – sensitive, Insen – insensitive) based on exposure to selected rates of fluopyram on agar medium.

				EC_{50} value (µg a.i. mL^{-1})		
Isolate	**Host**	**Province**	**Year**	**Conidial germination**	**Mycelial growth**	**Sensitivity classification**
225106	Oat	SK	1995	2.57×10^{14}	1.91×10^{8}	Insen
GB7_13	Onion	ON	2019	n/a	6.35×10^{-3}	S
GED3	Garlic	ON	2019	2.89×10^{3}	0.45	Insen / S
E06	Onion	ON	2019	3.17×10^{4}	0.83	Insen / S
GB6_10	Onion	ON	2019	2.03×10^{15}	0.91	Insen / S
GED4	Garlic	ON	2019	6.70×10^{6}	0.91	Insen / S
GED9	Garlic	ON	2019	5.46×10^{4}	1.27	Insen / S
KG06	Onion	ON	2019	1.57×10^{3}	1.71	Insen / S
KG07	Onion	ON	2019	2.50×10^{125}	1.90	Insen / S
Z11	Onion	ON	2019	5.40×10^{8}	3.67	Insen / S
KG05	Onion	ON	2019	4.99×10^{4}	6.48	Insen / S
MS11	Onion	ON	2018	12.74	6.89	Insen / S
KG02	Onion	ON	2019	1.30×10^{11}	8.47	Insen / S
KG09	Onion	ON	2019	n/a	10.5	Insen
GED7	Garlic	ON	2019	2.88×10^{11}	13.0	Insen
GB6_9	Onion	ON	2019	7.23×10^{40}	14.7	Insen
GB7_4	Onion	ON	2019	2.91×10^{17}	37.4	Insen
GB7_3	Onion	ON	2019	n/a	38.4	Insen
GB4_10	Onion	ON	2019	n/a	39.1	Insen
GB6_1	Onion	ON	2019	3.21×10^{23}	39.4	Insen
EO1	Onion	ON	2019	4.21×10^{72}	52.1	Insen
EO9	Onion	ON	2019	n/a	55.3	Insen
GED1	Garlic	ON	2019	5.25×10^{106}	65.7	Insen
GB6_5	Onion	ON	2019	1.51×10^{76}	66.6	Insen
GB6_8	Onion	ON	2019	n/a	96.1	Insen
GB6_2	Onion	ON	2019	6.03×10^{12}	99.3	Insen
GB6_6	Onion	ON	2019	8.51×10^{47}	163.8	Insen
GB4_11	Onion	ON	2019	2.03×10^{31}	244.2	Insen
EO8	Onion	ON	2019	4.06×10^{38}	3.61×10^{3}	Insen
MS01	Onion	ON	2018	2.79×10^{13}	2.70×10^{6}	Insen

n/a – none of the tested concentrations resulted in inhibition so EC_{50} cannot be calculated. Where two classifications are presented, the first classification is for conidial germination and the second is for mycelial growth.

In the main fluopyram assessment, the sensitivity of 97 isolates was tested in both the mycelial growth assessment and the conidial germination assessment. This included 85 isolates from onion, 9 isolates from garlic, 2 isolates from oat, and 1 isolate from asparagus. The discriminatory concentration chosen was 10 µg a.i. mL^{-1}, based on a previous report for

S. vesicarium in New York State (Hay et al., 2019). Subsequent screening of fluopyram activity was based solely on mycelial growth assessments, as has been done in several other studies of Ascomycete fungi (Avenot et al., 2011; Sang et al., 2018). Fluopyram did not inhibit conidial germination, even at the highest concentration tested, except for one isolate (Figure 4.3).

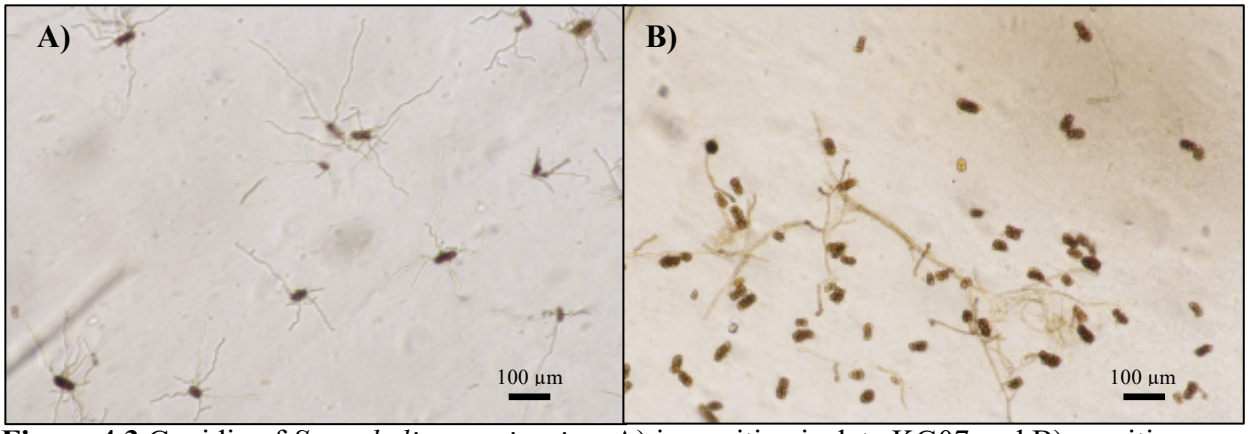

Figure 4.3 Conidia of *Stemphylium vesicarium* A) insensitive isolate KG07 and B) sensitive isolate MS11 PDA media amended with 100 µg a.i. mL^{-1} fluopyram.

Of the isolates from onion, the following were insensitive to fluopyram using the mycelial growth assessment: 0 of 1 from Keswick (0%), 4 of 12 from Exeter (33%), 16 of 39 from Grand Bend (41%), and 10 of 33 from the Holland Marsh (30%). Of 85 isolates collected from onion fields in Ontario in 2018 and 2019, 16 isolates (19%) were insensitive to the discriminatory concentration of fluopyram, and 1 of 9 (11%) isolates from garlic was insensitive using mycelial growth alone. However, the conidial germination assessment identified another 14 isolates from onion and 4 isolates from garlic that where insensitive to fluopyram. The one isolate from asparagus was sensitive to fluopyram.

In total, 60 of 95 isolates (63%) collected between 2012 and 2019 were sensitive to the discriminatory concentration of fluopyram with both methods of assessment. One of the baseline isolates (225106) was sensitive and the other was insensitive with the conidial germination assessment but sensitive with the mycelial growth assessment (225105) (Appendix 4.2). The asparagus isolate collected in 2012 was also sensitive, and 13 of 14 isolates (93%) collected in 2018 were sensitive. This dropped to 57% sensitive in 2019 and to 0% in 2020 (Stricker et al., 2021b).

4.3.5 Insensitivity to more than one active ingredients

There were 32 isolates of *S. vesicarium* collected from onion and garlic that were tested against both azoxystrobin and difenoconazole, which are the active ingredients in Quadris Top fungicide. All of these isolates were insensitive to azoxystrobin, and none were insensitive to difenoconazole using the mycelial growth assessment. However, the highest rate of difenoconazole did not inhibit conidial germination, so 100% of these isolates could be considered insensitive to both active ingredients.

For the 41 isolates tested against fluopyram and pyrimethanil, which are the two fungicides in Luna Tranquility, only two isolates were insensitive to both active ingredients, but only two isolates were sensitive to both active ingredients with both assessment methods. The remaining 37 isolates (90%) were insensitive to at least one ingredient with either or both the mycelial growth and conidial germination assessment methods.

Of the 39 isolates tested against both azoxystrobin and pyrimethanil, 100% were insensitive to at least one of the active ingredients, but 26 isolates (67%) were sensitive to pyrimethanil with the conidial germination assay. Of the 39 isolates tested against azoxystrobin and fluopyram, all of the isolates were insensitive to at least one active ingredient and five isolates were insensitive with both assessment methods. Of the 21 isolates tested against pyrimethanil and difenoconazole, only one isolate was sensitive to both active ingredients.

4.3.6 *In vitro* mycelial growth fitness cost

The mycelial growth rates on unamended media of insensitive isolates were compared to those that had been classified as sensitive to each active ingredient. The classification of sensitivity or insensitivity was based on the inhibition of growth or germination on fungicide-amended compared to the non-amended control; assessment of fitness compared growth on non-amended media only.

Mycelial growth was not affected by insensitivity to azoxystrobin (Table 4.7), based on 50 insensitive isolates but only one sensitive isolate. Isolates insensitive to difenoconazole, pyrimethanil, and fluopyram grew more slowly than sensitive isolates (Table 4.7). Slower growth of insensitive isolates could indicate that there was a fitness cost associated with insensitivity.

Table 4.7 Mean mycelial growth rate (mm h^{1}) on non-unamended agar medium of *Stemphylium vesicarium* isolates characterized as sensitive or insensitive to four fungicide active ingredients.

Classification	Azoxystrobin (n = 51)	Difenoconazole (n = 114)	Pyrimethanil (n = 49)	Fluopyram (n = 95)
Sensitive	0.19 ns[1]	0.35 a[2]	0.27 a	0.30 a
Insensitive	0.22	0.16 b	0.22 b	0.24 b

[1] ns – means do not differ based on Tukey's test at $P = 0.05$.
[2] Means in column followed by the same letter do not differ based on Tukey's test at $P = 0.05$.

4.4 Discussion

The sensitivity of isolates of *S. vesicarium* collected from onion in Ontario to four fungicide active ingredients with different modes of action was evaluated. Sensitivity was initially assessed based on mycelial growth and conidial germination for each fungicide relative to a non-amended control. However, only the mycelial growth assessment was used for difenoconazole, and fluopyram because these active ingredients did not inhibit conidial germination of *S. vesicarium* even at high concentrations.

The pattern of fungicide insensitivity in the *in vitro* assessments helped to explain the results of the field fungicide trials over the past decade, where none of the fungicides tested provided effective suppression of SLB, despite previous indications of efficacy (Stricker et al., 2020). Given the lack of efficacy of fungicides containing azoxystrobin reported by local growers and in small-plot trials at the MCRS (Stricker et al., 2020), it was not surprising to discover that 94% of the *S. vesicarium* isolates assessed were insensitive to azoxystrobin. In addition, 18% of the isolates were insensitive to fluopyram, and 61% were insensitive to pyrimethanil in at least one of two assessments. Also, 38 out of 39 isolates exhibited insensitivity to both azoxystrobin and fluopyram according to the mycelial growth assessment. This indicated that the population of *S. vesicarium* in southern Ontario is strongly trending towards insensitivity to these two fungicides. The only fungicide where a low level of insensitivity was found was difenoconazole, a demethylation inhibitor (DMI; FRAC group 3); only 1% of isolates were insensitive to a discriminatory concentration of 0.5 µg a.i. mL^{-1} based on mycelial growth. Also, conidia of *S. vesicarium* were able to germinate on difenoconazole-amended medium, even at the highest concentration tested.

Insensitivity to pyrimethanil was evenly distributed across the three onion production regions of Ontario when considering the conidial germination data (33% insensitive for Exeter,

50% for Grand Bend, 33% for the Holland Marsh). However, in the mycelial growth assay, there was 100% insensitivity for Exeter, 100% for Grand Bend, and 83% for the Holland Marsh. This highlights the importance of using both assessment methods to assess fungicide sensitivity.

The small study on the addition of SHAM to azoxystrobin-amended media indicated that there was little or no direct impact on *S. vesicarium*, but that SHAM reduced the variability of QoI-sensitivity reactions. However, this assessment was conducted with a small sample size (6 isolates) and would benefit from additional study using more *S. vesicarium* isolates from a wider diversity of sampling locations and plant hosts.

The fungicide timing trials presented in the previous chapter used fluopyram + pyrimethanil alternated with azoxystrobin + difenoconazole as the foliar fungicide treatments in 2018. However, seven weekly applications using this fungicide program did not reduce SLB severity. These are the main fungicides used for management of SLB in the Holland Marsh. Both were recommended based on registration, results of efficacy trials, and results from other jurisdictions. However, these fungicides and others were not effective in trials from 2013 to 2019 (Stricker et al., 2020).

The commercial fungicide Luna Tranquility contains fluopyram and pyrimethanil. Pyrimethanil causes some inhibition of spore germination and germ tube growth. However, spore germination data can be highly variable, as demonstrated in a study on *Botrytis cinerea* (Fritz et al., 2003). In the current study, the spore germination on pyrimethanil-amended media was highly variable even within one experimental unit for a single isolate. The proportion of isolates that were insensitive to pyrimethanil in the current study was even higher than the 33% of insensitive *S. vesicarium* isolates recently reported in New York (Hay et al., 2019). The pyrimethanil-sensitive isolates in the current study appeared to be quantitatively insensitive; isolate reaction ranged from highly sensitive ($EC_{50} < 0.1$ µg a.i. mL^{-1}) to insensitive ($EC_{50} < 5$ µg a.i. mL^{-1}) to extremely insensitive ($EC_{50} > 100$ µg a.i. mL^{-1}). However, since the mode of action of this active ingredient is unknown, there is limited data to confirm if insensitivity is qualitative or quantitative. The relatively small sample size assessed in the current study due to research restrictions province-wide associated with the COVID 19 lockdown in 2020 likely contributed to this high variability.

Fluopyram, the second ingredient in Luna Tranquility, is a succinate-dehydrogenase inhibitor (SDHI; FRAC group 7). Field trials in New York in 2017 demonstrated that fungicides

containing fluopyram suppressed the SLB score by 69% relative to the unsprayed control (Hoepting, 2018a). *In vitro* studies in New York also demonstrated a high level (> 90%) of sensitivity to fluopyram in the *S. vesicarium* population (Hay et al., 2019). However, fungicides containing fluopyram did not suppress SLB in recent field trials in the Holland Marsh (Stricker et al., 2020) and 40% of isolates tested exhibited insensitivity in at least one of the *in vitro* assessments. This difference may be due to lower sensitivity in the local population, lower disease pressure in Ontario, or due to a difference in disease assessment methods between the studies.

The *S. vesicarium* isolate collected from asparagus in 2012 was sensitive to fluopyram. This suggests that the population may have initially been sensitive, which is supported by the results of the initial field trials in the region (Stricker et al., 2020). In an experiment using *Alternaria alternata*, a mixture of fluopyram plus pyrimethanil was more effective at reducing *in vitro* mycelial growth compared to each active ingredient alone, which suggests a possibility of synergy between the two products (Fairchild et al., 2013). Of the 41 isolates tested against these two active ingredients, only two isolates were completely sensitive to both active ingredients.

In the current study, the EC_{50} values for conidial germination were often much higher (> 100×) than those estimated using mycelial growth. This may be associated with the assessment method. In another study, *Alternaria alternata* was also more sensitive to boscalid in mycelium assays than conidial germination, and conidial germination was not affected by concentrations > 50 µg a.i. mL^{-1} (Vega and Dewdney, 2015). Conversely, the fluopyram EC_{50} values of *Fusarium* spp. estimated using the mycelial growth inhibition were higher than those calculated using a conidial germination assay (Wang et al., 2017). Similarly, the EC_{50} values for *Venturia inaequalis* were higher for fluopyram and benzovindiflupyr (another SDHI fungicide) for mycelial growth (2.02 and 0.043 µg a.i. mL^{-1}, respectively) than for germ tube growth (0.176 and 0.0016 µg a.i. mL^{-1}) (Villani et al., 2016). Differences in reduction of germination of conidia as compared to inhibition of mycelial growth in different fungicides is common. The mode of action of fungicides can affect certain life stages differently; DMI fungicides may have little to no impact on spore germination since the mode of action is preventing sterol production and fungal spores contain sterol reserves (Mueller et al., 2013).

In another pathosystem, the degree of fungicide-insensitive respiration of *Fusarium oxysporum* increased over time, starting at germination and becoming up to 60% insensitive to

the active ingredient in the stationary growth phase (Pereira et al., 1997). This suggested that growth stage can have an important impact on sensitivity to synthetic fungicides. DMI fungicides such as difenoconazole inhibit cytochrome P450 and thus prevent the conversion of lanosterol to ergosterol which inhibits cell division and thus mycelial growth (Bossche et al., 1983). In one study, *Aspergillus ochraceus* Wilhelm mycelium consisted of 52% ergosterol and 37% lanosterol during the germination phase, but this shifted to 95% ergosterol in the vegetative growth phase (Gutarowska and Żakowska, 2009).

Azole fungicides block the production of ergosterol, which may explain why difenoconazole inhibited mycelial growth but not conidial germination. A complete lack of conidial germination inhibition by DMI fungicides has been previously reported for other fungal pathogens (Jaspers, 2001). In fact, conidia of *Fusarium* spp. germinated after 48 h of incubation when exposed to 2.5–250 μg mL^{-1} difenoconazole, but after 72 h of incubation the germ tubes had collapsed and elongation ceased (Masiello et al., 2019). However, this is not always the case for this fungicide group; 0.48 μg mL^{-1} difenoconazole inhibited conidial germination of *A. solani* after 24 h of incubation (Issiakhem and Bouznad, 2010) and other DMI fungicides (myclobutanil, prochloraz, prochloraz + epoxiconazole, and tebuconazole) inhibited conidial germination of *Diplodia mutila* Slippers with mean EC_{50} values ranging between 0.3–3.2 μg mL^{-1} (Torres et al., 2013). DMI fungicides inhibit production of sterols and since fungal spores contain sterol, the inhibition of conidial germination will vary by active ingredient, length of exposure, and the pathogen in question (Mueller et al., 2013).

The majority of tip-dieback symptoms of SLB may be caused by release of phytotoxins by *S. vesicarium* (Singh et al., 1999). Some systemic fungicides reduce the rate of lesion expansion but fungicides do not reduce or eliminate existing lesions (Ranzi and Forcelini, 2013). Infections that occurred early in onion growth could continue to produce toxins, even if additional infection was prevented by fungicide application. Also, systemic products would not be translocated into dead leaf tissue. The necrotic leaf tissue could then provide a location for new infections and would have to be protected by fungicides applied to the surface.

In populations where there is a fitness cost associated with fungicide insensitivity, the population will generally trend back towards sensitivity when the active ingredient is removed from the production system (Mikaberidze and McDonald, 2015). Therefore, assessment of the fitness associated with insensitivity can be an important component of a fungicide management

package. A number of fitness assessments have been used to assess the pathogenicity, virulence, and / or aggressiveness of pathogen isolates (Dyakov, 2007; Pariaud et al., 2009; Elliott et al., 2015). However, assessment of fitness costs was not a primary objective of the current study, so only a comparison of growth rate on unamended media was assessed. Growth rate on artificial media can approximate growth rate on the host, but it is not a good indicator of aggressiveness or fitness. There was a slight reduction in mycelial growth associated with insensitivity to difenoconazole, pyrimethanil, and fluopyram, but not for azoxystrobin. In fact, azoxystrobin-insensitive isolates had a slightly higher (numerically but not statistically) growth rate than sensitive isolates.

Overall, the current study provided evidence of fungicide insensitivity to three of four fungicides commonly used in Ontario to suppress SLB. The high rates of insensitivity reported in the current study for the first time in Canada should be very concerning for onion growers in Ontario. High levels of insensitivity were found to azoxystrobin. The other active ingredient in Quadris Top, difenoconazole, did not inhibit spore germination even at the highest concentration. This can explain why these commercial products were no longer effective against *S. vesicarium* in the field (Stricker et al., 2020). *In vitro* testing showed that insensitivity to azoxystrobin was very high (94%), to fluopyram was high (63%), to pyrimethanil was lower (41%), and to difenoconazole using the mycelial growth assessment only was very low (1%). Of 30 *S. vesicarium* isolates collected from the Holland Marsh in 2020, 97% were insensitive to azoxystrobin and 100% were insensitive to fluopyram (Stricker et al. 2021). This information is valuable to help growers and integrated pest management practitioners avoid fungicides that will not provide effective disease suppression in the field. Future research should investigate the *in vitro* sensitivity of other active ingredients and screen the *S. vesicarium* isolates from Ontario for mutations linked to fungicide insensitivity.

If a mutation causing fungicide insensitivity results in a fitness cost of some kind, there is hope that the fungal population will trend back towards sensitivity if growers stop using that product. Other research indicates that once reduced sensitivity to azoxystrobin has been established in a pathogen population, the phenotype is stable and will persist even if the selection pressure is removed (Primiano et al., 2017). Fluopyram is a relatively new active ingredient, developed by Bayer CropScience and released in 2010 (Hopkins, 2009) so information on insensitivity to this active ingredient is limited. In a study on *Botrytis cinerea*, some insensitive

mutants exhibited lower aggressiveness, spore production, mycelial growth, and sclerotia viability (Veloukas et al., 2014). However, no fitness cost of mutations conferring insensitivity to SDHI fungicides was observed in other studies (Scalliet et al., 2012). Pyrimethanil is another relatively new active ingredient; studies with pyrimethanil-insensitive isolates of *B. cinerea* have reported lower mycelial growth rates, spore germination, and competitive ability (Ren et al., 2017). Similarly, difenoconazole-insensitive isolates of *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., Bull. exhibited reduced virulence and mycelial growth (Li et al., 2020).

It would be interesting to determine if the insensitivity observed in this study is qualitative (conferred by single gene mutations with large effect) or quantitative (conferred by multiple mutations with small effects). Insensitivity to azoxystrobin is often qualitative, conferred by a single mutation such as F129L or G143A in the cytochrome *b* protein (Kim et al., 2003). The two mutations have not been documented in the same isolate (Li et al., 2021). Insensitivity to SDHI fungicides such as fluopyram can result from several mutations in three of the four sub-units of succinate dehydrogenase (Sun et al., 2016). Some mutations result in greater insensitivity than others, but multiple mutations within a single isolate of a pathogen are non-existent or very rare (Rehfus, 2018). Difenoconazole insensitivity typically builds over time in a multi-step process through many small-effect mutations (Brent and Hollomon, 2007a). The genetic cause of pyrimethanil-insensitivity has not been determined (Kanetis et al., 2010).

Based on the results of this study, the use of azoxystrobin, fluopyram, or pyrimethanil for in-field suppression of SLB on onion in Ontario is not recommended. Based on *in vitro* studies, difenoconazole may offer some protection from SLB, but field trials did not demonstrate disease suppression by this active ingredient. The fitness cost assessment in this study suggests that if growers can avoid use of products containing fluopyram and pyrimethanil, the *S. vesicarium* population may regain sensitivity to these fungicides in the future. However, the length of time this would take is unknown, especially if several similar fungicides are applied on the rotation crop, carrots. For example, insensitivity to pyrimethanil was stable after 10 generations of *B. cinerea* (Ren et al., 2017). Future research is needed to assess fitness costs of fungicide-insensitive *S. vesicarium* isolates collected in Ontario, and to perform genetic screening for known or new mutations conferring insensitivity to fungicides.

Chapter 5 Factors affecting epidemics of Stemphylium leaf blight

5.1 Introduction

The management of SLB might be improved through understanding the effects of abiotic factors on the epidemiology of this pathosystem. Determining conducive weather conditions for sporulation of *S. vesicarium* could be useful for scheduling fungicide applications, potentially reducing the number of applications needed, or identifying seasons where fungicide sprays are not necessary.

The concentration of air-borne fungal spores is often a critical factor in the development of plant disease epidemics and can often be directly linked to weather parameters such as precipitation, air temperature, and humidity. In temperate countries, rising temperatures have resulted in changes in fungal spore production throughout the growing season. For example, an earlier start date and later end date of *Alternaria* spp. spore production and increased spore production for *Stemphylium* spp. have been reported in Slovakia (Ščevková et al., 2016). The concentration of air-borne spores of *Cercospora beticola* increased with temperature when relative humidity exceeded 87% (Khan et al., 2009). In Germany, the concentration of air-borne spores (almost certainly conidia) of *S. vesicarium* increased steeply after 600 degree days (1 August 2013; 20 July 2014; 4 August 2015) (Bohlen-Janssen et al., 2018a).

Estimates of aerial spore concentrations from daily spore trap counts have been used to schedule fungicide applications on onion for the management of *Botrytis squamosa* (Carisse et al., 2005, 2008); fungicide applications begin once a critical threshold of 8–15 conidia per m^3 of air has been met. In this host-pathogen system, biological factors such as the concentration of air-borne spores were better predictors of the disease caused by this pathogen on onion than weather factors when disease pressure was low (Carisse et al., 2008). Another study used Rotorod samplers to collect air-borne spores of *Erysiphe necator* Schw. in grape vineyards and recommended fungicide applications when the spore numbers exceeded 10 spores per day based on qPCR analysis. This resulted in 2.3 fewer fungicide applications than the calendar method of fungicide timing (Thiessen et al., 2017).

Bioaerosols have been analysed using microscopy to visually identify and quantify fungal spores for many years. However, identifying a pathogen based on spore morphology is not always reliable because the target spores may be nondescript or easily confused with other

species. Recent studies have highlighted the difficulty of differentiating species in the genus *Stemphylium* due to similarities in morphological characteristics (Das et al., 2019). Quantitative polymerase chain reaction (qPCR) has been used to detect and quantify several plant pathogens using species-specific primers, including *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev on cucumber (Bello et al., 2021), *Botrytis cinerea* on grape (Carisse et al., 2014), and *Claviceps purpurea* (Fr.) Tul. on grasses (Dung et al., 2018). A strong positive correlation between *Peronospora effusa* (Grev.) Rabenh. spore counts and DNA copy numbers from qPCR standard curves has been demonstrated (Klosterman et al., 2014), which indicated that qPCR could be used to speed up and standardize the estimation of spore numbers from spore traps. No species-specific primers for *S. vesicarium* are yet available, but the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), translational elongation factor EF-1 alpha (*TEf-A*), and cytochrome *b* (*cytB*) genes can be used together to differentiate *S. vesicarium* from other species (Inderbitzin et al., 2009; Woudenberg et al., 2017). A qPCR assay specific for *S. vesicarium* could be developed to improve the timing of fungicide application for the management of SLB on onion in the Holland Marsh. Reducing the number of fungicide applications per year could extend the useful life (Brent and Hollomon, 2007b) of the limited number of fungicides available to onion growers.

Climate change trends for southern Ontario have indicated that local temperatures may rise by 1.5–6.3 °C in the next century and summer precipitation is expected to decrease, with increased frequency of severe weather events including droughts and floods (Bush and Lemmen, 2019). Also, growers in the Holland Marsh region have reported that stress caused by drought (possibly relating to soil type or poorer soil condition) may be a contributing factor in SLB epidemics. Abiotic stresses such as drought are known to affect the interaction of hosts and plant pathogens. It could be useful to examine how changes in precipitation, or drought, may affect SLB in Ontario in the future.

The objectives of this study were i) to develop a method to estimate the air-borne concentrations of *S. vesicarium* spores using a qPCR-based assay of bioaerosol samples collected in the field, ii) to develop management recommendations for growers based on weather conditions associated with the production and release of *S. vesicarium* conidia, and iii) to examine the effect of drought stress on the severity of SLB.

5.2 Materials and methods

5.2.1 Sporometrics spore sampling

A Spornado bioaerosol collection unit provided by Sporometrics Inc. (Toronto, ON) was used for comparison to the Burkard multi-vial cyclone sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, United Kingdom) in 2020. The Spornado is a wind-powered, low-cost spore trap consisting of a cone-shaped funnel attached to a metal pole (Figure 5.1). Plastic cassettes containing specialized membranes snap into the cone; fungal spores that are passively carried by the wind are trapped on the membrane. The intake opening was 1.8 m from the ground. DNA was extracted from the membranes with the FastDNA Spin kit for Soil (MP Biomedicals, USA) and subjected to multi-target qPCR by Sporometrics Inc. The Spornado trap operated for multiple days between changes of the cassette, so the spore estimates are converted to spores per day by dividing the estimate by the number of days sampled.

Figure 5.1 A Spornado bioaerosol collection unit located adjacent to a newly planted onion field at the Muck Crops Research Station in 2020 (photo courtesy of Dennis Van Dyk).

5.2.2 Bioaerosol collection and DNA extraction

A Burkard Multi-Vial Cyclone Sampler was placed at the Muck Crops Research Station (MCRS) of the University of Guelph in a 10 m × 60 m onion plot in 2018, 2019, and 2020. Each day, the bioaerosol was collected into a 1.5 mL microcentrifuge tube at an air movement rate of 16.5 L

min^{-1} from 6:00 am to 12:00 pm (Figure 5.2). The time for sampling was chosen based on previous research, which demonstrated that the majority of spores of *S. vesicarium* were released in the morning time period (Gossen et al., 2021). The intake opening of the Burkard sampler was 0.95 m above the soil surface. DNA was directly extracted from the microcentrifuge tubes, making this air sampler highly compatible with qPCR pathogen detection and quantification methods (Williams et al., 2001). There were 48 samples collected in 2018, 42 in 2019, and 27 in 2020. The air samples were collected from 8 June–31 August 2018, 31 May–30 August 2019, and 2 June–27 July 2020. Several samples where multiple days of bioaerosol were collected in error were removed from the study. Samples were stored at -20 °C prior to DNA extraction.

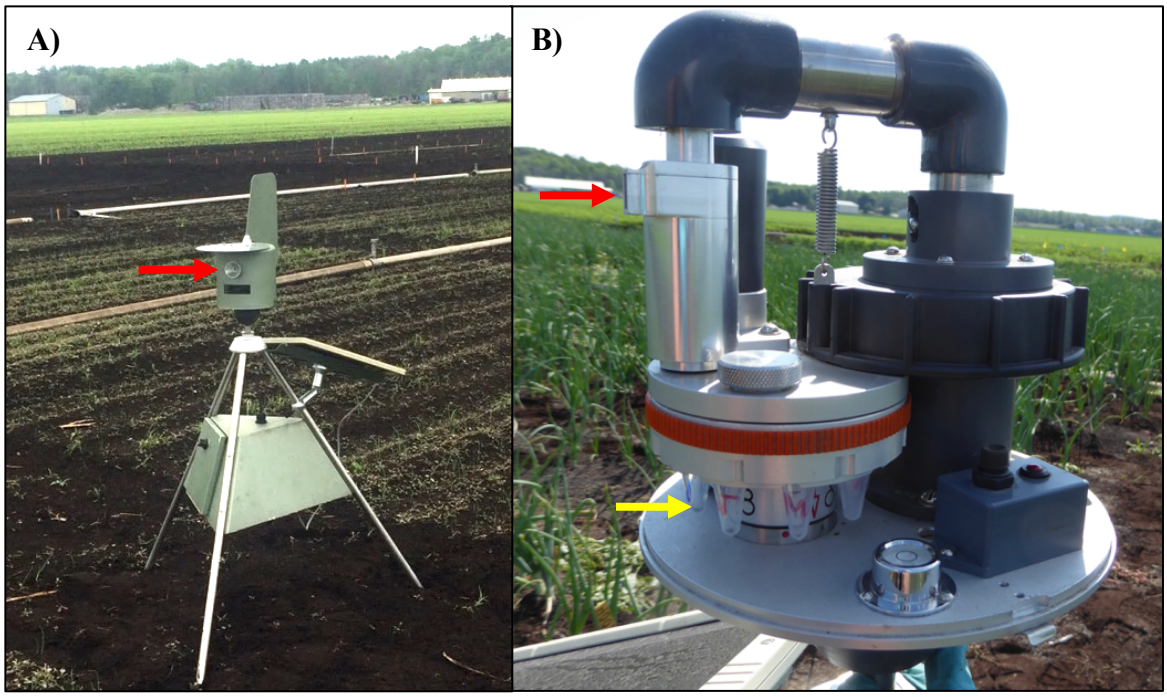

Figure 5.2 The Burkard multi-vial cyclone sampler A) in a field of seedling onion, and B) air is funnelled through the slotted opening (red arrow) by a battery-operated vacuum and bioaerosol is deposited into microcentrifuge tubes (yellow arrow).

The process to extract DNA from the samples was initiated by dislodging the bioaerosol from the inner surface of the tube walls and mechanically disrupting the samples as follows: 60 µL of C1 buffer from the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Toronto) was added to each collection tube and vortexed for 10 min, followed by three freeze–thaw cycles consisting of 2 min in liquid nitrogen followed by 2 min at 65 °C, with the final thaw extended to 30 min. The contents of one PowerBead tube was transferred into each collection tube, followed by DNA

extraction according to the manufacturer's protocol. In the final step, the DNA was eluted into 50 µL of solution C6 from the DNeasy PowerSoil DNA Isolation Kit. DNA samples were kept frozen at -80 °C. Nucleic acid concentration (ng μL^{-1}) was quantified using the QIAxpert system (Qiagen), which uses a microfluidic UV/VIS ratio absorption based on a spectrophotometer.

5.2.3 Primer design

Three primer and probe sets were designed for qPCR to target the internal transcribed spacer (ITS), translational elongation factor EF-1 alpha (*TEf-A*), and cytochrome *b* (*cytB*) genes of *S. vesicarium.* The primers and probes were designed using the free, on-line platform Benchling (benchling.com) based on contigs derived from previous gene sequencing of local *S. vesicarium* isolates (Foster et al., 2019). The specificity of the primers and probes was checked using the Primer BLAST form to ensure that the primers would amplify *S. vesicarium*. The TaqMan™ probes incorporated a 5' fluorescent reporter dye (FAM) and a 3' nonfluorescent quencher (NFQ-MGB). The quality of the primers and probes was confirmed using OligoAnalyzer (www.idtdna.com/calc/analyzer) to predict whether the primers would form hairpins and ensure that the melting temperatures were optimal. The glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) region was not selected because > 50% of the results yielded *Alternaria* species when searching GenBank accessions using the Basic Local Alignment Search Tool (BLAST) for primers (www.ncbi.nlm.nih.gov). The primers used by Sporometrics used a CAL Flour® Dye (CAL-560) that was quenched with Black Hole Quencher® (BHQ). The final primer sequences used for qPCR are listed in Table 5.1.

Table 5.1 Primers and probes used for quantitative PCR on DNA extracted from bioaerosol samples collected using a Burkard volumetric sampler in an onion field at the Holland Marsh, ON.

ITS	
Forward	5' TCGGTGAGGGCTCCAGCTTGTC 3'
Probe	5’ [6-FAM] ACAACGGATCTCTTGGTTCTGGCA [NFQ-MGB] 3’
Reverse	5' ATTGTGCTGCGCTCCGAAACCA 3'
TEf-A	
Forward	5' TGCACGGTGCTAACAAGCCTCA 3'
Probe	5' [6-FAM] CGAGCTCGGTAAGGGTTCCTTCA [NFQ-MGB] 3'
Reverse	5' TCGCGCTCAGCCTTCAACTTGT 3'
cytB	
Forward	5' GTAAGTTTGTATGTTCTTCCGTATG 3'
Probe	5' [6-FAM] ACTGTAGCACCTCATAATGACATTTGCC [NFQ-MGB] 3'
Reverse	5’ CAAGGAATAGCACTCATAAGGTT 3’
Primer used by Sporometrics (ITS region)	
Forward	5’ CCACCAGGACCAAACCATAAAC 3’
Probe	5’ [CAL-560] CGAATCTTTGAACGCACATTGCGCCCT [BHQ] 3’
Reverse	5’ GGTGTTGGGCGTCTTTGTCT 3’

5.2.4 Quantitative PCR assay

Quantitative PCR reactions were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using TaqMan fluorogenic probe detection at the University of Guelph Genomics Facility. All reactions were performed in 0.1 mL MicroAmp® Fast 96-Well Reaction Plates (Applied Biosystems) in a reaction volume of 20 µL containing 4 µL of DNA extract; 10 µL of TaqMan Universal PCR Mastermix (Applied Biosystems™, ThermoFisher Scientific), 2 µL of each primer, 0.2 µL of the corresponding probe, and 1.8 µL of PCR-grade sterile water. The amplification and quantification conditions were an initial denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 1 sec and annealing for 30 sec at 60 °C. Each qPCR reaction included the samples, a no-template negative control, and was performed in technical triplicates. Additionally, each plate contained a positive control of *S. vesicarium* conidia collected from known cultures (isolates GB8_1, EX06, EO15, and FA01 mixed together) as raw 10^{-5} conidia mL^{-1} spore suspension (DNA not extracted) to be used as an inter-run calibration control (one per plate, n = 6).

5.2.5 Dilution series to establish qPCR standard curve

To produce conidia, *S. vesicarium* colonies (isolates GB8_1, EX06, EO15, and FA01) were incubated at room temperature with 12 h UV light / 12 h dark. Conidia were harvested by

flooding the Petri dish with sterile water, gently scraping the colonies with a sterile scalpel, and collecting the loosened conidia with a pipette. Mycelial fragments were filtered out using sterile cheesecloth. The concentration of the spore suspension was adjusted to 1×10^5 spores mL^{-1} using a haemocytometer. The spore suspension was portioned into 200 µL subsamples to be used for qPCR and kept frozen at -20 °C until use. The DNA was extracted from these samples using the same protocol as the bioaerosol samples. After DNA extraction, the DNA was diluted using 10-fold serial dilutions to create DNA samples which represent 1×10^4, 1×10^3, 1×10^2, and 1×10^1 spores mL^{-1} samples. Fresh serial dilutions were made for each qPCR reaction. These samples were used in each qPCR reaction plate to generate a standard curve. The Applied Biosystems software calculated the slope of the standard curve samples (which should be between 90–110%) and the coefficient of correlation (R^2) value (values > 0.97 indicate good quality reactions). This method cannot differentiate between conidia and ascospores, so the qPCR values of the standard curves were used to determine the number of spores per collection tube. Each tube represented 6 h of sampling time per day. The number of spores per tube was converted to spores m^{-3} of air (number of spores per collection tube × 1,000 L m^{-3}) / (16.5 L min^{-1} × 60 min h^{-1} × 6 h).

5.2.6 Rotorod spore sampling

Rotating arm impactor spore samplers, trade name Rotorod samplers, have been used for routine monitoring of *Botrytis*, *Stemphylium*, *Peronospora*, *Alternaria*, and *Fusarium* species at the MCRS for many years. Bioaerosol was collected on a pair of 1.52 mm-wide polystyrene collector rods rotated at ~2,400 rpm by a simple motor powered by a 12V battery from 06:00 am to 12:00 pm on each sampling date in 2014, 2015, 2016, and 2020. The manufacturer states that a single rod sampled air at 22.6 m^{-3} min^{-1}; two rods were collected, resulting in a sampling volume of 45.1 m^{-3} min^{-1}. The leading edge of each rod was coated with a silicone grease adhesive, which served to trap impacted particles. The Rotorod sampler was placed at canopy height within an onion field at the MCRS (approx. 1.3 m).

For analysis, each rod was collected and mounted on a customized glass slide with an appropriately sized groove to hold it in place, and spores were counted under a compound light microscope by MCRS staff. The spores were identified to genus by a trained research assistant, producing discrete count data for each genus.

5.2.7 Burkard tape sampler

A Burkard 7-day recording volumetric tape sampler (Burkard Manufacturing Co. Ltd., Rickmansworth, UK) was used to trap air-borne spores from 20 May–16 September 2015 (120 days), and 21 April–6 September 2016 (139 days) by C.S. Tayviah (2017). The design of this Burkard tape was very similar to the Burkard multi-vial sampler, except that the opening orifice of the sampler was lower (only 0.7 m above the soil), the sampling airflow rate was lower (10 L min^{-1}), and the sampler was operated 24 h day^{-1}, 7 days a week. The spores were captured on a clear cellophane tape coated with an adhesive mixture (50 mL petroleum jelly, 6 g paraffin, 0.6 g phenol) mounted on a metal drum. The tape was changed weekly and 0.33-mm-long sections of tape, representing 2-h intervals, were examined under a light microscope at 250 × magnification to manually identify and count spores. The Burkard tape sampler was placed within an onion field at the MCRS.

5.2.8 Disease assessment

SLB was evaluated every 7–14 days throughout the growing season in four assessment plots of the unsprayed control in fungicide timing trials at the MCRS in 2018 and 2019, and in the fungicide efficacy trial conducted at the MCRS in 2020, using the disease assessment methods described in Chapter 4. The onion cultivars in these trials were LaSalle (2018), Fortress (2019), and Traverse (2020). The plot set-up and size were as described in Chapter 4. The disease incidence and severity (DSI) were plotted as disease progress curves for each year.

In addition, SLB incidence and leaf dieback data from the cultivar LaSalle from previous research at this site conducted by C.S. Tayviah (2017) was included in this study to make the analysis more robust and representative of the region. In the studies in 2015 and 2016, the incidence and severity of SLB was evaluated every 7–14 days from 29 June–14 August 2015 and 11 July–11 August 2016 in an onion cultivar trial.

5.2.9 Weather data collection

Hourly air and soil temperature (°C), relative humidity (%), rainfall (mm), and leaf wetness (%) were recorded using an Onset® automatic weather station (Onset Corporation, Bourne, MA) placed at the MCRS near an onion field (Figure 5.3) and maintained by MCRS staff. Air temperature and relative humidity (RH) were measured with a combined temperature and RH sensor (Model: S-THB-M00x). Rainfall was measured with a rainfall smart sensor (Model: S-

RGB-M002) and two leaf wetness smart sensors (Model: S-LWAM003) measured leaf wetness on a scale of 0–100% based on electrical resistance on grids of gold traces. The leaf wetness sensors were placed at an angled upright position (~45° from vertical). Where data were not available, the missing data were filled in using data from the nearby permanent weather station at the Muck Crops Research Station (LoggerNet, Campbell Scientific, Edmonton AB) maintained by MCRS staff. Solar radiation and wind speed were additionally gathered from the LoggerNet weather station.

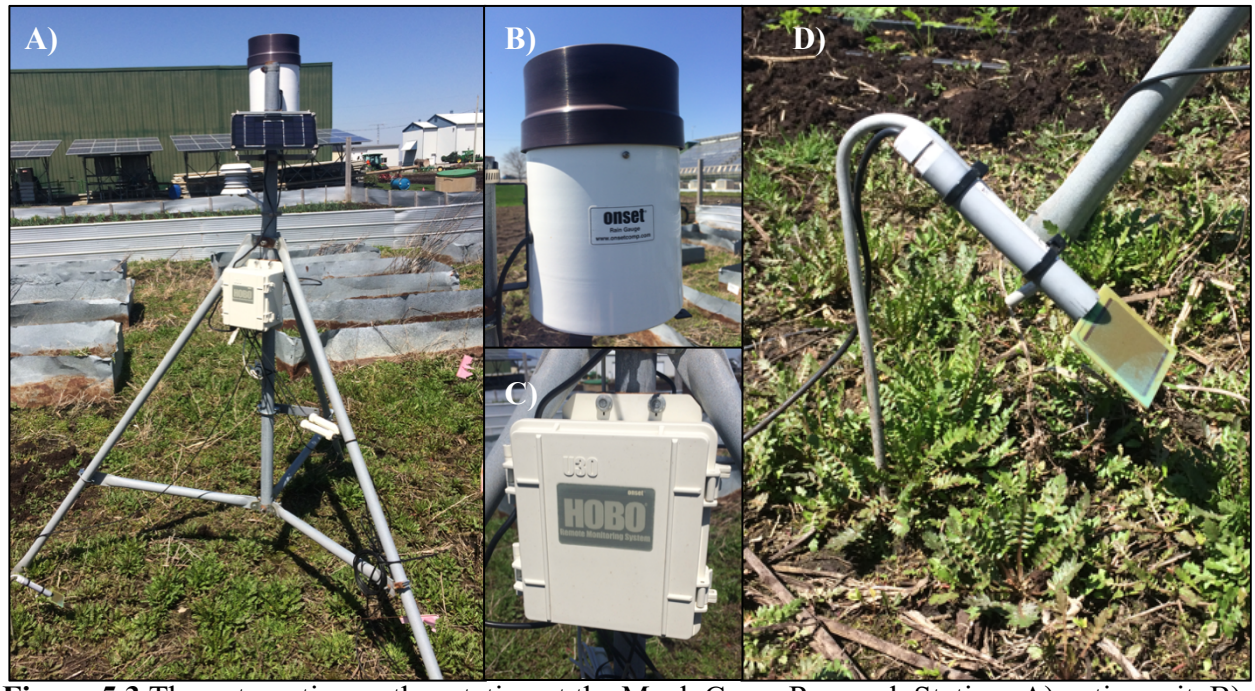

Figure 5.3 The automatic weather station at the Muck Crops Research Station: A) entire unit, B) rain gauge, C) digital data logger and D) leaf wetness sensors.

The average leaf wetness was calculated based on the data from the two sensors. Sensors were considered 'wet' if the average leaf wetness was > 75%. The daily average temperature, average relative humidity, total daily rainfall and daily number of leaf wetness hours (average of two sensors registering > 75% wetness) were recorded, in addition to other weather parameters chosen based on previous research conducted on *S. vesicarium* at the MCRS (Table 5.2) (Gossen et al., 2021). The leaf wetness sensors were not available in 2014 and were out of order from 21 April to 17 May 2016. It is important to note that since the leaf wetness sensors were placed within the crop canopy, irrigation events would be captured and logged as leaf wetness. However, irrigation events were not captured in the Rainfall and DryPeriod variables.

Table 5.2 Weather variables assessed in modelling air-borne spores of *Stemphylium vesicarium* trapped in an onion field at the Holland Marsh, ON in 2015, 2016, 2018, 2019, and 2020.

Variable	Unit	Description
Rad	Watts m^{-2}	Average solar radiation (calendar date, 24 h)
AvWind	km h^{-1}	Average wind speed (calendar date)
LWD	h	Daily leaf wetness duration (calendar date)
NightLWD	h	Duration of leaf wetness overnight (17:00–9:00)
LWD18h	h	Duration of leaf wetness over the day (5:00 to 23:00)
NightWetTemp	°C	Preceding overnight average temperature during leaf wetness
WTemp	°C	Average temperature during leaf wetness period on calendar date
DLWD*	d	Number of days with LWD ≥ 6 h
AvSoilT	°C	Average soil temperature on calendar date
Temp	°C	Average daily temperature on calendar date
NTemp	h	Number of hours daily with temperature ≥ 15 °C on calendar date
VPD	kPa	Average daily vapour pressure deficit on calendar date
NVPD	h	Number of hours daily with VPD ≤ 0.5 kPa
DVPD*	d	Number of days with average VPD ≤ 0.5 kPa
Rain	mm	Total daily rainfall on the calendar date
HRain	mm	Total rainfall from 5:00 to 23:00
TRain*	mm	Cumulative total rainfall in past 10 days
NRain*	d	Number of days with rainfall ≥ 2mm
DTemp*	d	Number of days with average temperature ≥ 15 °C
DryPeriod	h	Number of hours since last rainfall event
AvRH	%	Average daily relative humidity on calendar date
HumidLength	h	Number of hours relative humidity > 70% from 5:00 to 23:00

*Calculated for cumulative periods up to 10 days prior to spore trapping.

5.2.10 Drought study

Onion cultivar LaSalle was seeded in a 48-cell seeding tray (each cell = 6.0 cm long × 3.9 cm wide × 5.9 cm deep) filled with soil-less mix (Sunshine Mix LA4) and incubated in a growth room set at 24 °C day / 18 °C night, with 17 h day length, for approximately 6 weeks until they reached the 5-leaf growth stage. Muck soil from the Muck Crops Research Station was placed into 3.8-L black plastic pots, which were incubated in the growth room for 2 weeks to allow for

weed seedlings to be removed manually. Each pot filled with muck soil was watered to field capacity and weighed before transplanting one onion into each pot. There were four treatments: drought inoculated, drought non-inoculated, normal watering inoculated, normal watering non-inoculated, with eight pots per treatments for a total of 32 pots. An additional eight plants were grown so these could be destructively sampled to determine plant weight at a later date.

To determine the field capacity of the muck soil, five pots of muck soil were allowed to air-dry until the weight no longer changed (42 days), and the soil was further oven-dried at 60 °C for 4 days to determine the dry weight. This value was used to calculate the gravimetric soil water content (GSWC) using the following formula:

$$GSWC = \frac{(S - D)}{D} \times 100$$

where S = the weight of the soil and D = the mean dry weight of the same volume of soil after oven drying. Previous research on muck soil from this site determined that the wilting point occurred at 125% GSWC and field capacity occurred at 175% GSWC (Kora, 2004). Muck soil can hold an astounding amount of water (385% GSWC near saturation) (Kora, 2004), so it was important not to over-water the plants because it would take several days or weeks before the soil would dry enough to simulate drought.

The pots were weighed and watered to 175% GSWC each day for 2 weeks prior to the start of the drought treatment. Two weeks after transplanting, the pots were assigned into drought or control treatment groups. The additional plants were destructively harvested and weighed after removing as much soil from the roots as possible. The mean fresh weight of these eight plants was subtracted from the sample weight in the GSWC calculation to account for the weight of the plants when calculating how much water should be added. For the next 14 days, the drought treatment was watered only enough to maintain the GSWC at 110% (below the wilting point) and the control treatment was watered according to the previous watering schedule to maintain field capacity. Each experimental unit consisted of one plant, and there were eight replicates arranged in a randomized complete block design.

On the 14th day of the watering treatment, half the onions were inoculated, and the other half were mock-inoculated as a control. The leaves of the inoculated plants were rubbed with sterile cheesecloth and then sprayed with 2 mL of conidia suspension (~7×10^4 conidia mL^{-1} plus 0.01% Tween 20) using a plastic hand spray bottle. The mock-inoculated treatment was rubbed

with cheesecloth and sprayed with water plus 0.01% Tween 20. The inoculated and mock-inoculated treatments were sorted into two sub-plots on the bench to prevent cross-contamination by leaf contact. The plants were moved to a separate growth chamber with 85% relative humidity and misted for 30 sec every 30 min for 3 days.

After the 3-day incubation period, all of the treatments were maintained under optimal watering conditions and assessed for SLB symptoms over the next 3 weeks. The oldest five leaves (any leaf with > 5 cm green tissue remaining) for each plant were measured to assess total length and length of tip-dieback or chlorosis using a clear plastic ruler. These data were used to calculate percent chlorosis (chlorosis (%) = yellowed length / total length × 100%). The number of dead leaves (naturally senesced on the soil surface) was also counted. The study was repeated.

5.2.11 Data analysis

Spore quantification using qPCR

Kendall's tau-b (τb) was calculated using PROC CORR in SAS version 9.4 (SAS Institute, Cary, USA) to test for a correlation between QIAxpert values and qPCR values. No significant correlation was identified, so outliers of qPCR (higher or lower than 1.5× interquartile range) were removed, but the correlation was still not significant (Appendix 5.1).

The normality of the qPCR data was checked using PROC UNIVARIATE with and without outliers (Appendix 5.2). The effect of individual qPCR reaction plates was assessed using PROC GLIMMIX, with technical replicate as a random factor and qPCR plate as a fixed factor (Appendix 5.3). This was run again sorted by year (Appendix 5.4). PROC CORR in SAS was also used to test for a correlation between qPCR estimates (spores m^{-3}) obtained by the primer sets for three gene regions (*TEf-A*, *cytB*, and ITS). Pair-wise comparisons were made between each primer (Appendix 5.5).

The assumption of normality of the Sporometrics and *cytB* qPCR data were assessed using PROC UNIVARIATE, and then Kendall's tau-b (τb) was calculated using PROC CORR in SAS to test for a correlation between spores day^{-1} values from Sporometrics, *cytB* qPCR, and Rotorod (Appendix 5.6). Where sampling dates did not overlap between the two methods under comparison, the data were excluded. The analyses was assessed with and without removal of outliers that had been identified using a scatterplot.

Spore concentration modeling

Air temperature, total rainfall, and spore counts (*cytB* spores m^{-3}) were plotted to visually assess relationships among spores detected and these major weather parameters. Pairwise correlations among all weather variables were assessed using PROC CORR to create a correlation matrix. The spore dataset included Rotorod samplers that had been manually counted (variable names = Rotorod; 2014, 2015, 2016, 2020), a Burkard-type tape spore sampler with sticky tape that was manually counted (Burkard tape; 2015, 2016 from Tayviah, 2017), and estimates from *cytB* qPCR of samples from a Burkard bioaerosol sampler (*cytB*; 2018, 2019, 2020).

The *cytB* qPCR values were exponentially higher than the manual spore counts, so the *cytB* values were scaled to produce a range similar to that of the manual counts based on the season-long association between Rotorod spore counts and *cytB* qPCR estimates of samples collected on the same date. In this study, the assumption was made that the efficiency of the *cytB* qPCR data and the Rotorod sampling methods were equal. The qPCR data was scaled to the same range as the Rotorod spore counts from the same date using a linear regression (n = 19). The adjustment equation then applied to all other *cytB* qPCR estimates was as follows:

$$\text{Adjusted spore amount} = 1.016 + 0.00894 \times (\textit{cytB}\text{ qPCR estimate})$$

Multiple stepwise regressions of the effect of weather variables pooled across 5 years on spore estimates were conducted using PROG REG with an entry level of $F = 0.15$ and $F = 0.10$ to stay in the model (Appendix 5.7, Tayviah, 2017). The spore estimates were calculated as spores per day in order to match the Spornado sampling method. In addition to the weather variables listed in Table 5.2, the variables were also squared to assess quadratic relationships in the regression analyses. The analysis was conducted with and without the squared variables, and the R^2 was used to identify the model with the best fit. Stepwise regression was also conducted for each year individually to determine if certain variables were essential to the model across the different growing seasons, and to assess the three different spore sampling methods separately.

Cumulative log regression was conducted using PROG LOGISTIC with the option link = CLOGIT, with and without the squared variables (Appendix 5.8). This proportional odds model required an ordinal response variable, so spore estimates / counts were as follows: 0 = < 10 (few or no spores) and 1 ≥ 10 spores (many spores). This model was conducted stepwise with an entry level of $F = 0.15$ and $F = 0.1$ to stay in the model.

Regression tree analysis, also called classification tree analysis, was conducted using PROC HPSPLIT with cross-validation using a random seed number of 124 and the option CVMODELFIT to request a model assessment based on this cross-validation (Appendix 5.9). The seed number is a random number for the random number generator to start cross-validation of the model. The model was created using 10 folds, in which the data were divided randomly into 10 sets and 10 trees are built using 9 training sets, using all but one-fold (the holdout fold) that is used to calculate the average squared error for that tree. The final tree is grown from the parameter that has the minimum cross-validated error. The trees were pruned to include only 4 leaves, which represented categories of low, medium, high, and very high spore concentrations.

The models created using stepwise regression, cumulative log regression, and regression tree analysis, with and without the squared weather variables, with all years pooled or with each year analyzed separately, were compared in an excel document. The actual spore counts / estimates were graphed against the expected values from each model and graphed to examine trends. The error rate was calculated using the following formula:

$$(\text{Expected} - \text{Actual}) / \text{Actual} \times 100\%$$

Controlled environment drought study

For the drought study, variance was partitioned into random effects (replicates nested within repeated experiment) and fixed effects (treatment and inoculation) and analysed using PROC GLIMMIX procedure of SAS. Each date was analysed separately to investigate disease progress over time. Means were separated using Tukey's honest significance test a $P = 0.05$ (Appendix 5.10). Additionally, the entire data set was analysed as a repeated measured analysis with regression (Appendix 5.11). For each inoculation treatment (inoculated or non-inoculated), PROC GLIMMIX was used to create orthogonal regression partitions using a dummy variable. The covariance parameter estimates were used for the next step where regression coefficients were estimated using PROC GLIMMIX and the 'parms' statement with the 'noint' option. The regression model for each watering treatment was derived from the solutions for fixed effects with $Pr > |t| < 0.05$.

5.3 Results

5.3.1 Quantification of spore samples using Burkard multi-vial sampler and qPCR

The qPCR successfully amplified 132 samples for the *cytB* region primers, 129 samples for *TEf-A*, and 51 samples for ITS for the bioaerosol samples collected using the Burkard multi-vial sampler. When the inter-run calibration sample (containing 10^{-5} conidia mL^{-1}) was compared among reaction plates, runs from 2018 were much higher than runs from 2019 with the *TEf-A* primer. When sorted by year, the reaction runs for each primer set individually were not significantly different and thus could be compared with each other (Appendix 5.4). The increased spore values in 2018 is likely due to higher disease pressure in 2018.

The data were not normally distributed, so a non-parametric rank correlation, Kendall's Tau-b (τb), was used instead of Pearson's correlation. There was no correlation between DNA quantification by QIAxpert and the qPCR values (Appendix 5.1). However, the estimates from the three gene regions amplified by qPCR were correlated. There was a strong correlation between *cytB* and *TEf-A* (τb = 0.84, $P < 0.0001$, n = 135), and a weaker correlation between *cytB* and ITS (τb = 0.49, $P < 0.0001$, n = 56) and between *TEf-A* and ITS (τb = 0.49, $P < 0.0001$, n = 56) (Appendix 5.5). The *cytB* region amplified the most samples (n = 132), so the qPCR values from the *cytB* primer were used for subsequent analyses to assess the impact of weather and SLB severity on spore numbers. Whether the low estimates from qPCR for early-season sampling dates were due to poor DNA extraction or other human error is unknown. An internal amplification control (Deora et al., 2015) could be used in future studies to account for false negatives resulting from poor DNA extractions or PCR inhibition.

5.3.2 Comparison of spore sampling methods

Only 35 sampling dates were available for both the *cytB* qPCR from the Burkard multi-vial sampler and the Spornado methods. From these samples, there was a weak positive correlation (τb = 0.36, $P < 0.01$) between spore estimates from *cytB* qPCR and the Spornado cassettes (analyzed by Sporometrics using ITS qPCR). When outliers were removed (one each of the Sporometrics and *cytB* estimates), there was still a positive correlation (τb = 0.31, $P = 0.02$), but the relationship was not strong (Appendix 5.6). The data were not normally distributed, so a non-parametric analysis was conducted to compare the rank of the Sporometrics estimates and the

rank of *cytB* qPCR estimates. The line of best fit described a positive exponential relationship between the rank values of the two spore quantification methods (Figure 5.4).

The Sporometrics method estimated between 0.328 and 1,507 spores per day, whereas the *cytB* qPCR with the Burkard sampler estimated between 3.2 and 194,675 spores per day. The mean estimate was 86.2 spores day^{-1} for Sporometrics and 9,332 spores day^{-1} for *cytB*. However, since the Sporometrics Spornado sampled for several days at a time, it is impossible to say which date contributed more or less bioaerosol on each cassette and the estimates of spores day^{-1} represent the mean of spores captured in that time frame.

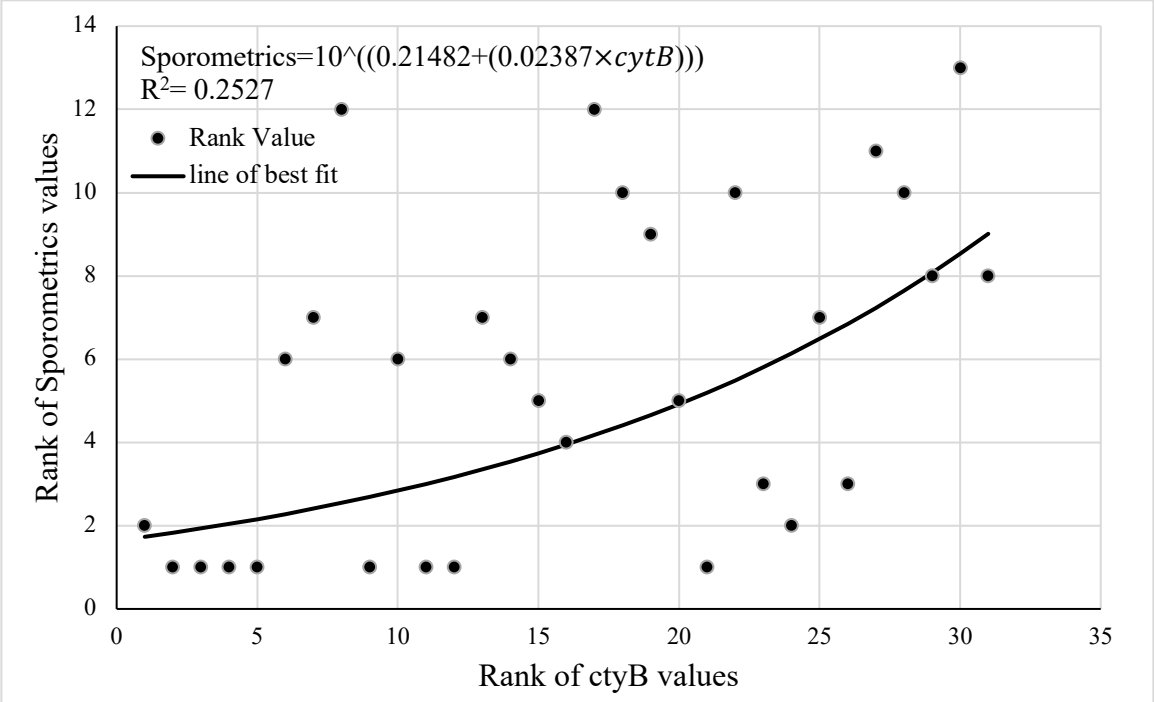

Figure 5.4 Scatterplot of rank analysis of spore estimates from the Burkard using *cytB* qPCR and Sporometrics Spornado using ITS qPCR sampling methods for samples collected on the same day within an onion field in the Holland Marsh in 2020.

Both molecular sampling methods (Burkard using *cytB* qPCR and Sporometrics Spornado using ITS qPCR) were significantly correlated with manual spore counts made from Rotorod samples on the same dates (Appendix 5.6). The manual counts ranged from 0 to 307 spores per day, with a mean of 36.6 spores day^{-1} (n = 31). The relationship between the Burkard and Rotorod estimates (R^2 = 0.56) was stronger than with the Sporometrics trap but not has high as desired.

5.3.3 SLB severity and spore concentration

SLB incidence increased to nearly 100% in all 3 years, but severity differed among years. Incidence, severity, and spore concentration using *cytB* qPCR increased during the growing season, as expected for a foliar disease. The destructive sample at the end of the season counted all of the leaves on the plant, as compared to the oldest three leaves in the other assessments, so severity in that assessment was lower than the final in-field assessment in two of the three years (Figure 5.5).

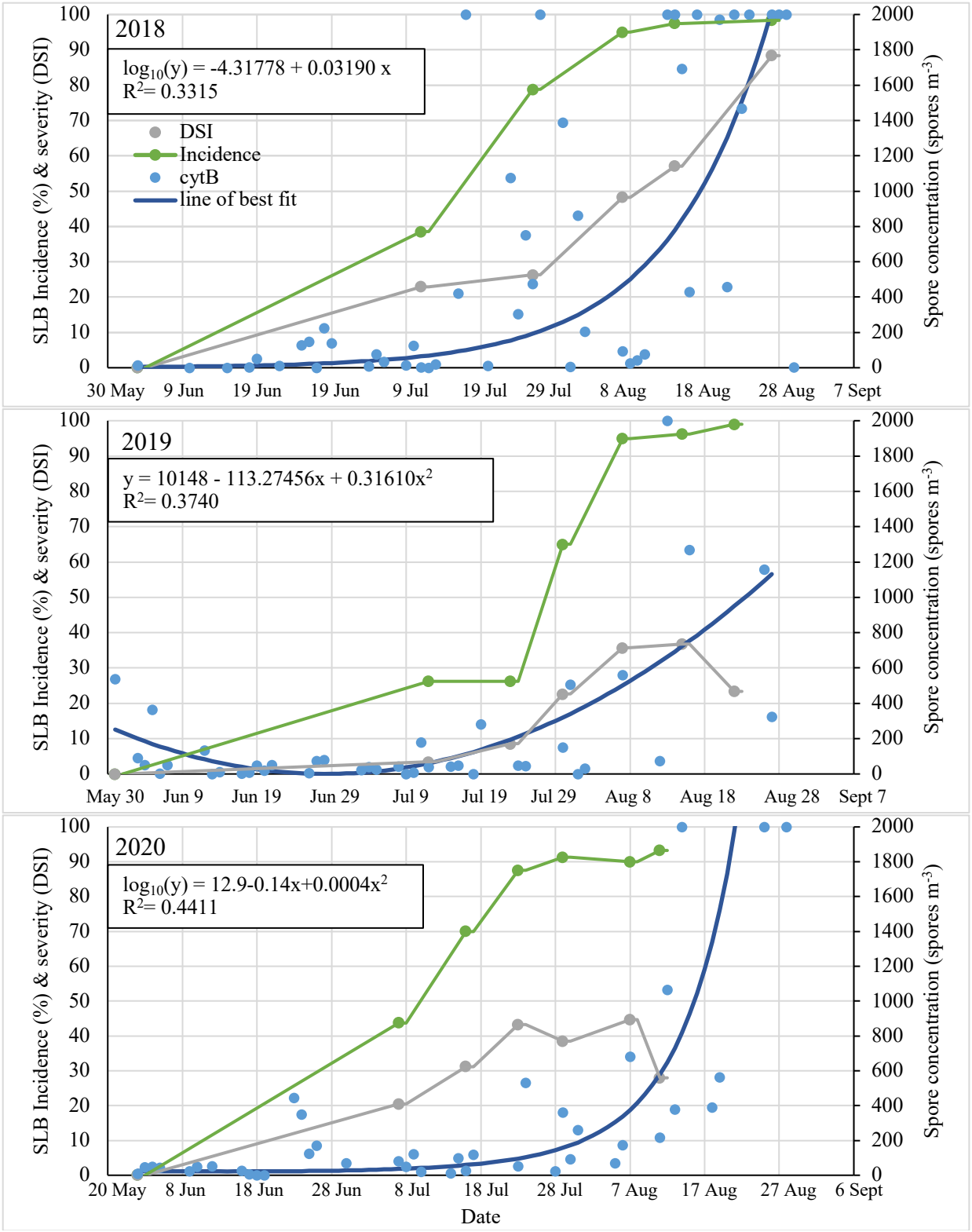

Figure 5.5 Stemphylium leaf blight (SLB) incidence and severity plotted with *cytB* qPCR estimates of air-borne spore quantity in 2018–2020. Extreme spore values at the top of the graph are (left to right): 2018) 2967, 2254, 7102, 4492, 25536, 2348, 2620, 6838, 62488, 59287; 2019) 2317; 2020) 4957, 32773, 4455.

5.3.4 Weather variables and air-borne spore concentrations

In 2018, 353 mm of precipitation fell during the sampling period (3 June–31 August: 90 days), 170 mm in 2019 (31 May–27 August: 89 days), and 356 mm in 2020 (2 June–30 August: 90 days). The mean daily temperature during the sampling period was 21.0 °C for 2018, 20.0 °C for 2019, and 21.0 °C for 2020. Air temperature and monthly rainfall compared to the previous 10-year average are presented in Table 5.3.

In 2015, the season began and ended with warm months with lower-than-average rainfall, although June was cooler with more precipitation. The growing season in 2016 and 2018 was warmer than usual, with 2016 being uncharacteristically dry. In 2019 and 2020, May was cooler than average, July was warm, and both seasons exhibited lower-than-average rainfall for several months.

Table 5.3 Average monthly temperatures and rainfall at the Holland Marsh, Ontario for 2018, 2019, and 2020 relative to the 10-year average. (orange cells higher, blue cells lower).

	Average temperature[1] (°C)					Monthly rainfall[2] (mm)				
Year	May	June	July	Aug	Sept	May	June	July	Aug	Sept
2015	15.9	17.7	20.5	19.5	18.9	40	171	36	79	27
2016	13.8	18.7	22.0	22.6	17.4	45	39	51	58	25
2018	15.8	18.4	22.0	21.9	17.5	82	59	104	109	20
2019	11.4	17.5	22.3	19.4	15.8	77	84	42	46	62
2020	11.6	19.2	23.3	20.6	15.0	38	77	58	140	65

[1]The 10-year average temperatures were:
2006–2015:May 13.4°C, June 18.9°C, July 20.9°C, August 19.6°C, and September 15.5°C.
2007–2016: May 14.1°C, June 18.7°C, July 21.0°C, August 19.8°C and September 15.8°C.
2009–2018 May 13.9°C, June 18.6°C, July 21.2°C, August 20.1°C, September 16.0°C.
2010–2019: May 14.3°C, June 18.4°C, July 21.1°C, August 20.2°C, and September 16.4°C.
2011–2020: May 14.2°C, June 18.5°C, July 21.5°C, August 20.3°C, and September 16.5°C.
[2]The 10-year rainfall averages were:
2006–2015: May 66 mm, June 75 mm, July 94 mm, August 69 mm, and September 85 mm.
2007–2016: May 68 mm, June 85 mm, July 96 mm, August 71 mm and September 82 mm.
2009–2018: May 74 mm, June 101 mm, July 97 mm, August 75 mm, and September 67.
2010–2019: May 77 mm, June 100 mm, July 93 mm, August 80 mm, and September 61.
2011–2020: May 73 mm, June 103 mm, July 84 mm, August 76 mm, and September 62 mm.

Spores of *S. vesicarium* were captured throughout the season in all 3 years, but the concentrations differed substantially. The average daily conidia concentration estimates were 59,287 spores m^{-3} in 2018, 2,317 spores m^{-3} in 2019, and 32,773 spores m^{-3} in 2020 with *cytB* qPCR. Spikes in spore concentrations did not consistently correspond with precipitation events or temperature fluctuations (Figure 5.6).

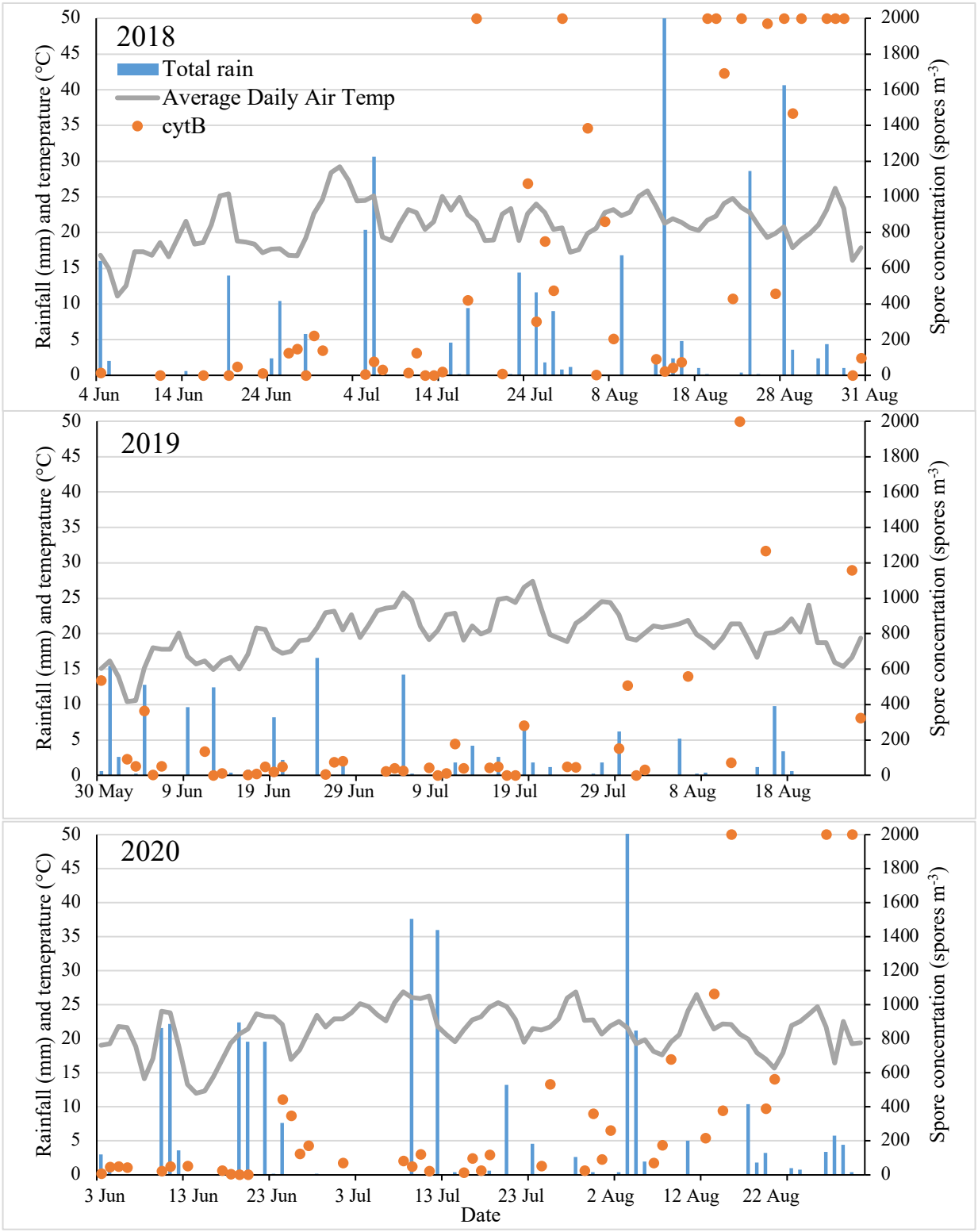

Figure 5.6 Scatterplots of daily air temperature, rainfall, and estimates of air-borne spore quantity of *Stemphylium vesicarium* based on *cytB* qPCR in the Holland Marsh in 2018, 2019, and 2020. Extreme spore values at the top of the graph left to right, are: 2018) 2967, 2254, 7102, 4492, 25536, 2348, 2620, 6838, 62488, 59287; 2019) 2317; 2020) 4957, 32773, 4455.

AvRH was positively correlated with leaf wetness duration (LWD), number of days with LWD ≥ 6 h in the past 10 days (DLWD), running total of duration of leaf wetness over past 18 h from 5:00 to 23:00 (LWD18h), humidity length (# hours RH > 70% from 5:00 to 23:00), and air-borne spore concentration estimated with *cytB* qPCR (Table 5.4). Rain was positively correlated with leaf wetness duration (LWD, LWD18h) and relative humidity (AvRH, HumidLength) (Table 5.4), which are also important for infection. DVPD was positively correlated with the number of hours daily with VPD ≤ 0.5 kPa (NVPD), but not correlated with average daily VPD (AvVPD) (Table 5.4.).

DryPeriod was positively correlated with several temperature variables (Temp, AvSoilT, NTemp, WTemp, DTemp, NightWetTemp), and as one would expect, DryPeriod was negatively correlated with rainfall variables (Rain, HRain, Train, NRain). Unexpectedly, DryPeriod was positively correlated with variables associated with leaf wetness duration (Night LWD, LWD, DLWD, LWD18h) and number of days with average VPD ≤ 0.5 kPa (DVPD) (Table 5.4). The correlation with leaf wetness may be due to the fact that the sensors were placed within the canopy of an onion plot that received irrigation during extreme drought periods, which represents commercial practice in the Holland Marsh.

Soil temperature (AvSoilT and AvSoilT^2) had a strongly positive correlation with leaf wetness (NightWetTemp, NightLWD, LWD, DLWD, LWD18h), relative humidity (AvRH), vapour pressure deficit (VPD, DVPD), and air temperature (Temp, NTemp, WTemp, DTemp). Soil temperature was negatively correlated with number of days with rainfall ≥ 2mm in the past 10 days (NRain) and average daily wind speed (Table 5.4).

Radiation was negatively correlated to leaf wetness variables (NightWetTemp, NightLWD, LWD, DLWD, LWD18h), humidity (AvRH, HumidLength), and total rainfall over past 18 h from 5:00 to 23:00 (HRain). As expected, solar radiation was positively correlated with average daily VPD and air temperature (Temp) (Table 5.4).

Increased wind speed can lead to higher concentrations of bioaerosol being trapped, which is reflected in the positive coefficient in several models for average daily wind speed (AvWind). However, there was a negative correlation between wind speed and spore concentration estimated with *cytB* qPCR (Table 5.4). Wind speed was also negatively correlated with leaf wetness (NightLWD, LWD, DLWD, LWD18h) (Table 5.4).

Table 5.4 Correlation matrix among weather variables pooled across the growing seasons at the Muck Crops Research Station of 2015, 2016, 2018, 2019, and 2020.

	NightWet Temp	NightLWD	LWD	DLWD	LWD18h	AvRH	Humid Length	Rain	HRain	TRain	NRain	DryPeriod	VPD	NVPD	DVPD	AvSoilT	Temp	NTemp	WTemp	Dtemp	Rad	AvWind	Spores
NightWetTemp	1.000	0.279	0.243	0.335	0.211	0.340	0.263	0.138	0.107	-0.167	-0.257	0.105	0.088	0.088	0.140	0.748	0.806	0.815	0.938	0.396	-0.245	0.159	0.166
		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.002	0.016	0.000	<0.0001	0.017	0.046	0.045	0.002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.002
	515	515	515	488	515	515	515	515	515	488	488	515	515	515	488	514	515	515	515	488	461	461	358
NightLWD	0.279	1.000	0.659	0.468	0.461	0.440	0.286	-0.004	-0.017	-0.075	-0.100	0.133	-0.246	0.082	0.304	0.266	0.111	0.166	0.160	0.316	-0.165	-0.289	0.369
	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.924	0.697	0.090	0.024	0.002	<0.0001	0.057	<0.0001	<0.0001	0.010	0.000	0.000	<0.0001	0.000	<0.0001	<0.0001
	515	538	538	510	538	538	538	538	538	510	510	538	538	538	510	537	538	538	526	510	483	483	378
LWD	0.243	0.659	1.000	0.413	0.932	0.591	0.573	0.206	0.238	-0.108	-0.147	0.113	-0.397	0.009	0.313	0.201	0.081	0.178	0.317	0.286	-0.504	-0.261	0.332
	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.015	0.001	0.009	<0.0001	0.844	<0.0001	<0.0001	0.060	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	515	538	541	510	541	541	541	541	541	510	510	541	541	541	510	540	541	541	529	510	486	486	378
DLWD	0.335	0.468	0.413	1.000	0.269	0.325	0.153	-0.015	-0.011	-0.021	-0.090	0.132	0.044	0.104	0.234	0.629	0.504	0.488	0.313	0.825	-0.057	-0.193	0.419
	<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	0.000	0.732	0.799	0.628	0.039	0.002	0.313	0.017	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.220	<0.0001	<0.0001
	488	510	510	528	510	528	528	528	528	528	528	528	528	528	528	527	528	528	499	528	473	473	395
LWD18h	0.211	0.461	0.932	0.269	1.000	0.578	0.625	0.256	0.292	-0.111	-0.157	0.086	-0.424	-0.018	0.252	0.116	0.031	0.130	0.304	0.187	-0.586	-0.136	0.256
	<0.0001	<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001	0.012	0.000	0.046	<0.0001	0.676	<0.0001	0.007	0.467	0.002	<0.0001	<0.0001	<0.0001	0.003	<0.0001
	515	538	541	510	541	541	541	541	541	510	510	541	541	541	510	540	541	541	529	510	486	486	378
AvRH	0.340	0.440	0.591	0.325	0.578	1.000	0.872	0.329	0.315	0.121	-0.024	0.045	-0.744	0.052	0.088	0.198	0.070	0.212	0.320	0.258	-0.713	-0.005	0.215
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001	0.003	0.557	0.249	<0.0001	0.179	0.031	<0.0001	0.070	<0.0001	<0.0001	<0.0001	<0.0001	0.896	<0.0001
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
HumidLength	0.263	0.286	0.573	0.153	0.625	0.872	1.000	0.386	0.385	0.129	-0.003	-0.023	-0.729	0.049	0.010	0.050	-0.042	0.111	0.265	0.108	-0.785	0.069	0.079
	<0.0001	<0.0001	<0.0001	0.000	<0.0001	<0.0001		<0.0001	<0.0001	0.002	0.942	0.552	<0.0001	0.205	0.811	0.200	0.287	0.004	<0.0001	0.008	<0.0001	0.088	0.103
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
Rain	0.138	-0.004	0.206	-0.015	0.256	0.329	0.386	1.000	0.921	0.059	0.033	-0.200	-0.282	0.123	-0.119	-0.008	0.000	0.082	0.141	-0.044	-0.340	0.187	-0.083
	0.002	0.924	<0.0001	0.732	<0.0001	<0.0001	<0.0001		<0.0001	0.149	0.415	<0.0001	<0.0001	0.002	0.004	0.830	0.996	0.035	0.001	0.279	<0.0001	<0.0001	0.084
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
Hrain	0.107	-0.017	0.238	-0.011	0.292	0.315	0.385	0.921	1.000	0.079	0.036	-0.183	-0.269	0.141	-0.092	-0.013	-0.005	0.067	0.118	-0.034	-0.346	0.101	-0.070
	0.016	0.697	<0.0001	0.799	<0.0001	<0.0001	<0.0001	<0.0001		0.053	0.384	<0.0001	<0.0001	0.000	0.024	0.745	0.888	0.088	0.007	0.407	<0.0001	0.013	0.147
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
Train	-0.167	-0.075	-0.108	-0.021	-0.111	0.121	0.129	0.059	0.079	1.000	0.762	-0.363	-0.220	0.052	-0.220	-0.037	-0.179	-0.068	-0.178	-0.012	-0.048	-0.050	-0.032
	0.000	0.090	0.015	0.628	0.012	0.003	0.002	0.149	0.053		<0.0001	<0.0001	<0.0001	0.202	<0.0001	0.368	<0.0001	0.095	<0.0001	0.775	0.261	0.248	0.502
	488	510	510	528	510	600	600	600	600	600	600	600	600	600	600	599	600	600	499	600	545	545	430
NRain	-0.257	-0.100	-0.147	-0.090	-0.157	-0.024	-0.003	0.033	0.036	0.762	1.000	-0.437	-0.155	0.052	-0.117	-0.175	-0.284	-0.202	-0.253	-0.133	0.015	-0.014	-0.070
	<0.0001	0.024	0.001	0.039	0.000	0.557	0.942	0.415	0.384	<0.0001		<0.0001	0.000	0.205	0.004	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.735	0.739	0.150
	488	510	510	528	510	600	600	600	600	600	600	600	600	600	600	599	600	600	499	600	545	545	430
DryPeriod	0.105	0.133	0.113	0.132	0.086	0.045	-0.023	-0.200	-0.183	-0.363	-0.437	1.000	0.071	-0.047	0.185	0.118	0.153	0.127	0.107	0.142	0.052	-0.062	0.357
	0.017	0.002	0.009	0.002	0.046	0.249	0.552	<0.0001	<0.0001	<0.0001	<0.0001		0.069	0.223	<0.0001	0.002	<0.0001	0.001	0.014	0.001	0.206	0.128	<0.0001
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
VPD	0.088	-0.246	-0.397	0.044	-0.424	-0.744	-0.729	-0.282	-0.269	-0.220	-0.155	0.071	1.000	-0.068	-0.012	0.351	0.554	0.321	0.143	0.150	0.648	-0.113	-0.076
	0.046	<0.0001	<0.0001	0.313	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.000	0.069		0.083	0.763	<0.0001	<0.0001	<0.0001	0.001	0.000	<0.0001	0.006	0.117
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
NVPD	0.088	0.082	0.009	0.104	-0.018	0.052	0.049	0.123	0.141	0.052	0.052	-0.047	-0.068	1.000	0.316	-0.054	-0.085	-0.088	0.067	-0.054	-0.034	0.006	0.124
	0.045	0.057	0.844	0.017	0.676	0.179	0.205	0.002	0.000	0.202	0.205	0.223	0.083		<0.0001	0.163	0.029	0.024	0.124	0.190	0.407	0.888	0.010
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
DVPD	0.140	0.304	0.313	0.234	0.252	0.088	0.010	-0.119	-0.092	-0.220	-0.117	0.185	-0.012	0.316	1.000	0.168	0.099	0.094	0.133	0.050	0.038	-0.136	0.531
	0.002	<0.0001	<0.0001	<0.0001	<0.0001	0.031	0.811	0.004	0.024	<0.0001	0.004	<0.0001	0.763	<0.0001		<0.0001	0.015	0.021	0.003	0.224	0.374	0.001	<0.0001
	488	510	510	528	510	600	600	600	600	600	600	600	600	600	600	599	600	600	499	600	545	545	430
AvSoilT	0.748	0.266	0.201	0.629	0.116	0.198	0.050	-0.008	-0.013	-0.037	-0.175	0.118	0.351	-0.054	0.168	1.000	0.840	0.784	0.759	0.683	0.038	-0.192	0.235
	<0.0001	<0.0001	<0.0001	<0.0001	0.007	<0.0001	0.200	0.830	0.745	0.368	<0.0001	0.002	<0.0001	0.163	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001	0.345	<0.0001	<0.0001
	514	537	540	527	540	659	659	659	659	599	599	659	659	659	599	659	659	659	529	599	605	605	431
Temp	0.806	0.111	0.081	0.504	0.031	0.070	-0.042	0.000	-0.005	-0.179	-0.284	0.153	0.554	-0.085	0.099	0.840	1.000	0.889	0.838	0.612	0.103	-0.060	0.183
	<0.0001	0.010	0.060	<0.0001	0.467	0.070	0.287	0.996	0.888	<0.0001	<0.0001	<0.0001	<0.0001	0.029	0.015	<0.0001		<0.0001	<0.0001	<0.0001	0.011	0.143	0.000
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
NTemp	0.815	0.166	0.178	0.488	0.130	0.212	0.111	0.082	0.067	-0.068	-0.202	0.127	0.321	-0.088	0.094	0.784	0.889	1.000	0.810	0.595	-0.027	-0.015	0.215
	<0.0001	0.000	<0.0001	<0.0001	0.002	<0.0001	0.004	0.035	0.088	0.095	<0.0001	0.001	<0.0001	0.024	0.021	<0.0001	<0.0001		<0.0001	<0.0001	0.501	0.708	<0.0001
	515	538	541	528	541	660	660	660	660	600	600	660	660	660	600	659	660	660	530	600	605	605	432
WTemp	0.938	0.160	0.317	0.313	0.304	0.320	0.265	0.141	0.118	-0.178	-0.253	0.107	0.143	0.067	0.133	0.759	0.838	0.810	1.000	0.386	-0.262	0.084	0.130
	<0.0001	0.000	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.007	<0.0001	<0.0001	0.014	0.001	0.124	0.003	<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	0.067	0.013
	515	526	529	499	529	530	530	530	530	499	499	530	530	530	499	529	530	530	530	499	475	475	367
Dtemp	0.39631	0.31593	0.28565	0.82503	0.187	0.258	0.108	-0.044	-0.034	-0.012	-0.133	0.142	0.150	-0.054	0.050	0.683	0.612	0.595	0.386	1.000	-0.035	-0.105	0.268
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.008	0.279	0.407	0.775	0.001	0.001	0.000	0.190	0.224	<0.0001	<0.0001	<0.0001	<0.0001		0.412	0.014	<0.0001
	488	510	510	528	510	600	600	600	600	600	600	600	600	600	600	599	600	600	499	600	545	545	430
Rad	-0.245	-0.165	-0.504	-0.057	-0.586	-0.713	-0.785	-0.340	-0.346	-0.048	0.015	0.052	0.648	-0.034	0.038	0.038	0.103	-0.027	-0.262	-0.035	1.000	-0.168	0.047
	<0.0001	0.000	<0.0001	0.220	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.261	0.735	0.206	<0.0001	0.407	0.374	0.345	0.011	0.501	<0.0001	0.412		<0.0001	0.353
	461	483	486	473	486	605	605	605	605	545	545	605	605	605	545	605	605	605	475	545	605	605	388
AvWind	0.159	-0.289	-0.261	-0.193	-0.136	-0.005	0.069	0.187	0.101	-0.050	-0.014	-0.062	-0.113	0.006	-0.136	-0.192	-0.060	-0.015	0.084	-0.105	-0.168	1.000	-0.108
	0.001	<0.0001	<0.0001	<0.0001	0.003	0.896	0.088	<0.0001	0.013	0.248	0.739	0.128	0.006	0.888	0.001	<0.0001	0.143	0.708	0.067	0.014	<0.0001		0.034
	461	483	486	473	486	605	605	605	605	545	545	605	605	605	545	605	605	605	475	545	605	605	388
Spores	0.166	0.369	0.332	0.419	0.256	0.215	0.079	-0.083	-0.070	-0.032	-0.070	0.357	-0.076	0.124	0.531	0.235	0.183	0.215	0.130	0.268	0.047	-0.108	1.000
	0.002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.103	0.084	0.147	0.502	0.150	<0.0001	0.117	0.010	<0.0001	<0.0001	0.000	<0.0001	0.013	<0.0001	0.353	0.034	
	358	378	378	395	378	432	432	432	432	430	430	432	432	432	430	431	432	432	367	430	388	388	

Green cells indicate positive significant relationships and orange cells indicate significant negative relationships ($P < 0.05$).

The models where data was pooled across years captured the most observations (n = 386 for stepwise regression, n = 539 for cumulative log regression). Substantially fewer observations were available for models where LWD and related terms were included (n = 314 for stepwise regression, n = 428 for cumulative log regression). The cumulative log models without LWD exhibited similar but 0.8–2.3% lower concordant responses, and the stepwise regression models exhibited lower adjusted R^2 values.

The model with the highest adjusted R^2 (0.50) of all stepwise regression models was selected, although the models for 2018 alone were slightly higher (R^2 = 0.52 and 0.60 for models with and without quadratic functions included, respectively). The selected model had the lowest error rate (12.6% difference from expected). The model with the highest average error rate was 38% above the actual spore values.

5.3.5 Weather model observations

The selected model was as follows:

$$\text{Spores} = -430.51 + (8.89 \times \text{AvSoilT}) + (8.62 \times \text{AvRH}) - (6.26 \times \text{NightLWD}) + (4.35 \times \text{DVPD}) + (0.41 \times \text{AvWind}) + (0.38 \times \text{TRain}) + (0.06 \times \text{DryPeriod}) + (0.35 \times \text{NightLWD}^2) - (0.05 \times \text{AvRH}^2) + (0.00002 \times \text{Rad}^2) - (0.24 \times \text{AvSoilT}^2) + (0.08 \times \text{LWD}^2) + (0.30 \times \text{DLWD}^2) - (0.09 \times \text{HumidLength}^2)$$

The model contained several variables related to leaf wetness duration (LWD^2, $DLWD^2$, NightLWD, $NightLWD^2$), and relative humidity (Average RH, $AvRH^2$, $HumidLength^2$), as well as average daily wind speed (AvWind), cumulative rainfall over 10 days (TRain), number of hours since a rainfall event (DryPeriod), the number of days with VPD < 0.5 over the past 10 days (DVPD), average daily radiation (Rad), and soil temperature (AvSoilT, $AvSoilT^2$) (Table 5.5). The final R^2 was 0.53 and the adjusted R^2 was 0.50.

Table 5.5 Model overview, standard error (SE) and summary of stepwise selection.

Variable	Parameter	SE	Pr > F	Partial R^2	Model R^2
Intercept	-430.512	104.876	<0.0001		
DVPD	4.353	0.580	<0.0001	0.29	0.29
$DLWD^2$	0.296	0.089	0.0011	0.06	0.35
$DryPeriod^2$				0.05	0.40
Remove- $DryPeriod^2$				-0.0005	0.40
TRain	0.381	0.118	0.001	0.03	0.43
$NightLWD^2$	0.347	0.118	0.003	0.02	0.44
VPD^2*				0.02	0.45
Remove-VPD^2				-0.004	0.45
NightLWD	-6.265	2.336	0.008	0.01	0.46
$AvRH^2$	-0.052	0.022	0.02	0.009	0.47
AvRH	8.617	2.882	0.003	0.008	0.48
LWD^2	0.083	0.023	0.0004	0.008	0.49
AvSoilT	8.893	4.139	0.03	0.007	0.49
AvWind	0.414	0.191	0.03	0.006	0.50
$AvSoilT^2$	-0.241	0.100	0.02	0.006	0.50
Rad^2	0.00002	0.000	0.006	0.005	0.51
DryPeriod	0.064	0.011	<0.0001	0.005	0.51
$HumidLength^2$				0.01	0.52
Remove-$HumidLength^2$				-0.004	0.52
$HumidLength^2$	-0.087	0.051	0.09	0.005	0.53

*Variables in bold were selected for removal from the model when entry level was changed to $F < 0.05$.

DVPD – Number of days with average VPD ≤ 0.5 kPa; DLWD – Number of days with LWD ≥ 6 h in past 10 days; DryPeriod – Number of hours since last rainfall event; TRain – Cumulative total rainfall in past 10 day; NightLWD – Duration of leaf wetness overnight (17:00–9:00); AvRH – Average daily relative humidity on calendar date; LWD – Daily leaf wetness duration (calendar date); AvSoilT – Average soil temperature on calendar date; AvWind – Average wind speed (calendar date); Rad – Average solar radiation (calendar date, 24 h); HumidLength – Number of hours relative humidity > 70% from 5:00 to 23:00.

The positive coefficients for many variables associated with moisture, such as LWD^2, $DLWD^2$, Average RH, $NLWD^2$, and TRain, demonstrated there was a positive association between spore concentrations and rainfall. However, there is also a small negative relationship between spore concentration and NightLWD, $AvRH^2$, and $HumidLength^2$. The large positive coefficient of AvRH (8.617) was counteracted by the small negative coefficient of $AvRH^2$ (-0.052). Likewise, the small positive effect of $NightLWD^2$ (0.347) was made larger by the fact that $NightLWD^2$ was squared, and the negative coefficient (-6.265) for NightLWD decreased background noise and moderated the maximum expected value from the model. The impact of AvRH plus $AvRH^2$ and NightLWD plus $NightLWD^2$ on the model was examined by manually

removing one or the other from the model. Since both versions of each variable had an effect on the overall outcome, all four variables were retained in the model. AvRH was later removed from the simplified model.

Average daily relative humidity (AvRH) was the variable most often included in models (2016, 2018, and both multi-year models). Similarly, cumulative total rainfall in the past 10 days (TRain) or the quadratic form ($TRain^2$) was commonly included in the models for individual years (2015, 2016, 2018) and in both multi-year models. This indicated that rainfall is an important factor in predicting high spore concentrations. The number of days with average VPD ≤ 0.5 kPa over the past 10 days (DVPD) was included in the cumulative log, stepwise regression, and regression tree models from 2014, 2015, 2016, 2018, and both multi-year models. AvVPD or the squared form ($AvVPD^2$) was occasionally included in stepwise regression models or the cumulative log models but was frequently included along with DVPD. DVPD contributed the largest partial R^2 in this model (0.29) followed by $DLWD^2$ (0.06) and $TRain^2$ (0.03); all other variables contributed less than 2% to the overall model R^2, with $HumidLength^2$ being the lowest (0.5%).

DryPeriod (number of hours since last rainfall event) or the quadratic form ($DryPeriod^2$) was a major factor in 2020, when there was a very long drought (873 h without rainfall event) and no rainfall recorded late in the season (August and September). DryPeriod was also included in the stepwise regression models for 2015 and 2019, and in the regression tree for the pooled-year model without the quadratic variables. In 2015, the maximum DryPeriod length was 354 h (15 May – 25 May) although the soil is typically highly saturated at this time of year, and the overall precipitation was the highest of all 6 years (348 mm). In 2019, the longest DryPeriod (167 h) stretched from 30 July to 6 August, but overall, the year exhibited moderate rainfall (133 mm total) overall.

The model described spore amounts for 2015, 2016, 2018, 2019, and 2020 accurately based on visual observation (Figures 5.7 and 5.8). The model chosen not only had the highest R^2 value, but also achieved the lowest range of error (Minimum [Expected – Actual] – Maximum [Expected – Actual] = -168.9) and the median error rate closest to 0% (-0.02). In addition, the model was able to accurately detect low spore concentration, which was associated with low SLB incidence and severity in 2016 (Figure 5.7). However, in 2019 and 2020, there were high

levels of spores but severity did not exceed 50 DSI (Figure 5.8). More data is needed to validate the model so that it can identify conditions where SLB severity will be low.

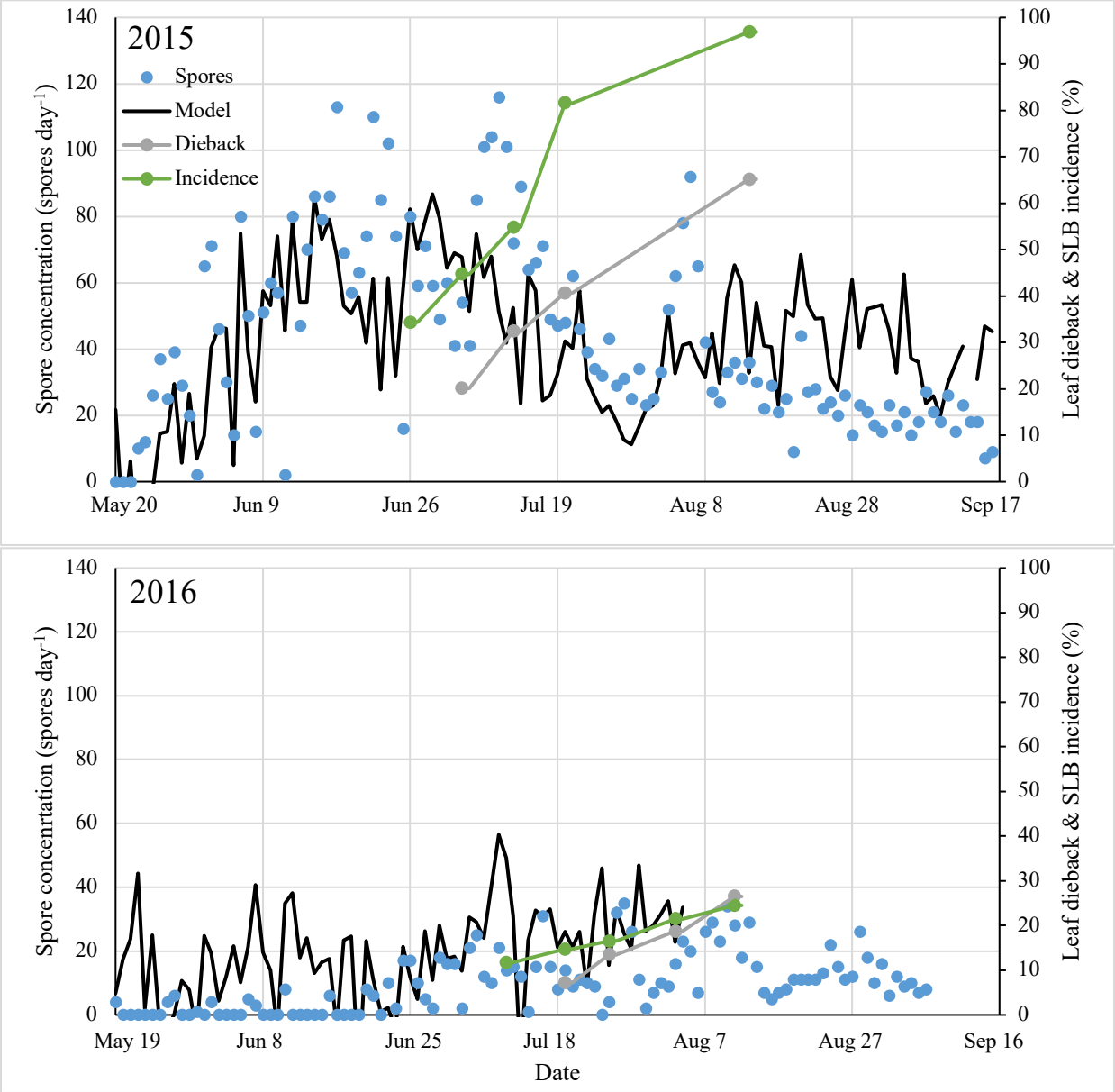

Figure 5.7 Spore prediction model versus actual spore concentrations (Burkard tape spore counts) and Stemphylium leaf blight (SLB) in 2015 and 2016 (data from Tayviah 2017).

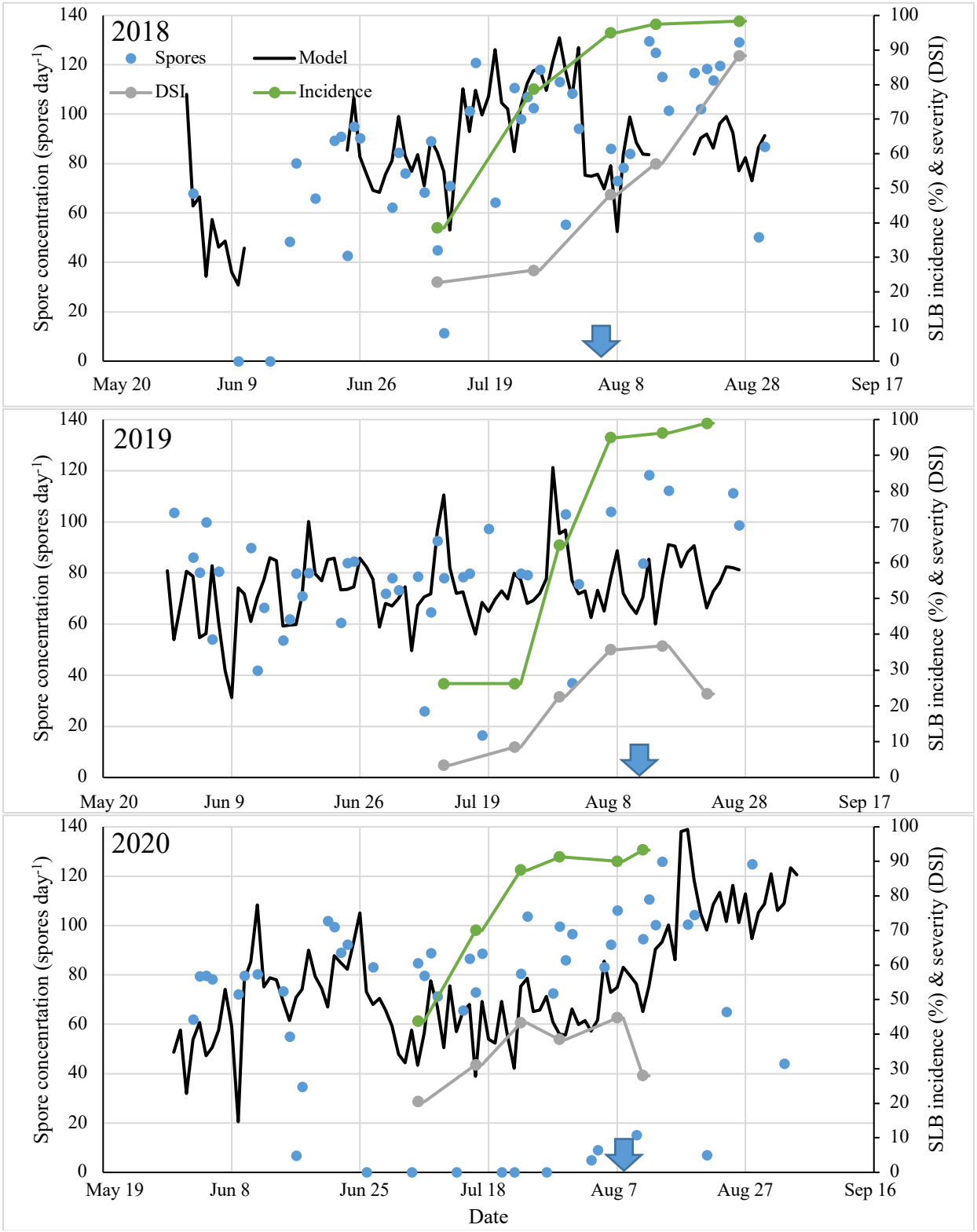

Figure 5.8 Spore prediction model versus actual spore concentrations (adjusted *cytB* qPCR estimates) and Stemphylium leaf blight (SLB) in 2018, 2019, and 2020. Blue arrow indicates approximate date when lodging began.

The model was simplified by removing the variables that produced a relatively small contribution. When the entry level of the model was reduced to $F = 0.05$ and $F = 0.05$ to stay in the model, it excluded AvSoilT, AvSoilT^2, AvRH, AvRH^2, AvWind, Rad^2, LWD^2, and HumidLength^2 (adjusted $R^2 = 0.44$). The cut-off point was chosen based on the commonly used cut-off point of $P < 0.05$ (Appendix 5.12). The simplified model was:

Spores = 10.70 − (5.95×NightLWD) + (4.45×DVPD) + (0.41×TRain) +
(0.39×NightLWD^2) + (0.36×DLWD^2) + (0.00008×DryPeriod^2)

5.3.6 Effect of drought

Chlorosis and tip dieback occurred in all treatments and increased over time from 7 days-post-inoculation (DPI) to 18 DPI (Figure 5.9). The symptoms in the inoculated treatment were somewhat atypical. Oval-shaped lesions were largely absent, possibly due to the lack of UV light to induce sporulation. Non-inoculated plants exhibited some chlorosis when grown under 'normal' watering conditions (~30% chlorosis) by the end of the experiment, but inoculated treatments had substantially more chlorosis (71–78%) at each assessment date. No cross contamination with inoculum (e.g., SLB symptoms on non-inoculated controls) was observed. At all four disease assessment time points, there was no difference between the proportion of chlorosis (%) of the normal watering treatment and the simulated drought treatment. When the data were analysed as repeated measures, the conclusions were the same (Appendix 5.10).

In the regression analysis, the quadratic term was not significant for inoculated or non-inoculated treatments, resulting in a linear relationship (Appendix 5.11). There was no interaction between drought treatment and inoculation. The main effect of inoculation was significant, with higher percent leaf chlorosis in the inoculated treatment, as expected. The main effect of drought was not significant. The regressions of watering treatment were plotted to visualise the relationships over time.

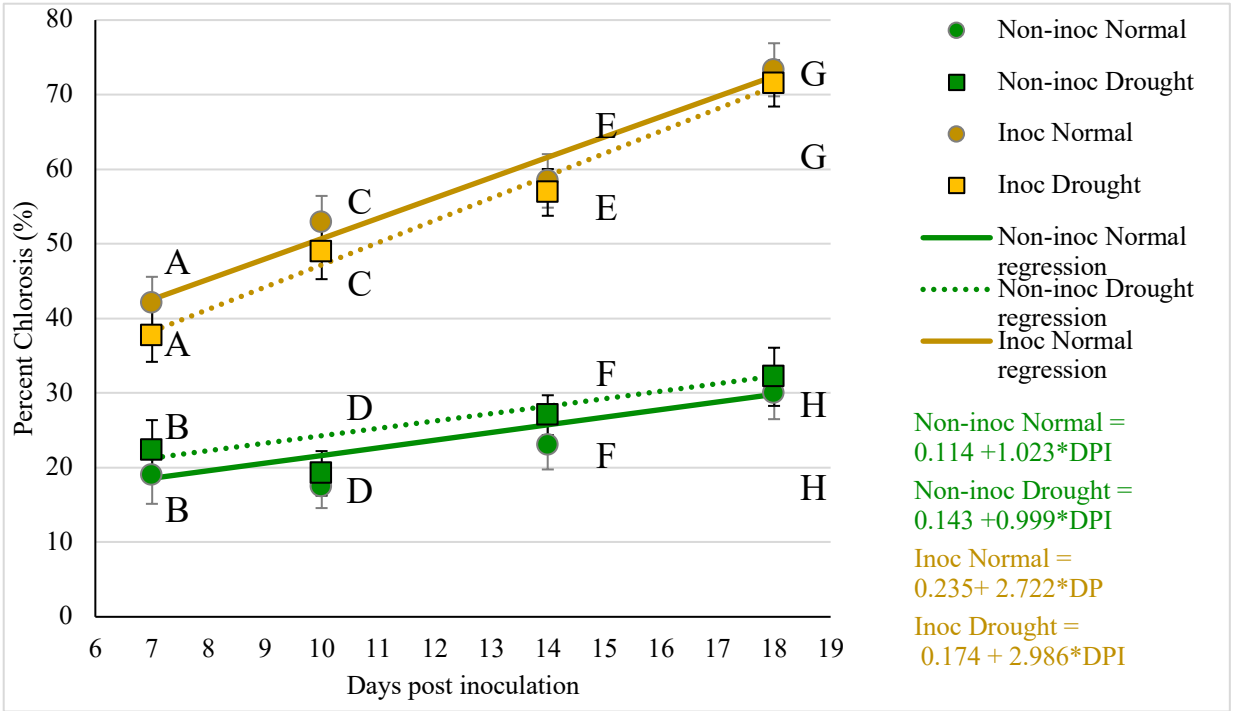

Figure 5.9 Chlorosis of onion plants subjected to 'normal' watering or 'drought' conditions, inoculated with *Stemphylium vesicarium* or mock inoculated at 7-, 10-, 14-, and 18-days post inoculation in a controlled environment. Values topped with the same letter do not differ based on Tukey's HSD at $P < 0.05$. Capped lines represent standard error.

5.4 Discussion

This study investigated the effects of weather variables on the concentration of spores of *S. vesicarium* in the air of an onion field and the relationship between air-borne spores and SLB incidence and severity on onion. Five years of data were included in the study, three from the current research and two from a previous study (Gossen et al., 2021). Air-borne spores were captured using different spore traps and quantified using microscopy and two qPCR methods.

No pre-existing species-specific primers for *S. vesicarium* were available, so quantitative PCR was conducted to estimate spore concentration in the samples from the current study. Primers from three gene regions were used and this ensured a reliable identification of *S. vesicarium*. The *cytB* primer was most closely correlated with other methods of quantifying spores and was selected for use in subsequent regression analysis to identify weather factors associated with spore production and release.

Overall, spore concentration estimates were correlated to several moisture-dependant variables: nighttime leaf wetness duration (NightLWD), number of days with VPD < 0.5 kPa

(DVPD), rain in the past 10 days (TRain), and number of days with leaf wetness duration > 6 h in the past 10 days (DLWD).

The use of molecular methods to detect and quantify air-borne spores of important plant pathogens has advanced dramatically in recent years and there is a great deal of interest in this approach. Even though it is still in the early stages of research and implementation, some companies already offer this service on a commercial basis. The current study compared an active spore trap that drew bioaerosols into a microcentrifuge tube for analysis via qPCR (Burkard multi-vial sampler), a passive spore trap provided by a private company (Spornado by Sporometrics), and a commonly used impact-based spore trap which is quantified via manual counting under a microscope (Rotorod).

To achieve an accurate estimate using qPCR analysis, the efficiency of spore collection, DNA extraction, collection, and quantification efficiencies for aerosol sampling devices must be consistent and uniform. qPCR analysis relies on the assumption that the number of gene copies of the target sequence in the pathogen's genomic DNA is constant and that the entire gene region was successfully amplified (Hospodsky et al., 2010). Depending on the organism and sampling material, qPCR is useful for determining dissimilarity between two samples only if the true values are greater than 1.3 to 3.2 fold different (Hospodsky et al., 2010). Quantitative PCR generally has the lowest observed limit of quantification ($\sim 10^{-3}$ ng μL^{-1}) compared to UV spectroscopy and fluorometry (Olson and Morrow, 2012). In a previous study, qPCR was able to detect the DNA of five cells of the bacterial *Bacillus atrophaeus* Nakamura, less than one spore of *Aspergillus fumigatus* Fresenius (Hospodsky et al., 2010), or two spores of *Botrytis squamosa* (Carisse et al., 2009).

A major limitation to using qPCR for measuring DNA concentration is that protocol development, validation, and execution are time-consuming and require skilled operators. Also, standard qPCR protocols cannot differentiate between living and dead cells, so it is impossible to know if the spores collected are viable or pathogenic. This is important because the pathogenicity of *S. vesicarium* is not uniform; of 44 *S. vesicarium* isolates collected as spores in a pear orchard, 89% were not pathogenic to pear (Moragrega et al., 2018). However, qPCR is able to measure the DNA concentration of a specific organism in a mixed sample (e.g., detection of a specific pathogen in bioaerosol). Unfortunately, no standard reference materials were available for the qPCR assays used in this study, so the associated uncertainty of the standard curve used in this study could not

be determined. In the current study, a serial dilution was used to create a standard curve that included concentrations as low as 10 spores μL^{-1}.

The methods used in this study represent those that are commonly used in pest diagnostic laboratories. More accurate DNA measurement methods are available, including digital drop PCR (Pinheiro et al., 2012) and phosphorus elemental analysis (Holden et al., 2007), which may be implemented in research and commercial systems in the future. Currently, the equipment needed for these methods is expensive and not commonly available in most research laboratories.

Differences in DNA extraction protocols may contribute to differences among the estimates from qPCR. In bioaerosol samples collected using cassettes, such as in the Spornado method, the accuracy is limited by cell extraction from sample filters and DNA extraction efficiencies from cells; some bioaerosol particles may not be released from the cassette filter material. For example, DNA extraction efficiency was only 3.4–13.3% in a study that used quartz and polycarbonate filters to quantify air-borne spores of *Escherichia coli* (Migula) Castellani and Chalmers, *B. atrophaeus*, and *A. fumigatus* (Hospodsky et al., 2010). One benefit of the Burkard sampler was that the DNA extraction occurred within the sampling container, which prevented any loss due to transferring bioaerosol from the collection device to the reaction vessel. The PowerSoil extraction kit has been previously used in combination with the Burkard multi-vial cyclone sampler for analysis of bioaerosol with satisfactory results (Parker, 2012; Rastrojo et al., 2018).

It was difficult to determine which spore sampling method used in the current study was the most accurate. The relationship between ranked values of spore estimates trapped using different methods at the same site over the same time period would normally be expected to be a straight line. The line of best fit ($r = 0.25$) between ranked values from the Sporometrics and Burkard methods was exponential, and the association was low. The exponential relationship may be due to the fact that the Sporometrics system sampled over several days and the spore estimate was divided by number of days sampled to provide spores per day for comparison with the Burkard sampler, resulting in several dates with the same estimated value. It is impossible to know which dates contributed more or less to the spore estimates. Also, it was difficult to determine if outliers contributed to the exponential relationship. Unfortunately, the Sporometrics and Burkard methods only overlapped for 23 sampling dates, so the sample size for this comparison was very low. Both of these molecular methods were positively correlated with

manual spore counts from the Rotorod sampler conducted by researchers at the MCRS, although the relationship was not very strong ($R^2 = 0.55$ for the Burkard method, $R^2 = 0.37$ for Sporometrics). The low values of the regression coefficient may be linked to the small sample size ($n < 30$), which occurred because the Rotorod counts, Burkard sampling, and Spornado methods did not always coincide with the same sampling dates.

The ability of air samplers to trap bioaerosol is affected by the equipment used and the target organism. In a previous study that compared a Burkard-type sampler to Rotorod sampler, the Rotorod was only 37% as efficient as the Burkard at capturing air-borne spores of *Venturia inaequalis* (Aylor, 1993) and 57% for pollen (Solomon et al., 1980). This may be due to the size and shape of the particles in question, with smaller particles more able to follow air streamlines around the moving rods of the Rotorod (Muilenberg, 2003). In another study of Urticaceae pollen, which was small but smooth and spherical, the efficiency of the Rotorod was 75% of the Burkard (Peel 2014). Non-spherical particles or those with rough surfaces result in greater drag than do smooth, spherical particles (Solomon, 2003). *Stemphylium vesicarium* spores are large and non-spherical, as are *V. inaequalis* spores, and not as smooth as Urticaceae pollen (Simmons, 1969; Raghavendra Rao and Pavgi, 1975). Large, non-spherical particles should be efficiently trapped by the Rotorod sampler, which sampled over twice as much air volume as the Burkard multi-vial cyclone sampler in 2020. However, spore estimates from the Rotorod were consistently lower than qPCR estimates. It is possible that the DNA copy number calculated by qPCR may be higher than the actual number of spores. This was the case for a study on *Peronospora effusa,* where demonstrated DNA copy values > 500,000 coincided with Rotorod spore trap samples on the same date of ~200 spores (Klosterman et al., 2014).

Even though spore concentration estimates from the Burkard multi-vial cyclone sampler were much higher than estimates from Sporometrics, there was a weak but significant correlation between the rank values of the sampling methods. The maximum value recorded by Sporometrics was 1,500 spores day^{-1} (14–17 August 2021), compared to 298,000 $spores^{-1}$ with the Burkard sampler (14 August 2020), and 17–307 spores day^{-1} for the Rotorod (14 and 17 August 2020). On another date, the Sporometrics method reported 9 spores day^{-1} and the Burkard method reported 194,000 spores day^{-1} (25 August 2020).

The large difference in estimates of spore concentration from the three sampling methods may be associated, at least in part, with differences in the volume of air sampled. The battery-

powered motor in the Burkard and whirling rods of the Rotorod sampler actively draw bioaerosol in, whereas the volume sampled in the passive sampling of the Sporometrics cassette relies on wind to deliver particles. However, the Spornado was operated for 24 h day^{-1} whereas the Burkard and Rotorod samplers ran for only 6 h day^{-1} in the morning, the time when *S. vesicarium* spores are most plentiful (Gossen et al., 2021). The Burkard sampled a total of ~5.9×10^3 L of air per day, the Rotorod sampled ~1.6×10^4 L of air per day, and the volume of air sampled by the Spornado sampled is unknown since it would be affected by the speed of wind that passed through the cassette filter over multiple days. However, collection by the Spornado would be expected to be higher (rather than lower) than the others since the sampling period for the Spornado was longer. It appears likely that battery-assisted methods sampled a larger volume of air over a shorter time period relative to a passive system.

The Burkard and the Spornado sampling devices are both gust-responsive, whereas the circular spinning path of Rotorod samplers is largely independent to wind direction (Solomon, 2003). All of these devices are affected by wind speed, with the Burkard operating best at low and high wind speed and the Rotorod most efficiently at moderate wind speed (Frenz, 2000). Conversely, a study on *A. porri* demonstrated that increased wind speed led to increased trapped spores using a Hirst volumetric sampler, which is similar to the Burkard model (Meredith, 1966). In this study, all three methods are able to trap spores of *S. vesicarium* and all methods demonstrated an increase in spore concentration over the growing season.

To make clearer comparisons between the Rotorod and the Burkard samplers, it would be useful to compare manual spore counts for both samplers. An undergraduate research project was previously conducted in 2011 using spore samples collected with the same Burkard air sampler (from 10:00–13:00) and *Stemphylium* spores were manually counted under a microscope (Stricker, *unpublished*). The values of the hand-counted bioaerosol samples ranged from 10 to 6317 spores per day in samples collected in 2009 and 2011. The sampler ran for a similar time period (although 3 h shorter and later in the season, 19 August–2 September 2009 and 29 August–21 September 2011), so it is not unreasonable that the quantity of spores captured would be comparable to the Burkard samples collected in 2018–2020 in the late-season time period. The average estimate was 84,900 spores day^{-1} (20–31 August 2018, n = 9), 4400 spores day^{-1} (26–27 August 2019), and 56,700 spores day^{-1} (18–28 August 2020, n = 4) for the Burkard sampler using qPCR for 2018, 2019, and 2020, respectively. The Burkard qPCR spore estimates

ranged from 587 to 371,000 spores day^{-1}. This further supports the hypothesis that the qPCR spore estimates from the Burkard sampler may be overestimations of the actual spore concentrations.

If users are looking for highly detailed data, the Burkard sampler would be the recommended method, but only if its use can be combined with improved qPCR protocols. Spore counting methods using the Rotorod, manually counting bioaerosol from the Burkard sampler, or an earlier version of the Burkard that trapped spores on sticky tape placed on a revolving drum, are both viable options that require minimal setup, calibration, and no genomics training, but are time consuming and may not be as species specific. There is also a commercial service that uses qPCR to quantify spores trapped using Rotorod samplers. The Spornado by Sporometrics is an alternative to the Burkard sampler that is easy to operate, but its efficacy must be confirmed with more sampling dates before it can be recommended for general use. We conclude that the existing qPCR method identified *S. vesicarium* to species but appeared to overestimate the spore concentration, so the manual counting methods (using the Rotorod or the Burkard volumetric sampler) would be recommended to get an estimate that more closely represented the actual spore concentration.

qPCR analysis with three primer regions showed that there was a strong correlation between the estimates generated by *cytB* and *TEf-A* primers, but only a weak correlation with the ITS primer. The ITS primer set did not amplify in many samples. This may have been the result of poor primer design, or that the ITS region was species specific whereas the *cytB* and *TEf-A* primers may have amplified the DNA of related fungi. The primers were designed based on the gene sequences of local isolates of *S. vesicarium* that were compared to GenBank accessions. The majority of the GenBank BLAST searches turned up *S. vesicarium* accessions, although some search results indicated other species (Foster et al., 2019). This may indicate that the gene regions are not species specific, or that some of the accessions on the worldwide database may be inaccurately identified. Even though each primer set was not necessarily species-specific, overall, the results from the three primer sets were correlated ($P < 0.05$) and followed similar trends. Therefore, we conclude that the spore capture data likely represents air-borne spores of *S. vesicarium.* One limitation of this approach was that it was not possible to differentiate between ascospores and conidia.

A confounding factor in the qPCR analysis could be difference in the calibration between qPCR reaction runs. To perform an inter-run calibration, there should be at least three repeat samples on each plate. An inter-run calibration was attempted using one identical sample of a *S. vesicarium* conidia suspension per run (raw spore; DNA not extracted). In this study, there was a significant effect of run (reaction plate) and there was a significant effect for all primers (Appendix 5.3) but not when sorted by year (Appendix 5.4) because the estimates from the plates containing samples from 2018 were higher than the other years (Appendix 5.12). These higher spore concentration estimates may be due to the higher disease pressure in 2018.

DNA concentration was measured using spectrofluorometry and qPCR. Bench-top spectrofluorometers, such as the QIAxpert, provide fast measurement of DNA concentration. However, spectrofluorometry does not quantify the DNA of specific species, so much of the DNA assessed may not be from the target pathogen, and the technique is unreliable at low concentrations of DNA. The QIAxpert system can detect between 1.5–2000 ng μL^{-1} dsDNA (Qiagen, 2017). The DNA quantity estimated by QIAxpert in these samples was low (average = 0.96 ng μL^{-1}), so most of these samples were below the threshold of detection. This likely accounts for the fact that QIAxpert measurements were not correlated to the qPCR estimates.

Based on the lowest error rate, the highest R^2 value (0.50), and the intention of maintaining leaf wetness sensors at the Muck Crops Research Station for future analyses, the spore forecasting model developed using stepwise regression with quadratic variables was selected. The models without leaf wetness duration exhibited lower adjusted R^2 values, which further indicated the important of this variable. The model was as follows, with variables in order from highest partial R^2 to lowest:

$$\text{Spores} = -430.51 + (4.35\times\text{DVPD}) + (0.30\times\text{DLWD}^2) + (0.38\times\text{TRain}) + (0.35\times\text{NightLWD}^2) - (6.26\times\text{NightLWD}) - (0.05\times\text{AvRH}^2) + (8.62\times\text{AvRH}) + (0.08\times\text{LWD}^2) + (8.89\times\text{AvSoilT}) + (0.41\times\text{AvWind}) - (0.24\times\text{AvSoilT}^2) + (0.00002\times\text{Rad}^2) + (0.06\times\text{Drought}) - (0.09\times\text{HumidLength}^2)$$

This model was further simplified by increasing the rigor at which variables entered the regression model. The simplified model exhibited a slightly lower R^2 value (0.44) but may be preferred due to ease of use. The simplified model was as follows:

$$\text{Spores} = 10.70 - (5.95 \times \text{NightLWD}) + (4.45 \times \text{DVPD}) + (0.41 \times \text{TRain}) + (0.39 \times \text{NightLWD}^2) + (0.36 \times \text{DLWD}^2) + (0.00008 \times \text{Drought}^2)$$

It was interesting to note that total daily rainfall (Rain) was not present in the model (along with several other variables related to rainfall, including Rain, HRain, and NRain). This is likely because rainfall washes air-borne spores out of the air and off lesions. Surface tension of rain or dew can also prevent liberation of fungal spores (Meredith, 1966). However, there was a positive relationship with rainfall in the 10 days before spore release (TRain) and high humidity (the number of days with average VPD < 0.5 in the past 10 days, DLWD). Rainfall creates the conditions required for infection, such as long leaf wetness periods, and infection is an essential precondition for subsequent sporulation events.

Based on previous research, a positive relationship was expected between rainfall and air-borne spore concentration of *S. vesicarium* (Tayviah, 2017). Similarly, peaks in ascospore release on leek and garlic have been linked to rainfall events (Suheri and Price, 2001; Prados-Ligero et al., 2003). The low SLB severity and spore capture observed in 2019 were likely associated with lower-than-average rainfall in June–August. However, plentiful early-season rainfall in 2020 did not produce an early-season spike in spore concentrations or SLB severity. Several rainfall events appeared to correspond with increases in spore concentration (14 July–28 August 2018), but this is not always the case (10 July–14 July 2020). In 2018 and 2020, the rainfall in August was above normal, and there was an increase in SLB severity at the end of the season. Unfortunately, spore estimates were not available for each consecutive date during these periods, so it is difficult to draw conclusions about the relationships between spore concentration and air temperature or rainfall based on the graphs alone. However, the stepwise regression, cumulative log regression, and regression tree analyses all included variables related to leaf wetness and rainfall, indicating an important relationship between *S. vesicarium* spore production and available moisture.

High relative humidity, which is an indicator of environmental conditions conducive to infection by fungal pathogens (Talley et al., 2002) was associated with low VPD. High relative humidity (AvRH) was included in the stepwise and cumulative log regression models when LWD was excluded, likely because they are concordant variables. Average VPD (VPD) was also included in some stepwise and cumulative log regression models, but both AvRH and VPD were removed from the simplified model because their inclusion did not substantially improve the

accuracy of the model. In garlic, ascospore production of *S. vesicarium* coincided with rainfall and periods with VPD < 0.5 kPa (Prados-Ligero et al., 2003), and VPD was negatively correlated with air-borne ascospore concentrations in asparagus (Granke and Hausbeck, 2010). In onion, low VPD was correlated with high conidial concentrations in onion fields in one of two years (Tayviah, 2017), and the number of days with average VPD ≤ 0.5 kPa over the past 10 days (DVPD). Nighttime leaf wetness duration (NightLWD) and DVPD were the most important variables in the stepwise regression models, and DVPD was a major factor in regression tree models.

In contrast to DVPD, NVPD was not a significant factor in the current study. The number of hours with vapour pressure deficit < 0.5 kPA in the past 10 days (NVPD) had a negative relationship with ascospore production in 2016, but did not impact conidia in either year (Tayviah, 2017). In a study on *S. vesicarium* in garlic fields in Spain, NVPD was positively correlated with ascospore production in 3 out of 4 years but was not linked to conidia concentration (Prados-Ligero et al., 2003). Based on other fungal foliar pathogens, a positive relationship between spore production and days with low VPD might be expected, and this was observed in 2018 and 2020. The lack of correlation in 2019 may be due to low disease pressure.

Leaf wetness has long thought to be an important factor for the development of foliar fungal diseases (Agrios, 2005). Interestingly, LWD was positively correlated with several temperature variables, such as average soil temperature (AvSoilT), number of hours daily with temperature ≥ 15 °C (NTemp), average temperature during leaf wetness period (WTemp), and the number of days with average temperature ≥ 15 °C over the past 10 days (DTemp). This relationship was not expected, since increased temperatures generally reduces leaf wetness duration due to increased evaporation. The stepwise regression model and the simplified model both included nightly leaf wetness duration (NightLWD) and number of days with leaf wetness duration > 6 h (DLWD). *Stemphylium vesicarium* requires > 16 h of leaf wetness at 5 °C or > 8 h at 10–25 °C for infection (Suheri and Price, 2000b). The first dates where temperatures exceeded 10 °C during at least 8 h of consecutive leaf wetness periods were: 25 May 2015, 26 May 2016, 24 May 2018, 23 May 2019, and 23 May 2020. This would be 2 weeks after most onion crops were seeded, with seedlings in the cotyledon or first-leaf stage. In general, leaf wetness duration increased over the growing season, and the average nightly leaf wetness exceeded 8 h for June–September 2015, July–September 2016, May–August 2018, May–August 2019, and June–

September 2020. The average temperature during nightly leaf wetness period was 12–20 °C, indicating that conditions suitable for infection by *S. vesicarium* occurred throughout the growing season.

Previous research at the MCRS demonstrated that there were positive relationships between air-borne conidia and temperature, temperature during leaf wetness and leaf wetness duration (Gossen et al., 2021). However, this was demonstrated for only one year, and the relationships were not significant for ascospores. The number of days with average temperature > 15 °C was significant in 2015 and 2016, but this is likely because the data recording in those years started earlier in the season, when mean temperatures were lower, than in 2018–2020. Air temperature was calculated as an average of 24 hourly measurements each day. The maximum average daily temperature did not exceed 29 °C on any date in the study, even though the maximum daily air temperatures occasionally exceeded 35 °C. A negative relationship between LWD and air temperature was expected but not observed. Conversely, there was a positive relationship between number of days with LWD ≥ 6 h in the past 10 days (DLWD) and air temperature. This correlation may be spurious, since average air temperature and DLWD both increased over time, with the majority of days in July–September exhibiting DLWD values of 9 or 10. The observed positive relationship between LWD and AvSoilT or NTemp may be explained by the increased capacity for air to hold moisture (increased dew point) at higher temperatures. The cut-off point for NTemp was any temperature ≥ 15 °C, which largely excluded early-season data collection points, and thus may have been linked to the general increase in air-borne spore concentration over the season. AvSoilT and AvSoilT^2 contributed only 0.007 and 0.055, respectively, to the overall model R^2, so these two variables were removed from the simplified model.

Non-temperature effects of climate change observed in the last two decades include decreased wind speed (Hartmann et al., 2013) and increased solar radiation (Wild et al., 2005), so it is especially important to understand the impacts of these two weather variables on the production of *S. vesicarium* inoculum. Increased wind speed can lead to increased concentrations of bioaerosol being trapped by Rotorod samplers (Frenz, 2000). It appears likely that the stepwise regression model selected was heavily influenced by data from the 2014–2016 growing seasons, since the coefficient for wind speed was positive (0.41) and these sampling years all included spore estimates from a Rotorod sampler. However, the direct correlation between wind

speed and spore concentration was negative (-0.11), which indicated that wind speed may have affected a related variable such as trapping efficiency. Wind speed was also negatively correlated with leaf wetness (NightLWD, LWD, DLWD, LWD18h), likely due to increased evapotranspiration at high wind speeds. Average wind speed contributed only 0.006 to the model R^2 and was removed from the simplified model.

Previous studies have reported that solar radiation over 164 W m^{-2} and wind speed < 3 m s^{-1} were linked to *S. vesicarium* sporulation (Prados-Ligero et al., 2003). This may be explained by increased solar radiation at the middle of the growing season around late-June and early-July, which corresponds to the summer solstice. When investigated individually, radiation was negatively correlated to spore concentration. The lowest average solar radiation occurred in 2014, which also exhibited fewer air-borne spores than other years. The coefficient for Rad was very small and it contributed only 0.005 to the overall model R^2, so it was removed from the simplified model.

Local temperatures in southern Ontario may rise by 1.5–6.3 °C in the next 100 years due to climate change and summer precipitation may decrease, which increases the risk of drought stress for field crops like onion (Bush and Lemmen, 2019). In the correlation matrix, DryPeriod was positively correlated with air-borne spores of *S. vesicarium*, most likely because several long drought events occurred late in the season when air-borne spores were plentiful, resulting in a spurious correlation. DryPeriod was included when modelling the impact of weather variables on spore concentrations because local growers had reported that drought increased SLB symptoms; it is not a variable that has been investigated previously. DryPeriod contributed only 0.005 to the stepwise regression model R^2, so it was removed from the simplified model.

Many disease management systems have used spore thresholds to time the initiation of foliar fungicide applications for disease management, such as 378 spores day^{-1} (8.6 spores m^{-3}) of *Bremia lactucae* Regel on lettuce (Dhar et al., 2020) and 446 spores day^{-1} (15 spores m^{-3}) of *B. squamosa* on onion (Carisse et al., 2009). This method could be used to set a threshold of when to begin fungicide applications for the management of SLB, if an effective fungicide can be identified in the future. The 2016 growing season exhibited less than 30% SLB incidence, low disease severity (20 sAUDPC), and the maximum spore count for *S. vesicarium* was 35 spores day^{-1} using the Burkard tape sampler with manual counting (Tayviah, 2017). In 2020, SLB incidence exceeded 80% and severity was 43 DSI before a single spore was detected using the

Rotorod sampler. Spore detection peaked late in the season, with a maximum of 307 spores day^{-1} on 17 August 2020, a time when some of the onion crop had already begun to lodge. At the end of the growing season, SLB incidence (91%) was relatively high but severity low (38 DSI) in unsprayed plots.

Overall, air-borne spores increased after SLB incidence was already high. The concentration of air-borne spores increased exponentially over time, which is not surprising for a pathogen with a polycyclic life cycle (Arneson, 2011). As more plants are infected, more lesions are available to sporulate, and increase the concentration of air-borne spores. In all three years in the current study, SLB incidence increased steadily over the season to nearly 100%, even when severity was relatively low. Therefore, the spore threshold to begin fungicide applications would be low. An increase in spore concentration early in the season did not generally coincide with high SLB severity at the end of the growing season. None of the years included in this study resulted in extreme disease severity, so it is not possible to extrapolate the data to make management recommendations that would apply in a high disease year.

Local growers have reported an association between plant stress and SLB. This may be because tip-dieback from SLB can be similar to symptoms of drought, or because *S. vesicarium* can infect necrotic tissues, resulting in a mistaken identification of the causal agent. However, drought did not predispose onion crops to infection by *S. vesicarium* in a controlled environment study. SLB symptoms in the study were atypical, with oval-shaped lesions being largely absent, possibly due to the lack of UV light to induce sporulation. Onion plants have a relatively shallow root system, so low soil moisture reduces plant growth and inhibits the uptake of plant nutrients. At the end of the controlled environment study, the plants used were root-bound within the pots, which may account for the chlorosis observed in the normal-watering treatment. An alternative explanation is that insufficient drought stress was applied to make plants susceptible to SLB. Also, pot-based experiments often provide a poor simulation of the complex interactions in soil.

Future research could validate and improve the simplified spore forecasting model developed in this study. Receiver-Operating Characteristic (ROC) analysis could be used for evaluating the predictive model. It would also be interesting to conduct an experiment where each sampler is sprayed with a conidia suspension of a known concentration; some samples using the Burkard sampler could be manually counted, and others could be used for qPCR to create a correction factor between the Rotorod sampler and qPCR. Another sampling method that

could be used is the Burkard sampler which uses sticky tape, since the tape can be cut longitudinally; half of the tape could be manually counted, and the other half used for qPCR to further improve the calculation of spore estimates. Other pathogen identification methods could be tested, such as immunochromatographic test strips, which have been used for on-site field immediate test results to identify the downy mildew pathogen *Peronospora destructor* (Wakeham et al., 2012). Additionally, other statistical methods such as receiving operating curves can be used in future studies.

Future studies could use standardized gene fragments purchased from commercial vendors (gBlocks), which can be used as standards in probe-based qPCR reactions as an alternative to creating a standard curve from manually-counted spore samples to improve the accuracy of the qPCR protocols (Conte et al., 2018). It would also be useful to screen the pathogenicity of local air-borne *S. vesicarium* spores (similar to Moragrega et al. (2018), since spores produced on alternative hosts may not be pathogenic to onion. Further experiments on the effect of drought in a controlled environment should use larger, deeper pots to allow for natural water percolation and avoid non-uniform soil moisture patterns (Pennypacker et al., 1990).

The current study indicated that high daily average relative humidity, low vapour pressure deficit for extended duration, and increased leaf wetness, especially at night, were correlated to an increase in air-borne spores of *S. vesicarium*. However, the highest concentration of air-borne spores occurred at the end of the growing season after the onion crop had lodged and the leaves had begun to naturally die and desiccate. Further research is needed to understand spore concentrations in years with high SLB severity, to verify if the spore prediction model developed in this study can predict high spore concentrations, and that a spore threshold could be identified to recommend when or if fungicide sprays are needed in a growing season.

Chapter 6 General discussion

Stemphylium leaf blight and other foliar diseases of onion can limit onion yield and storage quality. Growers in Ontario are concerned about the leaf dieback and the lack of disease suppression when applying foliar fungicides. To maintain the sustainable production of onion in the region, it is important to understand this host-pathogen system and to develop improved management strategies / recommendations. The presented research is the first study in Canada to confirm the identification of *S. vesicarium* isolates collected in onion fields in Ontario, confirm fungicide insensitivity to commonly used fungicides, confirm seed-borne inoculum from naturally infested flowers, outline the life cycle of *S. vesicarium* in this region, and propose a model to predict sporulation events in southern Ontario.

In Ontario, field studies on cultivar resistance and fungicide efficacy on onion have identified only small differences among cultivars and dwindling fungicide efficacy over the past decade. The assessment of forecasting models based on weather parameters in the current study, together with a previous study at the same site, (Tayviah, 2017) has demonstrated that the number of fungicide applications can be reduced, but the foliar fungicides applied did not provide effective SLB suppression compared to the unsprayed control. A mineral oil product with suspected induced systemic resistance properties was tested in one year but also did not provide SLB suppression. Clearly, more research is needed to provide growers with new tools to manage this disease.

Cultural management techniques, such as reducing overwintering inoculum, have shown some efficacy for the diseases caused by *S. vesicarium* on asparagus (Johnson, 1990) and pear (Llorente et al., 2010b). Inoculated onion leaf tissue that had been buried in a fabric bag and exposed to the moist environment decomposed over the winter. Re-isolation of the pathogen from these buried wet samples was not successful on 11 April, 1 May, or 30 May sampling dates, but pseudothecia were present. The current study demonstrated that the production and survival of overwintering structures on onion leaves can occur in Ontario. However, viable ascospores were only observed on samples which were wet and had not been buried, which suggests that burial of onion leaf residue can prevent the formation of ascospores by hastening decomposition and excluding light from pseudothecia. It also showed that many of the common weed species, such as redroot pigweed, yellow nutsedge, purslane, marshcress, sowthistle, and bull thistle are asymptomatic hosts of *S. vesicarium*. The importance of these as sources of inoculum is not yet

known, but these weeds could be another source of primary inoculum for onion crops. Onion growers in the region have many reasons to manage weeds in and around fields and to manage cull onions to avoid cull piles. This research provides additional reasons to support weed management and disposal of culls, which will reduce the carry-over of inoculum.

Three of four fungicide active ingredients commonly used in Ontario to suppress SLB exhibited levels of *in vitro* insensitivity against *S. vesicarium,* and none were effective in the field. Of the isolates collected in southern Ontario, 94% exhibited insensitivity to azoxystrobin, 63% to fluopyram, 41% to pyrimethanil, and only 1% to difenoconazole. Fungicide insensitivity seems to have developed relatively quickly, in less than 5 years in the Holland Marsh (Stricker et al., 2020). *Stemphylium vesicarium* isolates were 7% insensitive to fluopyram in 2018, 43% in 2019, and 100% by 2020 (Stricker et al., 2021b). Future research could investigate fitness of insensitive and sensitive *S. vesicarium* isolates to determine if halting the use of one or more fungicides would result in a reversion to the sensitive wild type.

This study confirmed that *S. vesicarium* can survive on seed and infect the seedlings that develop, which is consistent with the results of Aveling et al. (1993). However, the role of seed-borne inoculum in SLB epidemics remains to be elucidated. Most commercial seeds are heat-treated before sale for management of Botrytis leaf blight (Stokes Seed, *personal communication*), which greatly reduces the risk of primary inoculum from seed. If some commercial onion seed producers are currently not using seed heat treatments, incorporation of this practice into their production process could reduce seed infestation with *S. vesicarium* and other pathogens. This may be most important for growers of organic onions or in regions where onion growers save seed. Additionally, commercial onion seed is treated with one or more fungicides, which could also prevent seed-to-seedling transmission of seed-borne inoculum. It is possible that *S. vesicarium* was initially introduced to the Holland Marsh region via seed-borne inoculum. However, the wide host range of this pathogen makes determination of the initial source of the pathogen difficult or impossible. Since the pathogen can overwinter in onion debris and infect local weed species, the majority of primary inoculum in a growing season is likely from the mycelia, conidia, or pseudothecia in plant residue. The role of weed species in the overwintering of *S. vesicarium* has yet to be investigated.

The current study indicated that foliar fungicide sprays in combination with seed treatments may be the best strategy to manage SLB, but seed treatments or foliar sprays alone

did not effectively suppress symptoms. It is possible that the seed treatment increased overall plant health, thus reducing the end-of-season disease symptoms. Alternatively, the seed treatment may have provided early-season protection against infection, which delayed symptom development. The second option seems more likely. The fungicides tested did inhibit some mycelial growth and germination of 'insensitive' isolates (<50% inhibition compared to growth or germination on unamended media). This slight inhibition could explain why the combination of foliar sprays and seed treatment with penflufen provided SLB suppression in both field seasons; the seed treatment may have provided enough early-season suppression to result in statistical significance among treatments.

Weather variables relating to moisture (high daily average relative humidity, low vapour pressure deficit, and increased leaf wetness) were correlated with an increase in air-borne spores of *S. vesicarium*, which is not surprising for a foliar fungal pathogen. The simplified spore forecasting model included nighttime leaf wetness duration, number of days with LWD ≥ 6 h, number of days with average VPD ≤ 0.5 kPa, rainfall in the past 10 days, and the length of time without rainfall (DryPeriod). All of these variables, except for DryPeriod, were positively correlated with daily average relative humidity. The model requires validation with more data, especially from years with high SLB severity. More research needs to be done to develop effective qPCR methods to quantify ascospores and conidia and relate spore concentration to disease risk.

The goal of this research was to develop and improve the spore forecasting model so that the IPM offered through the Muck Crops Research Station can make reliable recommendations on when or if the local growers need to apply fungicides. The new spore forecasting model developed in this study could be used to predict high disease years. For example, if the predicted spore count in the early-season (May–June) exceeds 60 spores per day, the SLB incidence will likely be high. If it exceeds 120 spores per day, then severity will also be high. However, this recommendation is based on only 5 years of data, so the model and these thresholds need continued assessment.

Disease forecasting models have been successfully used against other pathogens of onion and for diseases of other crops. The model DOWNcast has been used to time fungicide applications for downy mildew of onion caused by *Peronospora destructor* (De Visser, 1998) and Botrytis leaf blight of onion caused by *Botrytis squamosa* (Sutton et al., 1986). The

forecasting models TOMcast and BSPcast used in this study reduced the number of fungicide applications based on weather parameters by 1 or 2 sprays. Unfortunately, until a product that can decrease SLB in the field is available, developing a disease forecasting model to recommend foliar spray applications is not helpful. Future research should focus on new fungicides, alternative products, and biocontrol agents.

Abiotic stresses, such as drought, were suspected to increase SLB severity, but a number of stresses can cause tip-dieback and *S. vesicarium* can easily invade the necrotic tissues. A controlled environment study did not find a relationship between susceptibility to SLB and drought. It is conceivable that the controlled-environment study was a poor estimation of field conditions, especially since growers have reported increased SLB under plant stress conditions.

There are still many questions left unanswered about the management of SLB on onion in Ontario, Canada. Even the effect of SLB on yield is unknown. Onion growers are hesitant to forgo fungicide applications entirely because heavy losses have occurred in the past. Also, none of the fungicides registered in Canada have curative effects against SLB.

Based on the findings of this research, growers are recommended to remove or bury onion leaf debris from the field because it can act as primary inoculum in the following spring. Additionally, hand-pulled weeds should be removed from the field and thoroughly composted or buried away from onion fields to reduce the risk of inoculum from alternative hosts. Fungicides containing azoxystrobin, pyrimethanil, fluopyram, and difenoconazole are no longer effective at suppressing SLB symptoms and use of these products should no longer be recommended. There is a possibility that sensitivity to pyrimethanil, fluopyram, and difenoconazole may return if use is discontinued. Azoxystrobin insensitivity, however, is likely to remain within the population indefinitely. If onion growers are using foliar fungicide applications, the TOMcast model is a user-friendly option that can be calculated based on local weather conditions and can reduce fungicide applications, resulting in savings to the grower and reduced selection pressure for fungicide insensitivity. Additionally, resistance management recommendations should be followed such as rotating between fungicides with different modes of actions, and not applying a foliar FRAC group 7 fungicide as the first application if the seed has been treated with penflufen. Research on SLB will continue at the Muck Crops Research Station to address growers' needs.

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Appendices for Chapter 2

Appendix 2.1 Layout of the trial to examine survival of *Stemphylium vesicarium* on onion leaves overwinter at the Muck Crops Research Station. Paired 'wet' and 'dry' samples were pinned to the soil surface (S) or buried (B). The study was arranged in five blocks, one for each monthly sampling date.

Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
S1	(B3)	(B1)	S4	S3	(B1)	S3	(B4)	(B1)	S4
(B4)	S3	(B3)	(B5)	S5	S2	(B2)	B3	(B4)	(B5)
(B5)	S2	S3	(B4)	(B4)	S1	S1	S5	(B2)	S2
S5	(B1)	(B2)	S5	S4	(B5)	(B1)	(B5)	S3	S5
(B2)	S4	S2	S1	(B2)	(B3)	S2	S4	(B3)	S1

Appendix 2.2 Analysis of variance for alternative weed host assessment of *Stemphylium vesicarium*.

Covariance Parameter	**Estimate**		**Standard Error**	
Rep	-0.04		0.048	
Experiment	-0.02		0.060	
Residual	1.30		0.298	
Source	**Num df**	**Den Df**	**F Value**	**Pr>F**
weed	6	116	1.57	0.16
sterile	1	116	5.42	**0.02**
weed*sterile	6	116	2.11	0.06
Effect of Sterilization by Species	**Num df**	**Den Df**	**F Value**	**Pr>F**
Onion	1	25	10.37	**0.003**
Bull thistle	1	7	0.08	0.79
Marshcress	1	21	11.31	**0.003**
Nutsedge	1	15	4.86	**0.04**
Pigweed	1	15	3.70	0.07
Purslane	1	7	0.10	0.76
Sowthistle	1	15	0.04	0.85

Appendix 2.3 Analysis of variance for alternative weed host assessment of *Stemphylium vesicarium* – sorted by surface sterilization.

Surface Sterilized tissue only

Covariance Parameter	**Estimate**	**Standard Error**
Rep	-0.01	0.168
Residual	1.67	0.498
Fixed Effect	**F value**	**Pr>F**
Species	0.39	0.88

Difference of least squared means compared to Onion (Dunnet)	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adj P**
Bull Thistle	0.17	0.78	55	0.21	0.83	1.00
Marshcress	0.76	0.86	55	0.88	0.38	0.91
Nutsedge	-0.09	0.73	55	-0.13	0.90	1.00
Pigweed	-0.54	0.81	55	-0.67	0.50	0.97
Purslane	0.39	0.74	55	0.52	0.61	0.99
Sowthistle	0.09	0.71	55	0.12	0.90	1.00

Non-Sterilized tissue only.

Covariance Parameter	**Estimate**	**Standard Error**
Rep	-0.01	0.137
Residual	1.23	0.350
Fixed Effect	**F value**	**Pr>F**
Species	4.83	**0.0003**

Difference of least squared means compared to Onion (Dunnet)	**Estimate**	**Standard Error**	**DF**	**t Value**	**Pr > |t|**	**Adj P**
Bull Thistle	-1.68	0.68	81	-2.45	0.02	0.09
Marshcress	-3.69	0.85	81	-4.36	<0.0001	**0.0002**
Nutsedge	-0.46	0.50	81	-0.93	0.36	0.91
Pigweed	-1.43	0.56	81	-2.56	0.01	0.07
Purslane	-1.48	0.65	81	-2.27	0.03	0.13
Sowthistle	-1.82	0.61	81	-3	0.004	**0.02**

Appendix 2.4 Analysis of variance for production *Stemphylium vesicarium* colonies by onion seeds naturally inoculated and collected in the Holland Marsh, ON.

Covariance Parameter	**Estimate**		**Standard Error**	
Rep	0.006		0.0185	
Residual	190.99		77.950	
Source	**Num df**	**Den Df**	**F Value**	**Pr>F**
time collected (early/end season)	1	12	0.28	0.61
sterile (yes/no)	1	12	0.18	0.68
time*sterile	1	12	0.23	0.64

Appendix 2.5 Analysis of variance for germination of onion seeds naturally inoculated with *Stemphylium vesicarium*, collected in the Holland Marsh, ON.

Covariance Parameter	**Estimate**		**Standard Error**	
Rep	-0.009		0.008	
Residual	3.70		0.531	
Source	**Num df**	**Den Df**	**F Value**	**Pr>F**
time collected (early/end season)	1	156	4.46	**0.04**
sterile (yes/no)	1	156	8.07	**0.01**
time*sterile	1	156	1.04	0.31
Infected with SV (yes/no)	1	156	0.37	0.55
time*infect	1	156	8.68	**0.004**
sterile*infect	1	156	1.37	0.24
time*sterile*infect	1	156	0.25	0.62

Appendix 2.6 Analysis of variance for fungal infestation of onion seeds collected in the Holland Marsh, ON after heat treatments.

Covariance Parameter	**Estimate**		**Standard Error**	
Rep	0.12		0.173	
Residual	9.86		3.137	
Source	**Num df**	**Den Df**	**F Value**	**Pr>F**
time in water bath (10, 20, 30, or 40 min)	3	24	1.61	0.21
temperature of water bath (40, 50, or 60 °C)	2	24	36.39	**<0.0001**
time*temperature	6	24	0.33	0.92

Appendix 2.7 Analysis of variance for germination on Petri dishes of onion seeds collected in the Holland Marsh, ON after heat treatments.

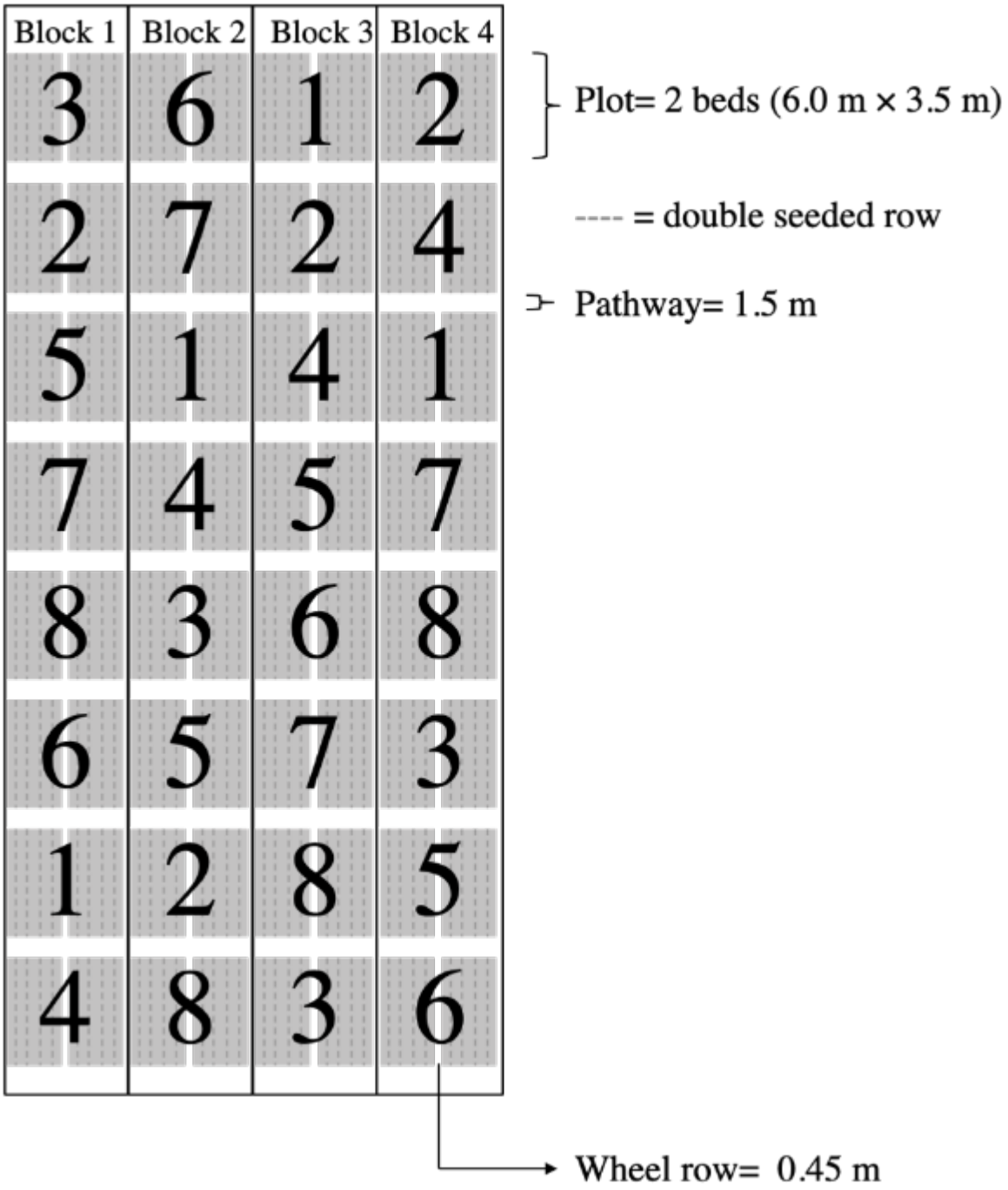
Covariance Parameter	**Estimate**		**Standard Error**	
Rep	0.23		0.261	
Residual	10.43		3.300	
Source	**Num df**	**Den Df**	**F Value**	**Pr>F**
time in water bath (10, 20, 30, or 40 min)	3	24	3.51	**0.03**
temperature of water bath (40, 50, or 60 °C)	2	24	2.62	0.09
time*temperature	6	24	3.71	**0.009**

Appendix 2.8 Analysis of variance for emergence of onion seedlings in a controlled environment after heat treatments.

Covariance Parameter	**Estimate**		**Standard Error**	
Rep	0.004		0.043	
Residual	343.26		99.329	
Source	**Num df**	**Den Df**	**F Value**	**Pr>F**
time in water bath (10, 20, 30, or 40 min)	3	24	40.34	**<0.0001**
temperature of water bath (40, 50, or 60 °C)	2	24	43.60	**<0.0001**
time*temperature	6	24	20.91	**<0.0001**

Appendices for Chapter 3

Appendix 3.1 Field diagram and randomized complete block design for Stemphylium leaf blight fungicide timing field trials.



Appendix 3.2 Restricted maximum likelihood covariate parameter estimates for Stemphylium leaf blight (SLB) in fungicide timing trails in 2018

SLB incidence, 14 Aug 2018.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		-0.03	0.017	
Residual		28.70	10.860	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	14	3.97	0.01
SLB disease severity, 14 Aug 2018.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.005	0.014	
Residual		45.21	14.262	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	6.54	0.0004
SLB disease severity in the destructive sampling, 27 Aug 2018.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.01	0.316	
Residual		21.91	29.366	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	24	4.77	0.002
SLB sAUDPC in 2018				
Covariance parameter		**Estimate**	**Standard Error**	
Block		3.81	3.29	
Residual		1.66	0.51	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	31.23	<0.0001

Appendix 3.3 Restricted maximum likelihood covariate parameter estimates for Stemphylium leaf blight (SLB) in fungicide timing trails in 2018

SLB incidence, 15 Aug 2019.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.29	0.288	
Residual		14.25	4.698	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	2.10	0.09
SLB disease severity, 15 Aug 2019.				
Covariance parameter		**Subject**	**Estimate**	**Standard Error**
Block			0.0008	0.010
Residual (VC)		trt*azoxy	0.07	0.060
Residual (VC)		trt*azoxySpray	0.04	0.037
Residual (VC)		trt*BSPcast	0.01	0.010
Residual (VC)		trt*control	0.04	0.040
Residual (VC)		trt*PenSpray	0.03	0.025
Residual (VC)		trt*Penflufen	0.07	0.038
Residual (VC)		trt*TOMcast	0.05	0.044
Residual (VC)		trt*Weekly2	0.002	0.003
Scale			1.01	.
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	4.05	0.006
SLB disease severity in the destructive sampling, 22 Aug 2019.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.00005	0.013	
Residual		53.60	16.89	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	1.90	0.12
SLB sAUDPC in 2019.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		-0.49	1.45	
Residual		16.61	5.12	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	1.60	0.19

Appendix 3.4 Restricted maximum likelihood covariate parameter estimates for onion yield in 2018.

Yield (t ha^{-1}) in 2018.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		-4.47	8.765	
Residual		110.76	4.183	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	0.87	0.55
Yield (bulb m^{-1}) in 2018.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		-0.72	0.411	
Residual		8.29	2.558	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	4.13	0.005
Bulb weight (g) in 2018.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		11.97	38.86	
Residual		270.92	83.61	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	2.15	0.08
% cull				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.02	0.041	
Residual		48.74	15.325	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	1.30	0.30
% marketable				
Covariance parameter		**Subject**	**Estimate**	**Standard Error**
Block		Treatment*block	0.20	0.058
Scale			9593776	.
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	24	1.68	0.16
% jumbo				
Covariance parameter		**Estimate**	**Standard Error**	
Block		-0.02	0.084	
Residual		12.36	4.109	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	1.55	0.21

Appendix 3.5 Restricted maximum likelihood covariate parameter estimates for onion yield in 2019.

Yield (t ha^{-1}) in 2019				
Covariance parameter		**Estimate**	**Standard Error**	
Block		10.77	16.971	
Residual		77.49	23.914	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	0.18	0.99
Yield (bulb m^{-1}) in 2019.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		1.51	0.496	
Residual		1.51	0.496	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	1.61	0.19
Bulb weight (g) in 2019.				
Covariance parameter		**Estimate**	**Standard Error**	
Block		14.69	33.956	
Residual		205.97	63.565	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	2.77	0.03
% cull				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.248	0.252	
Residual		135.67	42.075	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	3.00	0.02
% marketable				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.02	0.049	
Residual		27.48	8.751	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Treatment	7	21	1.44	0.244
% jumbo				
Covariance parameter		**Estimate**	**Standard Error**	
Block		0.004	0.052	
Residual		22.09	7.279	
Effect	Num DF	Den DF	F value	Pr>F
Treatment	7	20	1.39	0.26

Appendix 3.6 Restricted maximum likelihood covariate parameter estimates for end of season in-field DSI assessments for treatments in both 2018 and 2019.

Covariance parameter		**Estimate**	**Standard Error**	
Block		-0.003	0.002	
Residual		60.21	15.040	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Year	1	33	56.41	<0.0001
Treatment	5	33	11.36	<0.0001
Year * Treatment	5	33	3.10	0.02

Restricted maximum likelihood covariate parameter estimates for end of season in-field SLB incidence assessments for treatments in both 2018 and 2019.

Covariance parameter		**Estimate**	**Standard Error**	
Block		0.06413	0.08075	
Residual		0.06413	0.08075	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Year	5	30	7.33	0.0001
Treatment	1	6	0.76	0.4174
Year * Treatment	5	30	1.68	0.1689

Appendices for Chapter 4

Appendix 4.1 Fungicide-amended media recipes.

	Dilution 1 (D1)	Dilution 2 (D2)	Dilution 3 (D3)	Dilution 4 (D4)	Dilution 5 (D5)	Dilution 6 (D6)
Azoxystrobin 100,000 µg mL^{-1} [1]Stock: 0.1 g a.i. 1 mL acetone 100 mg mL^{-1} [1]SHAM: 0.3 g SHAM 3 mL Methanol **Azoxystrobin-amended media**	10,000 µg mL^{-1} 1 mL stock 9 mL water =10% solvent 5 mL D1 0.25 mL SHAM 294.75 mL PDA **200 µg mL^{-1} a.i.** 0.3% solvent	5,000 µg mL^{-1} 5 mL D1 0.5 mL acetone 4.5 mL water =10% solvent 6 mL D2 0.3 mL SHAM 293.7 mL PDA **100 µg mL^{-1} a.i.** 0.3% solvent	2500 µg mL^{-1} 3.5 mL D2 0.35 mL acetone 3.15 mL water =10% solvent 6 mL D3 0.3 mL SHAM 293.7 mL PDA **50 µg mL^{-1} a.i.** 0.3% solvent	250 µg mL^{-1} 0.7 mL D3 0.63 mL acetone 5.67 mL water =10% solvent 6 mL D4 0.3 mL SHAM 293.7 mL PDA **5 µg mL^{-1} a.i.** 0.3% solvent	25 µg mL^{-1} 0.7 mL D4 0.63 mL acetone 5.67 mL water =10% solvent 6 mL D5 0.3 mL SHAM 293.7 mL PDA **0.5 µg mL^{-1} a.i.** 0.3% solvent	2.5 µg mL^{-1} 0.7 mL D5 0.63 mL acetone 5.67 mL water =10% solvent 6 mL D6 0.3 mL SHAM 293.7 mL PDA **0.05 µg mL^{-1} a.i.** 0.3% solvent
Pyrimethanil 100,000 µg mL^{-1} [1]Stock: 0.25 g a.i. 2.5 mL acetone **Pyrimethanil-amended media**	10,000 µg mL^{-1} 1 mL stock 4 ml acetone 9 mL water =46% solvent 6 mL D1 294 mL PDA **200 µg mL^{-1} a.i.** 0.9% solvent*	5,000 µg mL^{-1} 5 mL D1 5 mL water =23% solvent 6 mL D2 294 mL PDA **100 µg mL^{-1} a.i.** 0.5% solvent	2500 µg mL^{-1} 3.5 mL D2 3.5 mL water =11.5% solvent 6 mL D3 294 mL PDA **50 µg mL^{-1} a.i.** 0.2% solvent	250 µg mL^{-1} 0.7 mL D3 0.61 mL acetone 5.69 mL water =10% solvent 6 mL D4 294 mL PDA **5 µg mL^{-1} a.i.** 0.2% solvent	25 µg mL^{-1} 0.7 mL D4 0.61 mL acetone 5.69 mL water =10% solvent 6 mL D5 294 mL PDA **0.5 µg mL^{-1} a.i.** 0.2% solvent	2.5 µg mL^{-1} 0.7 mL D5 0.61 mL acetone 5.69 mL water =10% solvent 6 mL D5 294 mL PDA **0.05 µg mL^{-1} a.i.** 0.2% solvent
Difenoconazole 100,000 µg mL^{-1} [1]Stock: 0.25 g a.i. 2.5 mL methanol **Difenoconazole-amended media**	10,000 µg mL^{-1} 2.5 mL stock 22.5 mL water =10% solvent 22.5 mL D1 202.5 mL PDA **1000 µg mL^{-1} a.i.** 1% solvent	1,000 µg mL^{-1} 2.5 mL stock 22.5 mL water =10% solvent 22.5 mL D2 202.5 mL PDA **100 µg mL^{-1} a.i.** 1% solvent	100 µg mL^{-1} 2.5 mL stock 22.5 mL water =10% solvent 22.5 mL D3 202.5 mL PDA **10 µg mL^{-1} a.i.** 1% solvent	10 µg mL^{-1} 2.5 mL stock 22.5 mL water =10% solvent 25 mL D4 225 mL PDA **1 µg mL^{-1} a.i.** 1% solvent		
Fluopyram 500 µg mL^{-1}Stock: 0.05 g a.i. 50 mL acetone 50 mL PDA **Fluopyram-amended media**	 90 mL Stock 360 mL PDA **100 µg mL^{-1} a.i.** 10% solvent*	 10 mL Stock 441 mL PDA **10 µg mL^{-1} a.i.** 1% solvent*	9.2 µg mL^{-1} 45 mL of 10 µg mL^{-1} a.i. media 4 mL acetone =10% solvent 49 mL D3 401 mL PDA **1 µg mL^{-1} a.i.** 1% solvent*	0.9 µg mL^{-1} 45 mL of 10 µg mL^{-1} a.i. media 4 mL acetone =10% solvent 49 mL D4 401 mL PDA 0.1 µg mL^{-1} a.i. 1% solvent*	0.1 µg mL^{-1} 45 mL of 10 µg mL^{-1} a.i. media 4 mL acetone =10% solvent 49 mL D5 401 mL PDA 0.01 µg mL^{-1} a.i. 1% solvent*	

*Pyrimethanil and fluopyram would not dissolve in lower solvent concentrations and precipitated out of solution at 1% solvent.

Appendix 4.2 Geographical origin, host plant and year collected of isolates of Stemphylium vesicarium from Ontario tested for sensitivity to the fungicides azoxystrobin and pyrimethanil. Phenotypes were classified as sensitive (S, inhibition at 5 µg a.i./mL), insensitive (Insen, no inhibition at 5 µg), or highly insensitive (Insen+, no inhibition at 100 µg a.i./mL).

Isolate	Location	Year	Response to:			
			Azoxy-strobin	Difeno-conazole[1]	Fluo-pyram	Pyrimeth-anil
Oat						
225105	Saskatchewan	1995	-	S	S / Insen	Insen
225106	Saskatchewan	1995	S	S	S	S / Insen
Onion						
OO69	Holland Marsh, MCRS	2016	Insen+	-		Insen
EX01	Exeter	2018	Insen	S	S	S / Insen
EX04	Exeter	2018	-	S		-
EX05	Exeter	2018	-	S		-
EX06	Exeter	2018	-	S	S	-
Onion1	Holland Marsh, Jane Street	2018	Insen+	-		-
Onion2	Holland Marsh, Jane Street	2018	Insen	-		S
HP01	Holland Marsh, Hillside Pilli's	2018	Insen+	-	S	S / Insen
SS0006	Holland Marsh, Farjani Field	2018	Insen+	-		S / Insen
AE02	Holland Marsh, 5 Acre Emma	2018	Insen	-	S	S
KK01	Keswick	2018	Insen+	-	S	S / Insen
FA01	Grand Bend, Field A	2018	Insen	-	S	S / Insen
FA04	Grand Bend, Field A	2018	-	S	S	-
FA05	Grand Bend, Field A	2018	Insen+	S	S	-
FC01	Grand Bend, Field C	2018	-	S		-
FC02	Grand Bend, Field C	2018	-	S	S	-
FC04	Grand Bend, Field C	2018	Insen	S	S	Insen
FC05	Grand Bend, Field C	2018	-	S		-
FC07	Grand Bend, Field C	2018	Insen+	-	S	S / Insen
FD01	Grand Bend, Field D	2018	-	S	S	-
FD02	Grand Bend, Field D	2018	-	S	S	-
FD03	Grand Bend, Field D	2018	-	S		-
FD04	Grand Bend, Field D	2018	-	S	Insen	-
FE01	Grand Bend, Field E	2018	-	S		-
FE02	Grand Bend, Field E	2018	-	S		-
FE03	Grand Bend, Field E	2018	-	S		-
FE04	Grand Bend, Field E	2018	Insen+	-	S	Insen
EO1	Exeter	2019	-	S	S / Insen	-
EO3	Exeter	2019	Insen+	S	S	Insen
EO4	Exeter	2019	Insen	S	S	S / Insen
EO5	Exeter	2019	Insen	S	S	S / Insen
EO6	Exeter	2019	Insen+	S	S / Insen	S / Insen
EO7	Exeter	2019	Insen	S	S	Insen
EO8	Exeter	2019	-	S	Insen	-
EO9	Exeter	2019	-	S	Insen	-

EO11	Exeter	2019	-	S		-
EO12	Exeter	2019	-	S		-
EO13	Exeter	2019	-	S		-
EO14	Exeter	2019	-	S	S	-
EO15	Exeter	2019	-	S	S	-
GB4.4	Grand Bend, Field 4	2019	-	S	S	-
GB4.6	Grand Bend, Field 4	2019	Insen+	S		S / Insen
GB4.7	Grand Bend, Field 4	2019	-	S	S	-
GB4.8	Grand Bend, Field 4	2019	Insen	S	S	Insen
GB4.9	Grand Bend, Field 4	2019	-	S	S	-
GB4.10	Grand Bend, Field 4	2019	-	-	S / Insen	Insen
GB4.11	Grand Bend, Field 4	2019	-	S	Insen	Insen
GB4.12	Grand Bend, Field 4	2019	-	S		-
GB6.1	Grand Bend, Field 6	2019	-	S	S / Insen	-
GB6.2	Grand Bend, Field 6	2019	-	-	Insen	-
GB6.3	Grand Bend, Field 6	2019	-	S	S	-
GB6.4	Grand Bend, Field 6	2019	Insen	S	S	S / Insen
GB6.5	Grand Bend, Field 6	2019	Insen	S	Insen	S / Insen
GB6.6	Grand Bend, Field 6	2019	Insen	S	Insen	Insen
GB6.7	Grand Bend, Field 6	2019	-	-	S	-
GB6.8	Grand Bend, Field 6	2019	Insen+	S	S / Insen	Insen
GB6.9	Grand Bend, Field 6	2019	-	S	S / Insen	-
GB6.10	Grand Bend, Field 6	2019	-	S	S / Insen	-
GB6.11	Grand Bend, Field 6	2019	-	S		-
GB6.12	Grand Bend, Field 6	2019	-	S		-
GB6.14	Grand Bend, Field 6	2019	-	S		-
GB6.16	Grand Bend, Field 6	2019	-	S	S	-
GB6.17	Grand Bend, Field 6	2019	-	S	S	-
GB7.2	Grand Bend, Field 7	2019	-	S	S	-
GB7.3	Grand Bend, Field 7	2019	Insen	S	Insen	S / Insen
GB7.4	Grand Bend, Field 7	2019	Insen+	S	Insen	S / Insen
GB7.5	Grand Bend, Field 7	2019	-	S		-
GB7.7	Grand Bend, Field 7	2019	Insen+	S	Insen	S / Insen
GB7.8	Grand Bend, Field 7	2019	-	S	S	-
GB7.9	Grand Bend, Field 7	2019	-	S	S	-
GB7.11	Grand Bend, Field 7	2019	-	S	S	Insen
GB7.12	Grand Bend, Field 7	2019	-	S	S / Insen	-
GB7.13	Grand Bend, Field 7	2019	-	S		-
GB7.14	Grand Bend, Field 7	2019	-	S		-
GB7.15	Grand Bend, Field 7	2019	-	S		-
GB7.18	Grand Bend, Field 7	2019	-	Insen		-
GB7.19	Grand Bend, Field 7	2019	-	S		-
GB7.20	Grand Bend, Field 7	2019	-	S		-
GB7.21	Grand Bend, Field 7	2019	-	S		-
GB8.1	Grand Bend, Field 8	2019	-	S	Insen	-
GB8.2	Grand Bend, Field 8	2019	-	S	Insen	-

GB8.3	Grand Bend, Field 8	2019	-	S	S	-
GB8.4	Grand Bend, Field 8	2019	-	S		-
GB8.5	Grand Bend, Field 8	2019	-	S		-
KG01	Holland Marsh, Strawberry Lane	2019	-	S	S	S
KG02	Holland Marsh, Strawberry Lane	2019	Insen	S	S / Insen	-
KG03	Holland Marsh, Strawberry Lane	2019	Insen+	S	S	S / Insen
KG04	Holland Marsh, Strawberry Lane	2019	-	S		-
KG05	Holland Marsh, Strawberry Lane	2019	-	S	S / Insen	-
KG06	Holland Marsh, Strawberry Lane	2019	-	S	S / Insen	-
KG07	Holland Marsh, Strawberry Lane	2019	-	S	S / Insen	-
KG08	Holland Marsh, Strawberry Lane	2019	Insen	S	S	S / Insen
KG09	Holland Marsh, Strawberry Lane	2019	-	S	S / Insen	Insen
KG10	Holland Marsh, Strawberry Lane	2019	-	S	S	-
KG11	Holland Marsh, Strawberry Lane	2019	-	S	Insen	-
KG12	Holland Marsh, Strawberry Lane	2019	-	S	Insen	-
KG14	Holland Marsh, Strawberry Lane	2019	-	S	S	-
KG15	Holland Marsh, Strawberry Lane	2019	-	S	S	-
KG16	Holland Marsh, Strawberry Lane	2019	-	S	S	-
KG17	Holland Marsh, Strawberry Lane	2019	-	S	S	-
KG18	Holland Marsh, Strawberry Lane	2019	-	S	S	-
MS01	Holland Marsh, MCRS	2019	-	S	Insen	-
MS02	Holland Marsh, MCRS	2019	-	S		-
MS05	Holland Marsh, MCRS	2019	-	S		-
MS06	Holland Marsh, MCRS	2019	-	S	S	-
MS11	Holland Marsh, MCRS	2019	-	S	S	-
MS12	Holland Marsh, MCRS	2019	Insen / S	S	S	Insen
MS13	Holland Marsh, MCRS	2019	Insen	S	S	S / Insen
MS14	Holland Marsh, MCRS	2019	Insen	S	S	S / Insen
MS15	Holland Marsh, MCRS	2019	Insen	-	S	S / Insen
MS16	Holland Marsh, MCRS	2019	Insen	-	S	Insen
OV04	Holland Marsh, MCRS	2019	-	S		-
OV10	Holland Marsh, MCRS	2019	-	-	S	-
OV11	Holland Marsh, MCRS	2019	Insen	S	S	Insen
Z01	Holland Marsh, Keel Street	2019	-	S		-
Z02	Holland Marsh, Keel Street	2019	Insen	S		-
z03	Holland Marsh, Keel Street	2019	Insen	S	S	S / Insen
Z04	Holland Marsh, Keel Street	2019	-	S		-
Z05	Holland Marsh, Keel Street	2019	Insen+	S	S	S / Insen
Z06	Holland Marsh, Keel Street	2019	Insen	S	Insen	-
Z11	Holland Marsh, Keel Street	2019	-	S	S / Insen	Insen
Z12	Holland Marsh, Keel Street	2019	-	S	S	-
Garlic						
GED1	Exeter, Dashwood Rd	2019	Insen	S	Insen	S / Insen
GED2	Exeter, Dashwood Rd	2019	-	S	S	-
GED3	Exeter, Dashwood Rd	2019	Insen+	S	S / Insen	S / Insen
GED4	Exeter, Dashwood Rd	2019	Insen+	S	S / Insen	Insen

GED6	Exeter, Dashwood Rd	2019	-	S	S	-
GED7	Exeter, Dashwood Rd	2019	-	-	S / Insen	-
GED9	Exeter, Dashwood Rd	2019	Insen+	S	S / Insen	-
GED10	Exeter, Dashwood Rd	2019	-	S		-
GED11	Exeter, Dashwood Rd	2019	Insen	-	S	Insen
GED18	Exeter, Dashwood Rd	2019	-	S	S	-
Leek						
Leek1	Holland Marsh, Jane Street	2018	Insen+	-		Insen
leek2	Holland Marsh, Jane Street	2018	Insen	-		S / Insen
leek3	Holland Marsh, Jane Street	2018	Insen	-		S / Insen
Asparagus						
OA03	Hemlock, Norfolk	2012	S / Insen	-	S	S / Insen
OA46	Gilbertville, Norfolk	2013	S	-		-
OA48	Harrow, Essex	2014	S	-		-
	S		3	114	57	20
	Insen		27	1	17	3
	Insen+		20			
	Insen / S		1	0	0	0
	S / Insen		1	0	19	28

- not tested.

[1]Difenoconaole information is for mycelial growth only.

[2]Where two classifications are presented, the first classification is for conidial germination and the second is for mycelial growth.

Appendix 4.3 Example of the Probit procedure for EC_{50} of *Stemphylium vesicarium.*

Probit Analysis on conc (isolate 225106, active ingredient= pyrimethanil)

Probability	EC_{50} (µg a.i. mL^{-1})	95% Fiducial Limits		
0.01	42571.0	898.57	9.29×10^{25}	
0.02	19034.0	562.93	2.53×10^{23}	
0.03	11422.0	416.47	6.01×10^{21}	
0.04	7779.0	331.00	3.61×10^{20}	
0.05	5691.0	273.96	3.68×10^{19}	
0.06	4362.0	232.78	5.27×10^{18}	
0.07	3454.0	201.46	9.60×10^{17}	
0.08	2803.0	176.75	2.10×10^{17}	
0.09	2319.0	156.70	5.26×10^{16}	
0.10	1947.0	140.07	1.47×10^{16}	
0.15	944.0	86.59	7.74×10^{13}	
0.20	531.1	57.50	1.23×10^{12}	
0.25	324.2	39.27	3.61×10^{10}	
0.30	208.1	26.90	1.58×10^{9}	
0.35	138.0	18.06	9.10×10^{7}	
0.40	93.5	11.59	6.48×10^{6}	
0.45	64.1	6.85	5.55× 105	
0.50	**44.2**	**3.52**	**5.72×10^{4}**	**EC_{50}: effective concentration to inhibit growth by 50%**
0.55	30.5	1.43	7467.00	
0.60	20.9	0.41	1324.00	
0.65	14.2	0.07	337.81	
0.70	9.4	0.01	120.66	
0.75	6.0	0.001	54.88	
0.80	3.7	2.00×10^{-5}	28.75	
0.85	2.1	3.791×10^{-7}	15.97	
0.90	1.0	2.26×10^{-9}	8.70	
0.91	0.8	6.47×10^{-10}	7.62	
0.92	0.7	1.66×10^{-10}	6.62	
0.93	0.6	3.68×10^{-11}	5.70	
0.94	0.4	6.84×10^{-12}	4.84	
0.95	0.3	9.98×10^{-13}	4.04	
0.96	0.3	1.03×10^{-13}	3.28	
0.97	0.2	6.33×10^{-15}	2.56	
0.98	0.1	1.53×10^{-16}	1.86	
0.99	0.05	4.27×10^{-19}	1.14	

Appendix 4.4 Mean mycelial growth of *Stemphylium vesicarium* isolated characterized as sensitive or insensitive to four fungicide active ingredients.

Active ingredient	Class	Mean mycelial growth (mm h^{-1})	
Azoxystrobin	Insensitive	0.22	ns
	Sensitive	0.19	
Pyrimethanil	Insensitive	0.22	B[2]
	Sensitive	0.27	A
Fluopyram	Insensitive	0.24	B
	Sensitive	0.30	A
Difenoconazole	Insensitive	0.16	B
	Sensitive	0.35	A

[1]ns – not significant. Means do not differ based on Tukey's test at $P = 0.05$.
[2]Means in a column and active ingredient followed by the same letter do not differ based on Tukey's test at $P = 0.05$.

Appendix 4.5 Restricted maximum likelihood covariate parameter estimates for fitness (assessed as mycelial growth rate) for isolates sensitive and insensitive to azoxystrobin, pyrimethanil, difenoconazole, and fluopyram fungicides.

Azoxystrobin

Covariance parameter		**Estimate**	**Standard Error**	
Rep		9.53×10^{-8}	.	
Isolate		0.000022	6.20×10^{-6}	
Residual		0.000033	3.57×10^{-6}	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Sensitivity classification	1	167	2.82	0.09

Pyrimethanil

Covariance parameter		**Estimate**	**Standard Error**	
Rep		-3.59×10^{-7}	.	
Isolate		0.00004	0.00001	
Residual		0.00004	5.04×10^{-6}	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Sensitivity classification	1	129	10.35	0.002

Difenoconazole

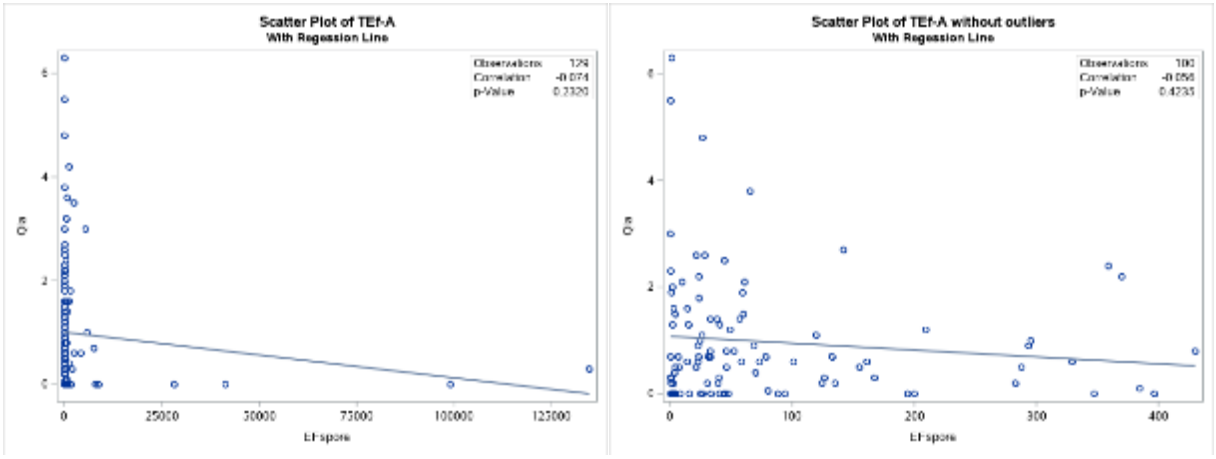
Covariance parameter		**Estimate**	**Standard Error**	
Rep	Isolate*rep	-0.00002	0.00004	
Residual		0.0003	0.00005	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Sensitivity classification	1	23	19.95	0.0002

Fluopyram

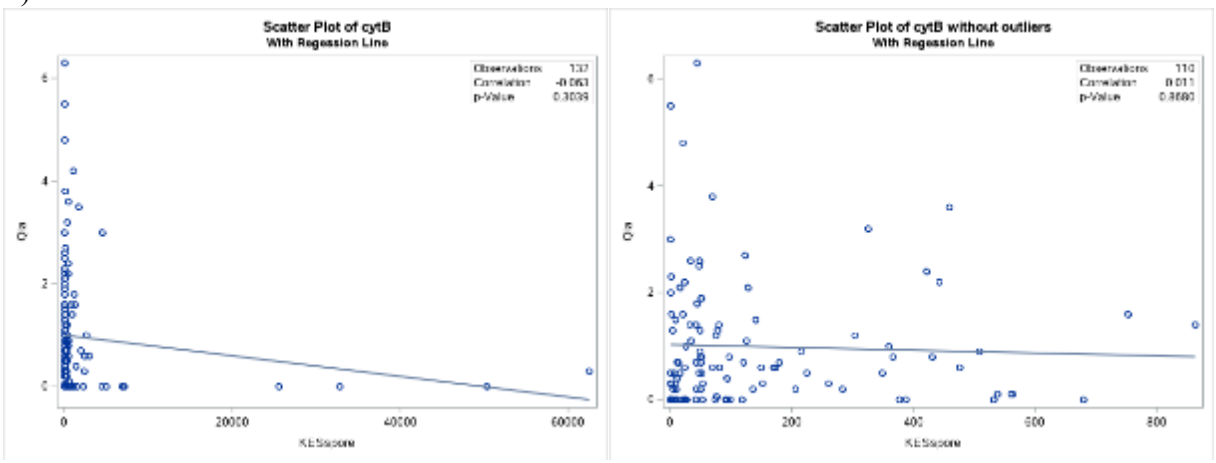
Covariance parameter		**Estimate**	**Standard Error**	
Rep		-0.00002	9.516×10^{-6}	
Isolate		0.004	0.0007	
Residual		0.003	0.0003	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Sensitivity classification	1	184	18.51	<0.0001

Appendixes for Chapter 5

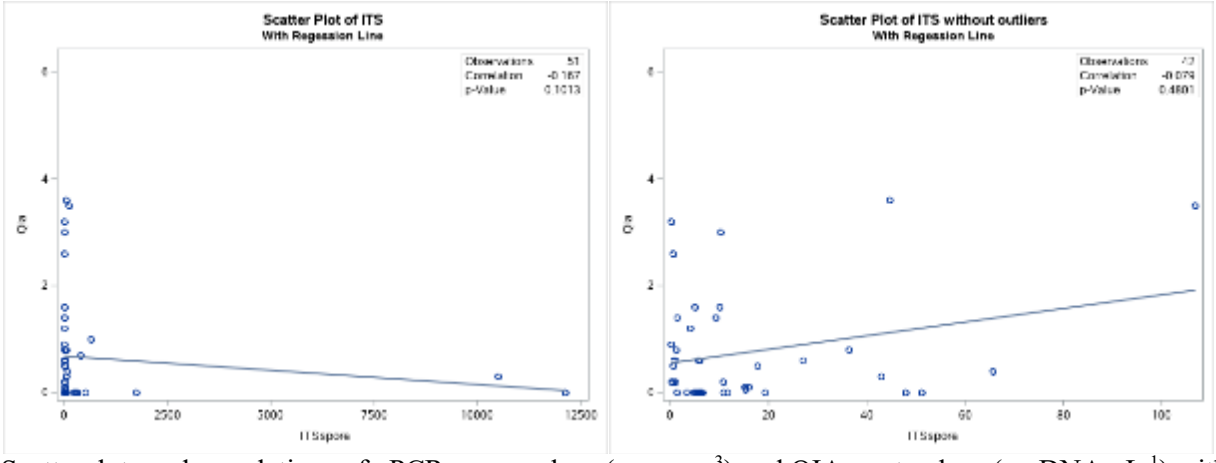
Appendix 5.1 Relationship between QIAxpert spectrofluorometry and qPCR spore estimates.
A)



B)



C)



Scatterplots and correlations of qPCR spore values (spore m^{-3}) and QIAxpert values (ng DNA μL^{-1}) with (left) and without (right) qPCR outliers based on 1.5 IQR using primers to amplify the A) EF-1 alpha (*TEf-A*=EF), B) cytochrome *b* (*cytB*=KES), and C) internal transcribed spacer (ITS) genes of *Stemphylium vesicarium.*

Appendix 5.2 Normality of qPCR with three primer sets (*TEf-A*, *cytB*, and ITS) and QIAxpert data with and without extreme outliers.

	Tests for Normality				
	Test	Statistic		p Value	
QIAxpert	Shapiro-Wilk	W	0.78	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.21	Pr > D	<0.0100
TEF-A spores	Shapiro-Wilk	W	0.18	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.43	Pr > D	<0.0100
cytB spores	Shapiro-Wilk	W	0.22	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.41	Pr > D	<0.0100
ITS spores	Shapiro-Wilk	W	0.24	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.42	Pr > D	<0.0100
	Tests for Normality – Outliers removed from qPCR values				
TEF-A spores	Shapiro-Wilk	W	0.72	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.25	Pr > D	<0.0100
cytB spores	Shapiro-Wilk	W	0.73	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.25	Pr > D	<0.0100
ITS spores	Shapiro-Wilk	W	0.68	Pr < W	<0.0001
	Kolmogorov-Smirnov	D	0.25	Pr > D	<0.0100

Appendix 5.3 Statistical analysis of qPCR analysis among reactions and between primer sets

Tukey-Kramer grouping for run least squares means ($P = 0.05$) for the effect of run/reaction on spore quantity estimated by three primer sets amplifying different genes

	Gene Amplified					
Run	*TEf-A*		ITS		*cytB*	
2018.1	2826.0	A[1]	39.4	A	3329.6	A
2018.2	913.7	B	24.6	AB	2622.5	A
2019.1	569.3	B	17.7	AB	565.6	B
2019.2	9.8	B	1.5	B	423.0	B
2020.1	418.7	B	-		454.4	B
2020.2	82.4	B	13.2	AB	553.4	B

Restricted maximum likelihood covariate parameter estimates for qPCR quantity for three primers compared to reaction run

TEf-A

Covariance parameter		**Estimate**	**Standard Error**	
Technical Rep		-3.69×10^{4}	4.1303×10^{4}	
Residual		3.02×10^{11}	3.02×10^{11}	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
qPCR run	5	7	10.30	0.004

ITS

Covariance parameter		**Estimate**	**Standard Error**	
Technical Rep		2.86	24.711	
Residual		65.60	44.108	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
qPCR run	4	4	8.68	0.03

CytB

Covariance parameter		**Estimate**	**Standard Error**	
Technical Rep		-1.04×10^{14}	1.20×10^{4}	
Residual		1.13×10^{10}	1.13×10^{10}	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
qPCR run	5	10	44.80	<0.0001

Note: This only includes the 10^{-5} conidia ml^{-1} calibration sample.

Appendix 5.4 Effect of run on spore estimates by qPCR for three primers sorted by year.

Effect of run on spore estimates from *TEf-A* qPCR for three years

	2018		2019		2020	
Covariance estimates						
	Estimate	**SE**	**Estimate**	**SE**	**Estimate**	**SE**
Rep	-63747	410921	-5506	54628	202	5321
Residual	641370	641370	32681	65793	7316	7935
Fixed Effects						
Effect	**F value**	**Pr>F**	**F value**	**Pr>F**	**F value**	**Pr>F**
Run	8.6	0.10	9.2	0.12	14.1	0.17

Effect of run on spore estimates from ITS qPCR for three years

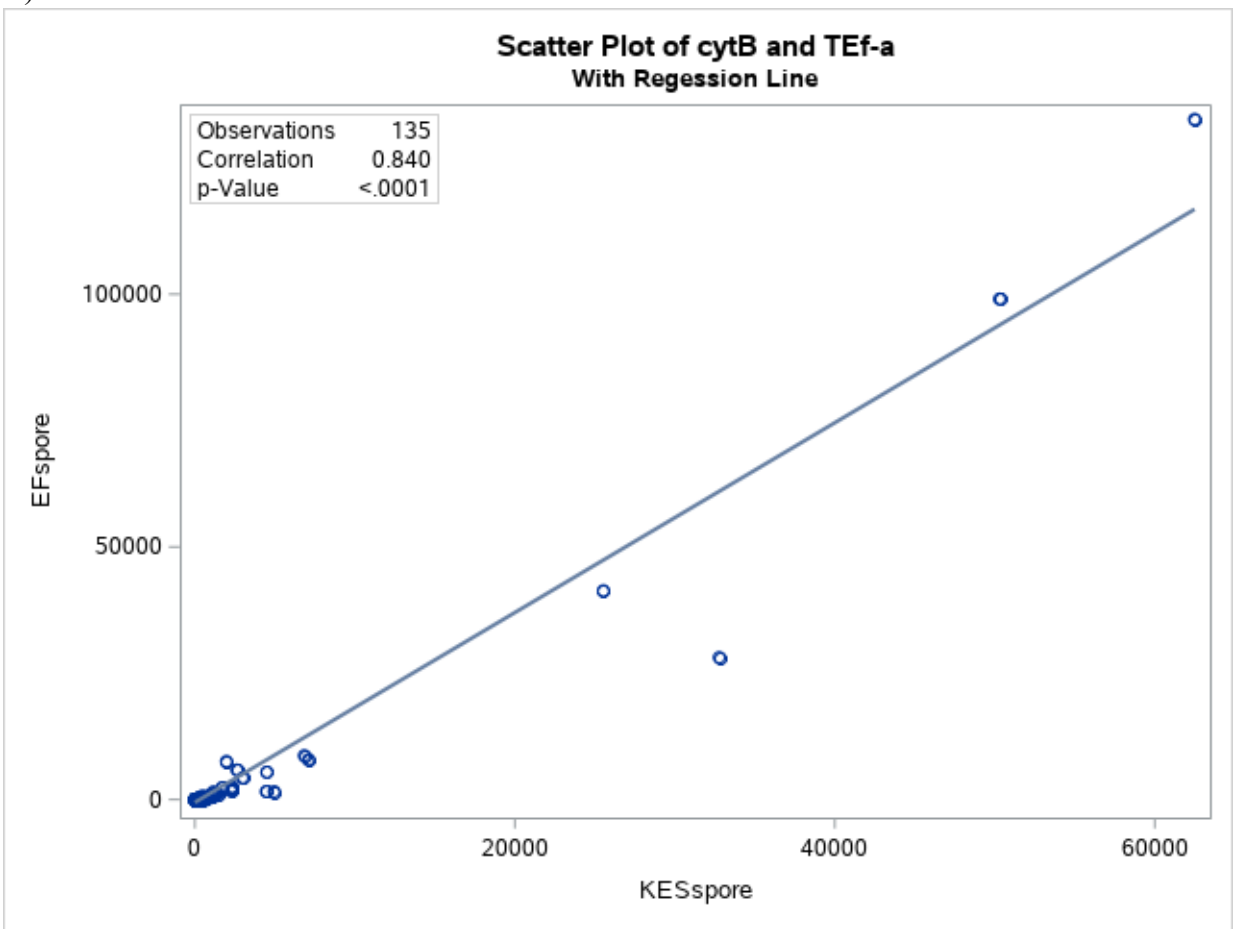
	2018		2019		2020	
Covariance estimates						
	Estimate	**SE**	**Estimate**	**SE**	**Estimate**	**SE**
Rep	6.1	7.00	-1.2	78.22	64.5	92.66
Residual	0.8	9.68	111.7	120.54	1.0	0.90
Fixed Effects						
Effect	**F value**	**Pr>F**	**F value**	**Pr>F**	**F value**	**Pr>F**
Run	104.1	0.99	2.9	0.34	7.3	0.43

Effect of run on spore estimates from *cytB* qPCR for three years

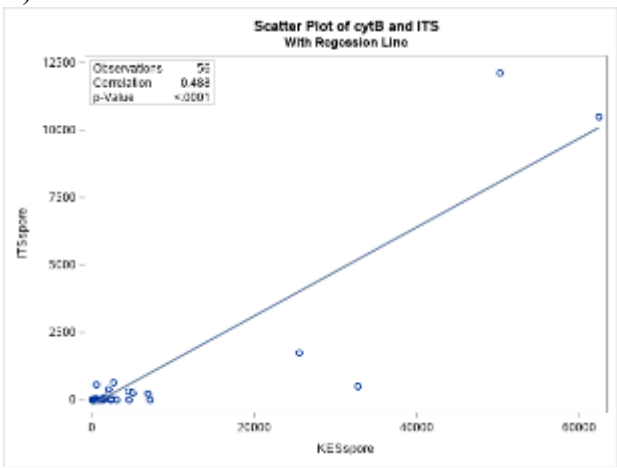
	2018		2019		2020	
Covariance estimates						
	Estimate	**SE**	**Estimate**	**SE**	**Estimate**	**SE**
Rep	-95075	221324	172	4503	-572	2621
Residual	393285	393285	6193	6194	4234	4234
Fixed Effects						
Effect	**F value**	**Pr>F**	**F value**	**Pr>F**	**F value**	**Pr>F**
Run	1.9	0.30	4.9	0.16	3.5	0.20

Appendix 5.5 Relationships between three primer sets amplifying different gene regions of *Stemphylium vesicarium* using a conidia suspension.

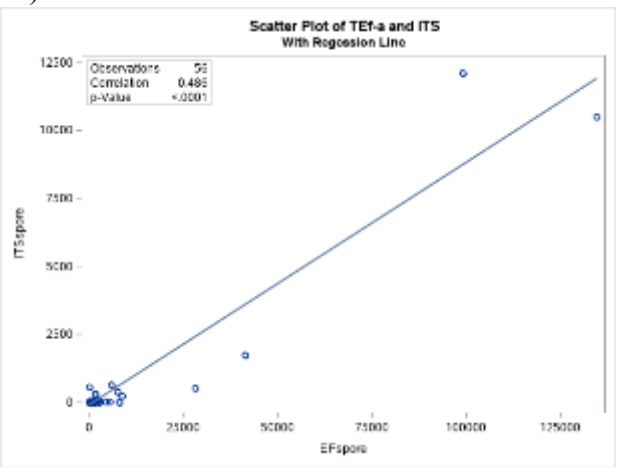
A)



B)

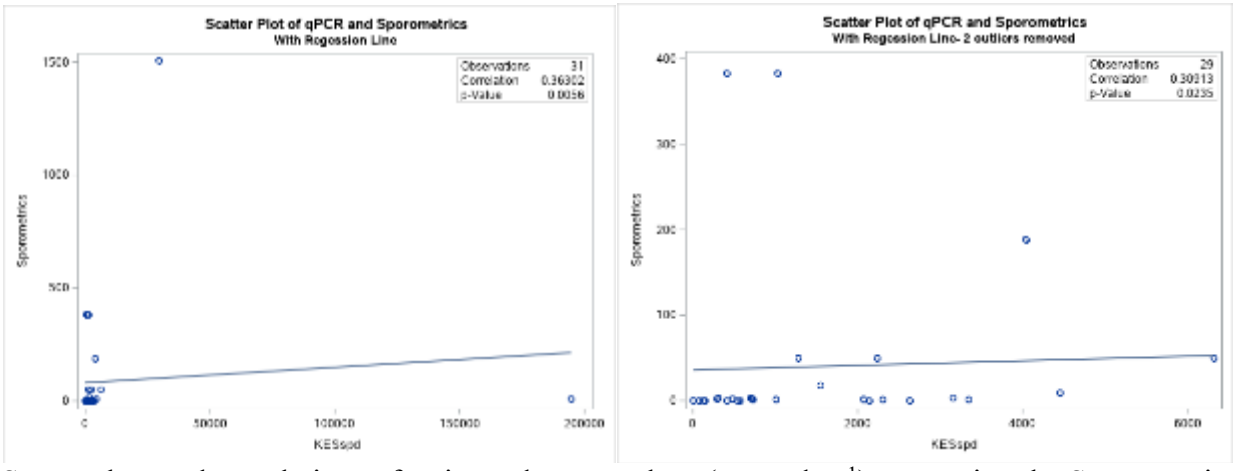


C)

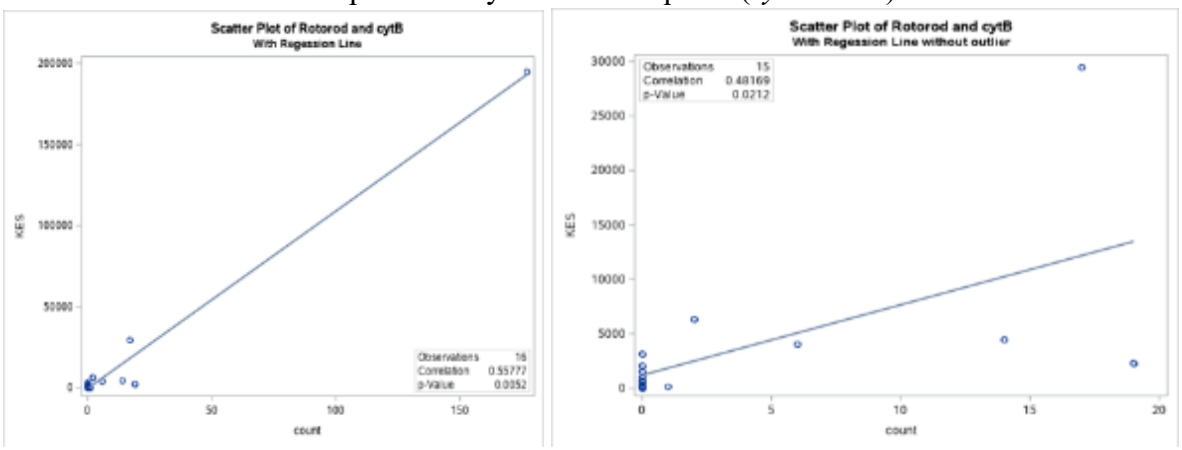


Scatterplots and correlations of qPCR spore values (spore m^{-3}) comparing primers used to amplify the EF-1 alpha (*TEf-A*=EF), cytochrome *b* (*cytB*=KES), and internal transcribed spacer (ITS) genes of *Stemphylium vesicarium.* A) *TEf-A* versus *cytB*, B) ITS versus *cytB*, C) ITS versus *TEf-A*. Note: This only includes the 10^{-5} conidia ml^{-1} calibration sample.

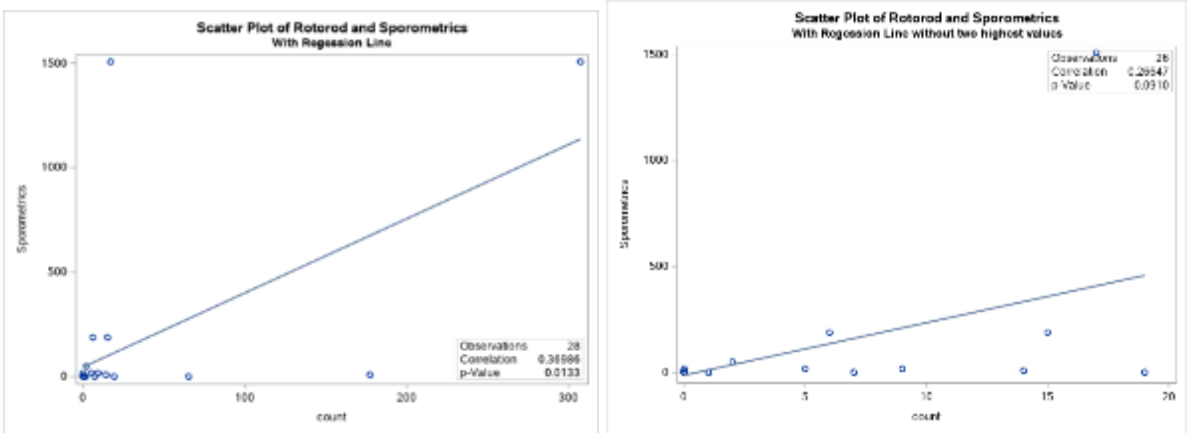
Appendix 5.6 Relationship between *cytB* qPCR, Sporometrics and Rotorod methods for estimating air-borne *Stemphylium vesicarium* spores.



Scatterplots and correlations of estimated spore values (spore day^{-1}) comparing the Sporometrics method to the Burkard sampler with cytochrome *b* qPCR (*cytB*=KES)



Scatterplots and correlations of estimated spore values (spore day^{-1}) comparing the Burkard sampler to cytochrome *b* qPCR (*cytB*=KES) with the Rotorod (count) method.



Scatterplots and correlations of estimated spore values (spore day^{-1}) comparing the Sporometrics method to the Rotorod (count) method.

Appendix 5.7 Results from the REG procedure for stepwise regression between *Stemphylium vesicarium* spore values and weather variables.

Model 3: Stepwise regression including quadratic weather variables

Number of observations used: 314

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F value	Pr>F
Model	14	313509	22393	23.68	<0.0001
Error	299	282757	946		
Corrected Total	313	596266			

Variable	Estimate	Standard Error	Type II SS	F Value	Pr>F
NightLWD	-6.26472	2.34	6802.0	7.2	0.01
AvRH	8.62	2.88	8451.2	8.9	0.003
AvWind	0.41	0.19	4431.2	4.7	0.03
AvSoilT	8.89	4.14	4365.6	4.6	0.03
DVPD	4.35	0.58	53348.0	56.4	<0.0001
Train	0.38	0.12	9863.9	10.4	0.001
Drought	0.06	0.01	34415.0	36.4	<0.0001
$NLWD^2$	0.34	0.12	8233.6	8.7	0.00
$AvRH^2$	-0.05	0.02	5528.4	5.9	0.02
Rad^2	0.00002	0.00	7392.3	7.8	0.01
$AvSoilT^2$	-0.24057	0.10	5477.1	5.8	0.02
LWD^2	0.08	0.02	12142.0	12.8	0.0004
$DLWD^2$	0.230	0.09	10335.0	10.9	0.001
$HumidLength^2$	-0.09	0.05	2738.0	2.9	0.09

Adjusted R^2 = 0.5036

Model 4: Stepwise regression without quadratic weather variables

Number of observations used: 314

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F value	Pr>F
Model	9	285596	31733	31.05	<0.0001
Error	304	310670	1021		
Corrected Total	313	596266			

Variable	Estimate	Standard Error	Type II SS	F Value	Pr>F
Intercept	-15.21	20.60	557.2	0.6	0.46
NightWetTemp	1.39	0.57	6174.5	6.0	0.01
Rad	0.03	0.01	5370.0	5.3	0.02
LWD	1.43	0.55	6902.8	6.8	0.01
VPD	-49.26	9.66	26569.0	26.0	<0.0001
DLWD	3.74	1.05	12979.0	12.7	0.0004
DVPD	4.36	0.58	57155.0	55.9	<0.0001
HumidLength	0.38	0.12	10607.0	10.4	0.001
Drought	-2.44	1.04	5652.6	5.5	0.02

Adjusted R^2 = 0.4635

Model 9: Stepwise regression including quadratic weather variables *NO LWD*

Number of observations used: 386

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F value	Pr>F
Model	9	360602	40067	40.64	<0.0001
Error	376	370691	986		
Corrected Total	385	731292			

Variable	Estimate	Standard Error	Type II SS	F Value	Pr>F
Intercept	-341.77	66.96	25680.0	26.1	<0.0001
AvRH	5.86	1.86	9736.4	9.9	0.002
Rad	0.03	0.01	12324.0	12.5	0.0005
AvSoilT	7.86	2.28	11728.0	11.9	0.0006
Dtemp	2.83	0.96	8642.4	8.8	0.003
DVPD	5.63	0.50	122761.0	124.5	<0.0001
Drought	0.07	0.01	41145.0	41.7	<0.0001
$AvRH^2$	-0.03	0.01	5706.4	5.8	0.02
$AvSoilT^2$	-0.24	0.06	17605.0	17.9	<0.0001
$Train^2$	0.00	0.00	5050.2	5.1	0.02

Adjusted $R^2 = 0.481$

Model 10: Stepwise regression without quadratic weather variables *NO LWD*

Number of observations used: 386

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F value	Pr>F
Model	8	346329	43291	42.40	<0.0001
Error	377	384963	1021		
Corrected Total	385	731292			

Variable	Estimate	Standard Error	Type II SS	F Value	Pr>F
Intercept	-149.53	24.03	39526.0	38.7	<0.0001
AvRH	1.56	0.28	32648.0	32.0	<0.0001
Rad	0.04	0.01	25227.0	24.7	<0.0001
AvSoilT	-3.18	0.87	13775.0	13.5	0.0003
Temp	2.07	0.85	6025.7	5.9	0.02
Dtemp	4.27	0.90	23005.0	22.5	<0.0001
DVPD	5.64	0.52	121572.0	119.1	<0.0001

Adjusted $R^2 = 0.4624$

Appendix 5.8 Results from the LOGISTIC procedure for cumulative log regression between *Stemphylium vesicarium* spore values and weather variables

Model 1: Stepwise regression including quadratic weather variables

Number of observations used: 428

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr>Chi Sq
Intercept	1	-2.17	0.93	5.5	0.02
AvSoilT	1	0.18	0.05	15.2	<0.0001
Dtemp	1	-0.22	0.09	5.2	0.02
DVPD	1	-0.23	0.04	34.5	<0.0001
Train	1	-0.05	0.03	3.7	0.06
VPD^2	1	-0.42	0.20	4.3	0.04
$DLWD^2$	1	0.03	0.01	17.8	<0.0001
$Train^2$	1	0.002	0.001	7.6	0.01

Association of Predicted Probabilities and Observed Responses

Percent Concordant	74.4	**Somers' D**	0.49
Percent Discordant	25.6	**Gamma**	0.49
Percent Tied	0	**Tau-a**	0.24
Pairs	44571	**c**	0.74

Model 2: Stepwise regression without quadratic weather variables

Number of observations used: 428

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr>Chi Sq
Intercept	1	-2.57	1.01	6.4	0.01
AvWind	1	-0.02	0.01	3.0	0.08
AvSoilT	1	0.19	0.05	16.0	<0.0001
VPD	1	-0.91	0.38	5.7	0.02
Dtemp	1	-0.23	0.10	5.2	0.02
DLWD	1	0.36	0.09	14.9	0.0001
DVPD	1	-0.22	0.04	31.6	<0.0001
Train	1	0.05	0.01	10.8	0.001
NRain	1	-0.28	0.16	3.3	0.07

Association of Predicted Probabilities and Observed Responses

Percent Concordant	73.6	**Somers' D**	0.47
Percent Discordant	26.4	**Gamma**	0.47
Percent Tied	0	**Tau-a**	0.23
Pairs	44571	**c**	0.74

Model 7: Stepwise regression including quadratic weather variables *NO LWD*

Number of observations used: 539

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr>Chi Sq
Intercept	1	-24.40	4.73	26.6	<0.0001
AvRH	1	0.51	0.13	15.9	<0.0001
AvWind	1	-0.02	0.01	4.2	0.04
Temp	1	0.30	0.05	41.9	<0.0001
$AvRH^2$	1	-0.003	0.001	11.5	0.001
Rad^2	1	0.000001	0.0000003	13.0	0.0003
$AvSoilT^2$	1	-0.01	0.001	32.0	<0.0001
$Train^2$	1	0.0003	0.0001	9.8	0.002

Association of Predicted Probabilities and Observed Responses

Percent Concordant	73.6	**Somers' D**	0.47
Percent Discordant	26.4	**Gamma**	0.47
Percent Tied	0	**Tau-a**	0.24
Pairs	72610	**c**	0.74

Model 8: Stepwise regression without quadratic weather variables *NO LWD*

Number of observations used: 539

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr>Chi Sq
Intercept	1	-8.21	1.56	27.8	<0.0001
AvRH	1	0.08	0.02	24.1	<0.0001
Rad	1	0.002	0.0005	14.7	0.0001
AvWind	1	-0.02	0.01	4.7	0.03
AvSoilT	1	-0.27	0.05	30.0	<0.0001
Temp	1	0.28	0.05	33.5	<0.0001
Dtemp	1	0.14	0.06	6.3	0.01
Train	1	0.02	0.01	8.6	0.003

Association of Predicted Probabilities and Observed Responses

Percent Concordant	71.3	**Somers' D**	0.43
Percent Discordant	28.7	**Gamma**	0.43
Percent Tied	0	**Tau-a**	0.21
Pairs	72610	**c**	0.71

Appendix 5.9 Results from the HPSLIT procedure for regression tree analysis of *Stemphylium vesicarium* spore values and weather variables

Model 5: Regression Tree including quadratic weather variables (n = 314)

Source	N leaves	ASE	RSS
Model based	4	1089.8	342183
Cross-validation	4	1357.0	

Variable	Relative	Importance	Count
DVPD	1.00	424.8	1
TRain	0.51	218.5	1
Drought^2	0.38	160.9	1

Mean Analysis Variable: Spores

Node #	N Obs	N	Mean	Std Dev	Min	Max
3	120	120	20.37	21.67	0	151
4	56	55	55.95	34.64	0	129
5	202	104	71.90	31.52	0	129
6	56	35	103.34	59.20	7	307

Model 6: Regression Tree without quadratic weather variables (n=314)

Source	N leaves	ASE	RSS
Model based	4	1089.8	342183
Cross-validation	4	1357.0	

Variable	Relative	Importance	Count
DVPD	1.00	424.8	1
TRain	0.51	218.5	1
Drought	0.38	160.9	1

Mean Analysis Variable: Spores

Node #	N Obs	N	Mean	Std Dev	Min	Max
3	120	120	20.37	21.67	0	151
4	56	55	55.95	34.64	0	129
5	202	104	71.90	31.52	0	129
6	56	35	103.34	59.20	7	307

Model 11: Regression Tree without quadratic weather variables *NO LWD* (n=386)

Source	N leaves	ASE	RSS
Model based	4	1144.6	441834
Cross-validation	4	1244.8	

Variable	Relative	Importance	Count
DVPD	1.00	441.1	1
TRain	0.55	242.3	1
Drought	0.43	190.3	1

Mean Analysis Variable: Spores

Node #	N Obs	N	Mean	Std Dev	Min	Max
3	120	120	20.37	21.67	0	151
4	56	55	55.95	34.64	0	129
5	202	104	71.90	31.52	0	129
6	56	35	103.34	59.20	7	307

Model 12: Regression Tree without quadratic weather variables *NO LWD* (n=386)

Source	N leaves	ASE	RSS
Model based	5	1063.5	410501
Cross-validation	5	1211.4	

Variable	Relative	Importance	Count
DVPD	1.00	441.1	1
Dtemp	0.55	242.3	1
TRain	0.43	190.3	1
Drought	0.4-	177.0	1

Mean Analysis Variable: Spores

Node #	N Obs	N	Mean	Std Dev	Min	Max
3	154	137	15.71	18.64	0	151
4	110	92	41.36	36.40	0	129
5	13	12	4.50	5.16	0	17
7	212	110	68.99	33.62	0	129
8	56	35	102.34	59.20	7	307

Appendix 5.10 Effect of drought (trt) and inoculation with *Stemphylium vesicarium* (inoc) for two repeated experiments (exp) under controlled environmental conditions

Restricted maximum likelihood covariate parameter estimates for effect of drought on percent chlorosis- 7 days post inoculation (DPI)

Covariance parameter		Estimate	Standard Error	
Block(exp)		-8.4	15.90	
Residual		163.0	34.93	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
exp	1	14	33.4	<0.0001
trt	1	44	0.02	0.89
inoc	1	44	40.97	<0.0001
trt*inoc	1	44	1.46	0.23
trt*exp	1	44	0.28	0.60

Restricted maximum likelihood covariate parameter estimates for effect of drought on percent chlorosis- 10 DPI

Covariance parameter		Estimate	Standard Error	
Block(exp)		7.0	16.33	
Residual		126.2	27.01	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
exp	1	14	17.31	0.001
trt	1	43	0.14	0.71
inoc	1	43	120.66	<0.0001
trt*inoc	1	43	0.99	0.33
trt*exp	1	43	1.03	0.32

Restricted maximum likelihood covariate parameter estimates for effect of drought on percent chlorosis- 14 DPI

Covariance parameter		Estimate	Standard Error	
Block(exp)		-16.4	13.95	
Residual		171.0	36.60	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
exp	1	14	4.97	0.04
trt	1	43	0.14	0.71
inoc	1	43	126.73	<0.0001
trt*inoc	1	43	0.70	0.41
trt*exp	1	43	1.11	0.30

Restricted maximum likelihood covariate parameter estimates for effect of drought on percent chlorosis- 18 DPI

Covariance parameter		Estimate	Standard Error	
Block(exp)		11.7715	23.9194	
Residual		167	36.0177	
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
exp	1	14	7.12	0.02
trt	1	44	0.00	0.96
inoc	1	44	145.66	<0.0001
trt*inoc	1	44	0.39	0.54
trt*exp	1	44	0.49	0.49

Restricted maximum likelihood covariate parameter estimates for effect of drought on percent chlorosis- repeated measures (all days pooled)

Covariance parameter		**Subject**	**Estimate**	**Standard Error**
block(exp)			-8.2	10.74
CS		trt*block*inoc*exp	63.0	24.00
Residual			128.0	13.00
Effect	**Num DF**	**Den DF**	**F value**	**Pr>F**
Repeat exp	1	13	6.7	0.02
Watering treatment	1	222	0.0	0.96
Inoculation	1	222	141.3	<.0001
trt*inoc	1	222	0.9	0.34
trt*exp	1	222	0.9	0.34
inoc*exp	1	222	1.8	0.18
DPI	3	222	43.5	<.0001
inoc*DPI	3	222	11.6	<.0001
trt*DPI	3	222	0.1	0.95
trt*inoc*DPI	3	222	0.1	0.97

Tukey-Kramer Grouping for trt*inoc Least Squares Means ($P = 0.05$)			
trt	inoc	Estimate	
Normal	Inoc	56.6	A[1]
Drought	Inoc	53.8	A
Drought	NI	25.2	B
Normal	NI	22.7	B

Appendix 5.11 Effect of drought on percent chlorosis regression over time.

Regression partition for non-inoculated treatment

Type I Tests of Fixed Effects: Non-inoculated				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Watering treatment (trt)	1	107	1.43	0.24
x	1	107	15.40	0.0002
x*x	1	107	1.98	0.16
x*DPI	1	107	1.51	0.22
x*trt	1	107	0.00	0.95
x*x*trt	1	107	0.00	0.97
x*DPI*trt	1	107	0.18	0.67

Regression partition for inoculated treatment

Type I Tests of Fixed Effects: Inoculated				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Watering treatment (trt)	1	109	1.49	0.22
x	1	109	99.37	<.0001
x*x	1	109	0.18	0.67
x*DPI	1	109	2.44	0.12
x*trt	1	109	0.21	0.65
x*x*trt	1	109	0.02	0.90
x*DPI*trt	1	109	0.04	0.85

Regression Equations:

Non-inoc Normal % chlorosis = (0.1141 +0.01023*DPI)*100
Non-inoc Drought% chlorosis = (0.1428 +0.00999*DPI)*100
Inoc Normal % chlorosis = (0.2348+ 0.02722*DPI)*100
Inoc Drought% chlorosis = (0.1735 + 0.02986*DPI)*100

Appendix 5.12 Simplified spore prediction model overview and analysis of variance

Variable	Parameter	SE	Pr > F	Partial R^2	Model R^2
Intercept	10.7	10.98	0.33		
NightLWD	-6.0	2.36	0.01	0.29	0.29
DVPD	4.5	0.59	<.0001	0.06	0.35
TRain	0.4	0.11	0.000	0.05	0.40
$NightLWD^2$	0.4	0.12	0.001	0.03	0.43
$DLWD^2$	0.4	0.08	<.0001	0.02	0.44
$Drought^2$	0.0	0.00	<.0001	0.01	0.45
Model Adjusted R^2					**0.44**

NightLWD - Duration of leaf wetness overnight (17:00–9:00)
DVPD - Number of days with average VPD ≤ 0.5 kPa
TRain - Cumulative total rainfall in past 10 days
DLWD -Number of days with LWD ≥ 6 h in past 10 days
Drought - Number of hours since last rainfall event

Simplified model Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	269974	44996	42.34	<0.0001
Error	307	326291	1063		
Corrected Total	313	596266			

Appendix 5.13 qPCR CT values, efficiency, and R^2 values for DNA of *Stemphylium vesicarium* extracted from bioaerosol samples using three gene regions (*TEf-A*, *cytB*, ITS)

	Sample	*TEF-A*		*CytB*		ITS	
Run	**YYMMDD**	**CT Mean**	**CT SD**	**CT Mean**	**CT SD**	**CT Mean**	**CT SD**
Jan-18	180603	34.75	0.16	31.68	0.22	Undetermined	
	180610	Undetermined		Undetermined		Undetermined	
	180615	Undetermined		Undetermined		Undetermined	
	180618	Undetermined		34.21	0.35	Undetermined	
	180619	36.39	0.69	30.11	0.06	Undetermined	
	180622	36.22	0.73	31.94	0.14	Undetermined	
	180625	32.83	0.16	28.92	0.10	Undetermined	
	180626	32.64	0.16	28.72	0.12	39.53	0.32
	180627	38.82		34.99	0.57	Undetermined	
	180628	31.86	0.13	28.19	0.11	33.76	0.21
	180629	32.9	0.43	28.8	0.14	Undetermined	
	180704	35.79	0.26	32.42	0.12	Undetermined	
	180705	33.26	0.11	29.57	0.09	Undetermined	
	180706	33.73	0.26	30.65	0.18	Undetermined	
	180709	35.72	0.25	31.63	0.12	Undetermined	
	180710	32.14	0.25	28.95	0.11	Undetermined	
	180711	36.11	0.46	34.63	0.02	Undetermined	
	180712	38.89	1.04	37.08		Undetermined	
	180713	34.45	0.46	31.29	0.13	Undetermined	
	180716	30.99	0.23	27.38	0.04	Undetermined	
	180717	28.41	0.19	24.87	0.09	33.72	0.29
	180720	35.9	0.19	32.16	0.07	Undetermined	
	180723	29.78	0.17	26.18	0.07	Undetermined	
	10^{-5} spores	30.73	0.37	27.01	0.06	35.5	0.12
	R^2 value	**0.898**		**0.961**		**0.972**	
	Efficiency	**161%**		**117%**		**75.80%**	
Feb-18	180724	29.05	0.5	26.53	0.09	36.5	0.32
	180725	27.93	0.3	25.42	0.06	34.96	0.06
	180726	28.51	0.37	26	0.26	38.69	0.46
	180727	26.66	0.1	24.11	0.26	36.31	0.22
	180730	26.98	0.19	24.68	0.10	31.91	0.35
	180731	32.43	0.51	31.76	0.24	39.8	
	180801	27.59	0.08	25.26	0.08	38.22	0.57
	180802	29.57	0.57	27.02	0.28	34.89	0.42
	180807	31.26	0.25	28.02	0.38	34.76	0.42
	180808	30.88	0.26	29.6	0.24	35.1	0.06
	180809	31.18	0.46	28.92	0.01	Undetermined	
	180810	30.17	0.69	28.27	0.39	34.28	0.16
	180813	24.95	0.04	22.69	0.15	35.74	0.71
	180814	25.38	0.19	23.25	0.03	34.93	0.02
	180815	26.33	0.33	24.44	0.13	31.09	0.15
	180816	28.06	0.16	26.14	0.34	32.86	0.24
	180817	23.13	0.27	21.13	0.13	26.57	0.54
	180820	25.07	0.49	24.25	0.03	28.94	0.15
	180821	27.64	0.17	26.05	0.28	32.54	0.36
	180822	26.51	0.24	24.05	0.18	32.58	0.01
	180823	27.5	0.55	24.62	0.21	32.29	0.02
	180824	25.34	0.44	23.9	0.05	28.22	0.65
	180827	24.85	0.2	22.74	0.15	29.82	0.36
	10^{-5}spores	29.32	0.12	26.1	0.33	36.43	
	R^2 value	**0.908**		**0.95**		**0.99**	
	Efficiency	**146%**		**127%**		**84%**	

	180828	23.48	0.13	20.36	0.11	24.71	0.31
	180829	23.83	0.05	20.66	0.33	24.5	0.16
	180830	36.3	0.62	33.61	0.93	38.2	1.23
	180831	31.97	0.19	28.62	0.07	33.93	0.04
	190531	29.94	0.21	26.45	0.06	Undetermined	
	190603	32.35	0.37	28.7	0.08	Undetermined	
	190604	32.66	0.24	29.45	0.06	Undetermined	
	190605	30.19	0.14	26.94	0.02	Undetermined	
	190606	36.92	0.37	32.8	0.19	Undetermined	
	190607	32.96	0.04	29.41	0.03	Undetermined	
	190612	31.65	0.27	28.21	0.08	Undetermined	
	190613	34.92	0.73	34.37	0.21	Undetermined	
	190614	33.6	0.06	31.23	0.09	Undetermined	
Jan-19	190617	36.54	0.05	32.91	0.51	Undetermined	
	190618	35.58	0.37	31.83	0.35	Undetermined	
	190619	33.25	0.42	29.52	0.14	Undetermined	
	190620	35.98	0.69	30.63	0.11	Undetermined	
	190621	32.51	0.18	29.47	0.10	Undetermined	
	190626	35.43	0.41	32	0.33	Undetermined	
	190627	32.74	0.23	28.97	0.05	Undetermined	
	190628	32.55	0.12	28.91	0.24	Undetermined	
	190703	34.12	0.42	30.52	0.17	Undetermined	
	190704	32.86	0.15	29.74	0.17	Undetermined	
	10^{-5}spores	32.09	0.38	28.68	0.25	36.15	1.73
	R^2 value	**0.952**		**0.976**		**0.93**	
	Efficiency	**135.50%**		**118%**		**110.50%**	
	190705	32.59	0.18	30.9	0.23	Undetermined	
	190708	36.66	0.05	30.22	0.53	Undetermined	
	190709	Undetermined		37.72	0.88	Undetermined	
	190710	34.72	0.22	32.13	0.00	Undetermined	
	190711	31.27	0.47	28.17	0.09	Undetermined	
	190712	32.83	0.16	30.23	0.08	Undetermined	
	190715	33.28	0.54	30.18	0.13	Undetermined	
	190716	32.95	0.49	29.97	0.12	Undetermined	
	190717	Undetermined		Undetermined		Undetermined	
	190718	Undetermined		38.9		Undetermined	
	190719	30.33	0.11	27.52	0.07	38.83	
	190724	32.67	0.42	30	0.27	36.51	0.18
2019-2	190725	32.49	0.22	30.07	0.29	Undetermined	
	190730	31.29	0.31	28.4	0.13	Undetermined	
	190731	30.31	0.28	26.7	0.08	39.6208839	
	190801	36.57	0.95	36.11	0.64	Undetermined	
	190802	33.38	0.45	30.55	0.00	37.39	0.01
	190807	29.65	0.03	26.56	0.06	32.66	0.01
	190812	32.54	0.56	29.47	0.48	Undetermined	
	190813	27.82	0.07	24.56	0.13	34.16	0.60
	190816	28.71	0.13	25.41	0.12	34.34	0.61
	190826	28.38	0.14	25.53	0.11	Undetermined	
	190827	29.76	0.12	27.33	0.07	39.02	0.09
	10^{-5}spores	33.23	0.1	29.47	0.07	38.69	0.43
	R^2 value	**0.954**		**0.976**		**0.973**	
	Efficiency	**137.70%**		**103%**		**97.60%**	
	200602	36.98	0.44	30.71	0.39	Undetermined	
	200603	33.40	0.51	28.35	0.24	Undetermined	
Jan-20	200604	33.26	0.32	28.29	0.13	Undetermined	
	200605	32.90	0.32	28.49	0.09	Undetermined	
	200609	33.21	0.28	29.32	0.32	Undetermined	

	200610	33.08	0.49	28.33	0.32	Undetermined	
	200611	Undetermined		Undetermined		Undetermined	
	200612	32.52	0.19	28.22	0.24	Undetermined	
	200616	33.17	0.20	29.16	0.17	Undetermined	
	200617	37.10	0.14	31.62	0.27	Undetermined	
	200618	Undetermined		38.0715828		Undetermined	
	200619	36.60	0.28	34.32	0.01	Undetermined	
	200623	30.41	0.21	25.33	0.06	Undetermined	
	200624	30.66	0.16	25.66	0.21	Undetermined	
	200625	31.42	0.41	27.07	0.28	Undetermined	
	200626	31.72	0.12	26.63	0.20	Undetermined	
	200630	32.27	0.05	27.83	0.17	Undetermined	
	200707	33.26	0.37	27.62	0.09	Undetermined	
	200708	33.68	0.20	28.30	0.10	Undetermined	
	200709	31.97	0.00	27.09	0.17	Undetermined	
	200710	33.69	0.33	29.42	0.08	Undetermined	
	200714	38.25	0.09	30.18	0.30	Undetermined	
	200715	32.52	0.19	27.38	0.18	36.70	0.09
	10^{-5}spores	32.23	0.42	27.69	0.14	Undetermined	
	R^2 value	**0.904**		**0.989**		**0.978**	
	Efficiency	**168.30%**		**110.90%**		**92.60%**	
Feb-20	200716	34.97	0.29	30.62	0.25	36.32	0.24
	200717	35.27	0.33	28.44	0.12	34.92	0.22
	200723	35.36	0.09	29.57	0.14	Undetermined	
	200724	31.80	0.55	26.34	0.14	34.34	0.45
	200728	36.18	0.30	30.68	0.13	Undetermined	
	200729	31.96	0.13	26.88	0.11	Undetermined	
	200730	33.62	0.16	28.80	0.13	34.42	0.36
	200731	32.74	0.19	27.33	0.15	Undetermined	
	200805	34.06	0.39	29.23	0.56	Undetermined	
	200806	32.81	0.29	27.91	0.21	34.11	0.19
	200807	31.58	0.37	26.05	0.50	32.37	0.28
	200811	34.06	0.66	27.61	0.35	Undetermined	
	200812	31.24	0.23	25.37	0.05	34.02	0.15
	200813	32.52	0.36	26.82	0.16	34.11	0.28
	200814	29.72	0.54	23.30	0.59	28.47	0.11
	200818	32.56	0.39	26.78	0.22	31.02	0.09
	200819	31.62	0.38	26.26	0.16	32.65	0.27
	200825	25.70	0.06	20.59	0.12	27.55	0.31
	200828	29.71	0.51	23.37	0.10	28.26	0.24
	10^{-5}spores	36.15	0.06	28.77	0.18	35.65	0.95
	R^2 value	**0.955**		**0.987**		**0.986**	
	Efficiency	**107%**		**104.90%**		**97.50%**	