Identification and management of wasabi pathogens in British Columbia

by

Emily C. Betz

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Name:	**Emily C. Betz**
Degree:	**Master of Science**
Title:	**Identification and management of wasabi pathogens in British Columbia**
Examining Committee:	**Chair: Vicki Marlatt** Assistant Professor
	Zamir K. Punja Senior Supervisor Professor
	Margo Moore Supervisor Professor Emerita
	Siva Sabaratnam Supervisor Plant Pathologist British Columbia Ministry of Agriculture
	Sherryl Bisgrove Internal Examiner Associate Professor
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Abstract

Wasabi (*Wasabia japonica*) plants in British Columbia are grown in moist conditions ideal for pathogens, and therefore, are prone to various diseases. Over 3 years, seven wasabi greenhouses were surveyed for pathogens. Prevalence and severity of diseases were documented. Pathogenic species including *Phoma wasabiae* (*Leptosphaeria biglobosa*), *Botrytis cinerea*, and *Erysiphe cruciferarum* were found in multiple greenhouses. A new disease of wasabi with symptoms of vascular blackening and wilt was discovered. Using morphological and molecular techniques, the causal organism was identified as *Verticillium isaacii*. Powdery mildew of wasabi caused by *E. cruciferarum* was prevalent in half the greenhouses surveyed. In order to evaluate management options for powdery mildew, 4 commercially available products, Actinovate®, Cueva®, Rhapsody®, and Regalia® were applied biweekly onto greenhouse plants. Both Cueva® and Regalia® significantly reduced the progression of powdery mildew on wasabi plants.

Keywords: *Wasabia japonica*; *Erysiphe cruciferarum*; *Verticillium isaacii*; powdery mildew; verticillium wilt; wasabi

Dedication

To my parents, Rick and Monika, who always cheered me on and at least pretended to be interested in whatever scientific concept I was blathering on about. I would not be where I am today without your continuing support.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1. The biology and production of *Wasabia japonica* in British Columbia

1.1.1. Introduction to wasabi

Wasabi (*Wasabia japonica* (Miq.) Matsumura, syn. *Eutrema japonicum* Matsum.) is a perennial plant in the Brassicaceae family. It produces a central, above ground, stem (commonly referred to as a rhizome) (Fig. 1.1a); older plants often produce offshoots from the central rhizome. At the crown of the rhizome, a multitude of petioles arise, each up to 50 cm long and ending with a single smooth, globose to cordate, leaf which can be up to 25 cm in diameter (Chadwick et al., 1993) (Fig. 1.1b). In spring, terminal single inflorescences may emerge from peduncles (Adachi, 1987; Chadwick et al., 1993). The flowers are white in colour and bracteate, raceme, and cruciform in shape (Fig. 1.1c). Due to self-incompatibility, cross-pollination is generally required (Chadwick, 1993; Palmer, 1990). Up to eight seeds are borne in seedpods (Fig. 1.1d) (Chadwick, 1993).

Wasabi is grown for its valuable rhizome, which is traditionally ground into a paste and used as a condiment in Japanese cuisine (Adachi, 1987). The leaves of the wasabi plant are also edible and can be eaten as salad greens (Chadwick et al., 1993). Both the leaves and rhizomes are approved by Health Canada for nutraceutical use as antioxidant supplements (pers. comm. with manufacturer). The rhizomes generally fetch a price between $250-350/kg (J. MacDonald, Agriculture and Agri-Food Canada, Summerland BC, pers. comm).

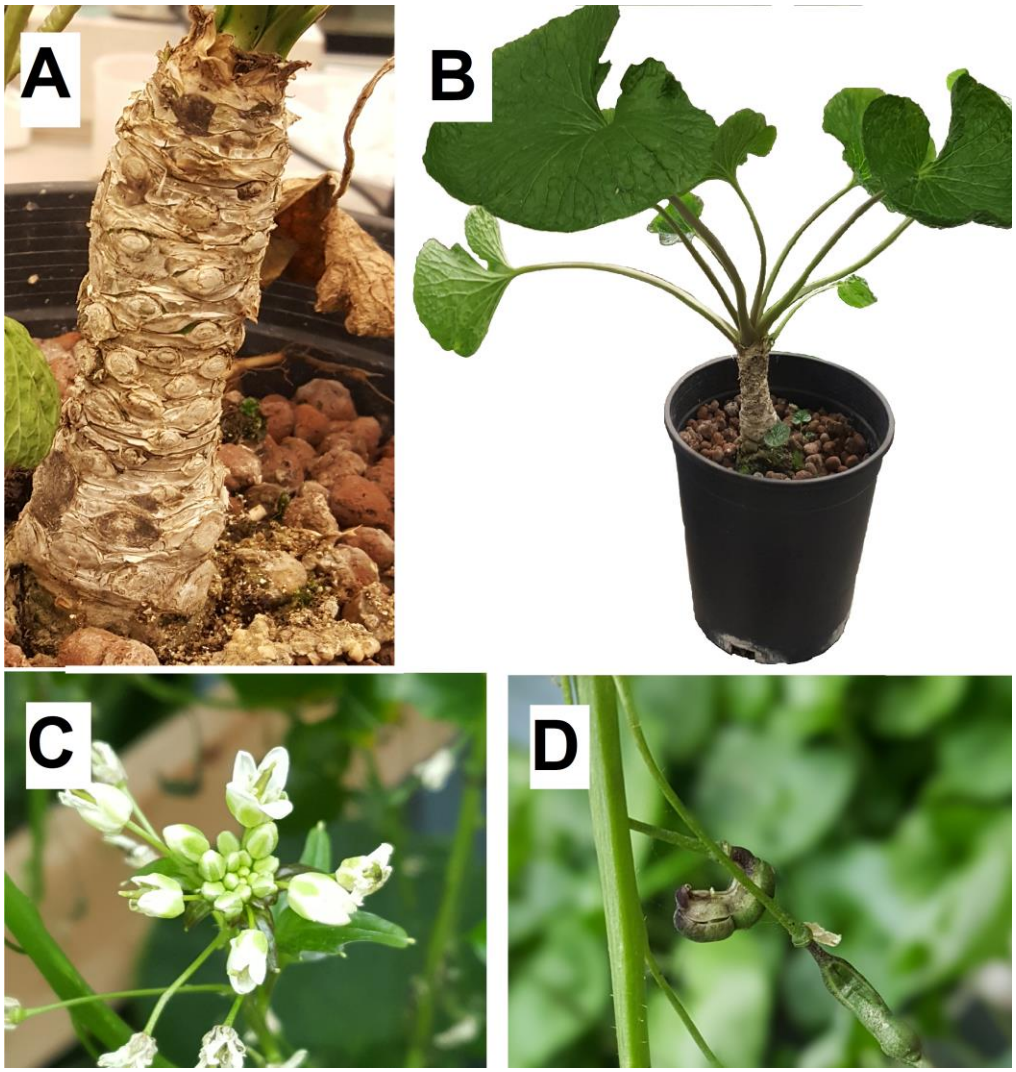


Figure 1.1 A) A mature wasabi rhizome, B) a mature wasabi plant with leaves arising from petioles at the rhizome crown, C) a terminal inflorescence, and D) wasabi seedpods.

The natural habitat of wasabi is that of mountain streambeds or riverbanks in Japan. It prefers cool, moist environments with ideal growing temperatures from 15-18 °C. In fact, the plant ceases growth at temperatures above 25 °C (Adachi, 1987; Chadwick et al., 1993).

Wasabi is traditionally a Japanese crop, but it is now grown in several countries including Taiwan, China, South Korea, New Zealand, the United States, Britain,

Northern Ireland, and Canada (Adachi, 1987; Palmer, 1990; Chadwick et al., 1993; Lo and Wang, 2000; Weng et al., 2010; Choi et al., 2014; MacDonald and Punja, 2016; MacDonald, 2018). The cool, wet environment required to grow wasabi makes the Pacific Northwest an ideal area for commercial production, and wasabi has been grown in British Columbia for over 20 years.

1.1.2. Greenhouse production in British Columbia

Commercial production of wasabi in Canada has been almost exclusively in the Pacific Northwest region of British Columbia. Wasabi in British Columbia (B.C.) is usually commercially grown in double layer polyethylene (double-poly) greenhouses, using semi-hydroponic overhead sprinkling systems (MacDonald and Punja, 2016). These double-poly greenhouses have vents to help manage temperature and aid with cooling in the summer, and the greenhouses can be heated in the winter (MacDonald, 2018). In order to help reduce UV radiation, these greenhouses are covered by 70% shade cloth for most of the year (MacDonald, 2018). The plants are sown into beds of river rock along the greenhouse floor. The sprinklers mist at short, regular intervals (sometimes less than 5 minutes apart) and are designed to provide water, nutrients, and cooling (MacDonald, 2018). This creates high moisture conditions, of up to 100% relative humidity, which are ideal for pathogen growth.

To combat the problem of pathogens, some greenhouses have switched to using drip fertigation. Other types of greenhouse production are being tested, including using glass greenhouses instead of double-poly. Substrates used in these production systems include gravel, hydroton (expanded clay pellets), and organic soil. Plants are grown in

either raised beds, or individual pots, to help reduce spread of pathogens. The hydroton and gravel is used to help reduce excessive moisture at the roots to control root rot, while organic soils have the potential to reduce pathogen development as they are microbially active. The drip fertigation provides water and nutrients but keeps the moisture level on the leaves down, reducing the likelihood of pathogen development.

Due to the difficulty in propagating plants from seed, most growers obtain new plants from either cuttings or tissue culture, regardless of production system.

1.2. Previously reported pathogens of wasabi

The conditions in which wasabi is grown are ideal for disease development as, in general, moist environments prevent pathogen desiccation and facilitate spore dispersal. Wasabi is known to be attacked by a multitude of disease-causing organisms of all different types, including fungi, oomycetes, bacteria, and viruses. These pathogens cause a variety of symptoms including leaf spots, mildew, wilt, and rot, which decrease the value of the wasabi crop, and in some cases make it unmarketable. Currently, there are 23 known pathogens of wasabi worldwide (in addition to 5 unconfirmed disease-associated bacteria), of which 12 have been reported in B.C., and 8 have been confirmed. In order to confirm that a pathogen causes disease, Koch's postulates must be proven: the pathogen must be isolated from symptomatic plants, cause disease when introduced to a healthy plant, then subsequently be re-isolated.

1.2.1. Fungi

Perhaps the most notorious disease of wasabi is blackleg caused by *Phoma wasabiae* (teleomorph, *Leptosphaeria maculans* or *Leptosphaeria biglobosa*). This fungus causes internal and external blackening of the wasabi rhizome, resulting in an unmarketable crop. It has been found on wasabi in Japan (Adachi, 1987), Taiwan (Lo *et al.*, 2002), China (Zhang *et al.*, 2004), New Zealand (Broadhurst and Wright, 1998), the United States, and Canada (Punja *et al*., 2017). In greenhouse grown wasabi in B.C., only *L. biglobosa* has been found. *Phoma wasabiae* also causes necrotic leaf spots which reduce plant productivity. *Leptosphaeria maculans* and *Leptosphaeria biglobosa* are part of a species complex, and both are ubiquitous pathogens of brassica species, most notably of oilseed crops including canola, causing blackleg or phoma stem canker (Fitt *et al.*, 2006). They likely spread to wasabi via nearby brassica crops or weeds (Punja *et al.*, 2017). In B.C., this may include crops such as broccoli and cauliflower.

Botrytis cinerea is another fungus previously reported on wasabi in B.C. (MacDonald and Punja, 2016). It causes leaf blight, resulting in chlorotic and necrotic leaf tissue. *Botrytis cinerea* is a necrotrophic pathogen with a very broad host range, affecting more than 200 plant species including brassica crops such as broccoli (Williamson *et al.*, 2007). Likely inoculum sources include nearby crops or weeds.

Erysiphe cruciferarum is a powdery mildew fungus that attacks crucifer species. It has been previously reported on wasabi in Korea (Park *et al.*, 2016) and Japan (Oku *et al*, 1993). It has been previously reported infecting wasabi in B.C. in 2013 and 2015 (Joshi *et al.*, 2014; Joshi and Jeffries, 2016).

Colletotrichum higginsianum has been reported in B.C. (MacDonald and Punja, 2016) and New Zealand (Martin *et al.*, 2002) causing anthracnose disease on wasabi leaves.

The literature from Japan also reports leaf spot diseases caused by *Ascochyta brassicae*, *Septoria wasabiae*, and *Alternaria brassicae* (Kishi, 1988; Chadwick, 1993). Also in the Japanese literature, cottony or watery soft rot caused by *Sclerotinia sclerotiorum* has been reported on seedlings, and damping off of seedlings caused by *Pellicularia filamentosa* (Pat.) Rogers and *Rhizoctonia solani* Kuhn has been reported (Adachi, 1987; Chadwick, 1993). *Rhizoctonia solani* was also associated with stem and crown rot of BC wasabi plants in 2009 and 2013 (Joshi and Jefferies, 2010; Joshi *et al.*, 2014).

1.2.2. Oomycetes and other members of SAR

Pythium spp. were first discovered as a causal agent of root rot on wasabi in B.C. (Rodríguez, 2007). Rodríguez and Punja (2007) were able to confirm pathogenicity of *P. dissotocum* and *P. intermedium* on wasabi. Both species have large host ranges and are fairly common pathogens (Rodríguez, 2007). *Pythium irregulare* has also been isolated from wasabi with root rot symptoms in B.C. (Joshi *et al.*, 2014; Joshi *et al.*, 2015).

Two *Phytophthora* spp. have been confirmed as pathogenic on wasabi – *P. drechsleri* in Japan causing root and rhizome rot (Minoshima *et al.*, 2017) and *P. cryptogea* in Michigan causing root and crown rot (Granke *et al.*, 2012). A *Phytophthora* sp. has also been isolated from wasabi with crown rot symptoms (Joshi *et al.*, 2014; Joshi

et al., 2015), and *P. cryptogea* was isolated from wasabi with symptoms of leaf and petiole blight (Joshi and Jeffries, 2016) in B.C.

Albugo spp. are obligate pathogens causing white blister rust on leaves. *Albugo* spp. (particularly *A. candida*) are cosmopolitan and attack plants of the Brassicaceae family. On wasabi, *A. wasabiae* has been reported in Japan and Taiwan (Adachi, 1987; Lo and Wang, 2000). *Albugo* spp. were first reported on wasabi in B.C. in 2009 (Joshi and Jeffries, 2010). MacDonald and Punja (2016) were able to confirm species identity as *A. candida*, which has also been recently reported on wasabi in Korea (Choi *et al*., 2014). In B.C., a genetically identical isolate was found on shepherd's purse (*Capsella bursa-pastoris*), suggesting that cruciferous weeds are an inoculum source of *A. candida* (MacDonald and Punja, 2016).

Plasmodiophora brassicae is a pathogen of crucifers causing the formation of root galls commonly referred to as clubroot. Galls can become large enough that they can restrict water intake via the xylem tissues, resulting in chlorosis of the leaves, and eventually death of the plant (Agrios, 2005). *P. brassicae* has been reported on wasabi in Japan (Adachi, 1987) and China (Chai *et al.*, 2014). Clubroot symptoms were found on B.C. wasabi in 2013 and 2014, but these samples could not be molecularly identified as *P. brassicae* and Koch's postulates were not confirmed (Joshi *et al.*, 2014; Joshi *et al.*, 2015; Z. Punja, Professor, Simon Fraser University, pers. comm.).

Peronospora alliariae f. sp. *wasabiae* causing downy mildew has been reported in Japan (Chadwick, 1993).

1.2.3. Bacteria

Pectobacterium spp. are often observed in tandem with black rot caused by *P. wasabiae* (Adachi, 1987; Rodríguez, 2007). They are soft rot-causing bacteria with a wide host range including many crop species such as potato (Toth *et al.*, 2002). *Pectobacterium* spp. produce extracellular pectinolytic enzymes which degrade pectin and cause cells to separate (Toth *et al.*, 2002). In wasabi, soft rot renders rhizomes completely unmarketable. *Pectobacterium carotovorum* subsp. *carotovorum, P. carotovorum* subsp. *wasabiae,* and *Pectobacterium rhapontici* have been isolated from wasabi in Japan (Goto and Matsumoto, 1986), but pathogenicity experiments were not conducted (Rodríguez, 2007). *Pectobacterium* spp. have also been isolated from symptomatic wasabi in New Zealand (Broadhurst and Wright, 1998). In 2009, Rodríguez and Punja were able to confirm *P. carotovorum* subsp. *carotovorum* as the causal agent of internal blackening and rot of wasabi rhizomes in Canada. Disease was caused when the rhizomes were wounded, either mechanically or through co-infection with *Pythium* sp., and the bacterium was introduced into the wound (Rodríguez and Punja, 2009). *Pectobacterium carotovorum* subsp. *carotovorum* was also isolated from wasabi in B.C. with soft rot symptoms in 2013 (Joshi *et al.*, 2014).

Various *Pseudomonas* spp. have also been associated with soft rot diseases. *P. marginalis* and *P. viridijlava* have been isolated from symptomatic plants in Japan (Goto and Matsumoto, 1986), and *Pseudomonas* spp. were also isolated from wasabi in New Zealand (Broadhurst and Wright, 1998). However, in neither case were pathogenicity tests conducted (Rodríguez, 2007). *P. syringae* pv. *maculicola* was also isolated from wasabi with leaf spot symptoms in B.C. in 2015 (Joshi and Jeffries, 2016).

Corynebacterium spp. have also been reported causing vascular wilts, blights, leaf spot and ring rot on wasabi in Japan (Adachi, 1987; Matsumoto *et al.*, 1985)

1.2.4. Viruses

Wasabi mottle virus (WMoV) has been previously reported in Japan (Shimamoto *et al*., 1998) and Taiwan. Recently, plants in B.C. have been found carrying the virus (MacDonald *et al.*, 2019). It is quite distinctive, and easily recognizable due to the leaf spots, mottle, and vein-clearing symptoms it produces. The strain found in B.C.is genetically similar to the strain reported in Taiwan, and as Taiwan is the source of the tissue-culture plants of cultivar 'Green Thumb' used in Canadian production (L. Benkrima, Lead Scientist, Your Wasabi Farms Ltd., pers. comm.), this is a possible point of entry for the virus. In B.C., it is believed that the virus is maintained in asymptomatic tissue culture plants at wasabi nurseries, and symptoms develop in plants that are heat stressed.

Other viruses previously reported on wasabi in Japan include *Tobacco mosaic virus* (TMV), *Turnip mosaic virus* (TuMV), and *Cucumber mosaic virus* (CMV) (Chadwick *et al.*, 1993). Additionally, CMV has been found on wasabi in Australia (Wilson, 1998). These viral diseases cause stunting on wasabi, along with leaf discolouration (Chadwick *et al*., 1993). *Alfalfa mosaic virus* (AMV) has been reported on wasabi in New Zealand with symptoms appearing as interveinal mottle, mosaic, and leaf crinkling (Fletcher, 1989). Currently, none of these viruses have been reported on wasabi in North America.

1.3. Current disease control options

1.3.1. Cultural methods

In Canada, the lack of approved pesticides on wasabi means that most control of diseases is managed through cultural methods. As such, monitoring for disease symptoms becomes especially important. Leaf diseases are controlled via pruning infected leaves. This is cost effective for small production systems, but in months when disease pressure is heavy, growers are easily overwhelmed (L. Benkrima, Lead Scientist, Your Wasabi Farms Ltd., pers. comm.). The potential to spread diseases, including rhizome diseases and viruses, through unsanitary pruning implements is also of concern.

Some growers have attempted using drip irrigation to reduce moisture levels in greenhouses, as opposed to the more traditional overhead sprinkling systems. Those greenhouses with drip irrigation tend to develop less rot, such as from *Botrytis cinerea*, but, conversely, encounter more powdery mildew as leaf conditions are more optimal.

Tissue culture plants are also used by some growers to obtain pathogen free plants. There is a tissue culture facility in the Lower Mainland which specializes in wasabi micropropagation. Tissue culture plants are more expensive of an option than vegetative cuttings, and there is also the concern that tissue culture plants may harbour viruses if mother plants are not tested for 'virus-free' status (MacDonald *et al.*, 2019).

1.3.2. Pesticides approved for use on wasabi in Canada

Currently, the only chemical-based insecticide approved for use on wasabi is Ambush 500EC (Permethrin), and there are no chemical fungicides that are approved to

manage pathogens (Table 1.1). There are only four bio-fungicides approved for use on wasabi diseases; BW240 WP and Rootshield PLUS WP have *Trichoderma* sp. as their active ingredient and are approved for use on specific root diseases, while Cease and Rhapsody ASO have *Bacillus subtilis* as their active ingredient and are approved for use on powdery mildew and some root rot (Table 1.1) (Health Canada, 2016). All of these are biological pesticides, and their efficacy on wasabi has not yet been proven.

Table 1.1 List of registered pesticides, including biocontrol products, for use on wasabi in Canada

Product	Active Ingredient	Pest/ Disease	Manufacturer
Ambush 500EC	Permethrin	• Cabbage looper • Diamondback moth larvae • Crucifer flea beetle	AMVAC Chemical Corp.
Bioprotec CAF	*Bacillus thuringiensis kurstaki*	• Cabbage looper • Alfalfa looper	AEF Global, Inc.
Bioprotec PLUS	*Bacillus thuringiensis kurstaki*	• Cabbage looper • Alfalfa looper	AEF Global, Inc.
Botanigard 22WP	*Beauveria bassiana* GHA	• Aphids	LAM International Corp.
Botanigard ES	*Beauveria bassiana* GHA	• Aphids	LAM International Corp.
BW240 WP	*Trichoderma harzianum* KRL-AG2 and *Trichoderma virens* G-41	• Fusarium root rot • Phytophthora root rot • Pythium root rot and damping off • Rhizoctonia root rot and damping off	BioWorks, Inc.
Cease®	*Bacillus subtilis* QST 713	• Powdery mildew • Pythium root rot • Phytophthora crown rot and root rot	BioWorks, Inc.
Dipel® 2X DF	*Bacillus thuringiensis kurstaki*	• Cabbage looper • Alfalfa looper	Valent BioSciences Corp.
Rhapsody® ASO™	*Bacillus subtilis* QST 713	• Powdery mildew • Pythium root rot • Phytophthora crown rot and root rot	Bayer CropScience Inc.
Rootshield® PLUS WP	*Trichoderma harzianum* KRL-AG2 and *Trichoderma virens* G-41	• Fusarium root rot • Phytophthora root rot • Pythium root rot and damping off • Rhizoctonia root rot and damping off	BioWorks, Inc.

1.4. Verticillium wilt

1.4.1. Taxonomy and identification

Verticillium species are hemibiotrophic, soil-borne fungi in the Class Sordariomycetes, Order Hypocreales, and Family Plectosphaerellaceae (Depotter et al., 2016). Of the 10 species currently recognized in the genus, seven are broadly recognized as wilt pathogens of higher plants: *V. dahliae* Kleb., *V. longisporum* (C. Stark) Karapapa, Bainbr. & Heale, *V. albo-atrum* Reinke & Berthold, *V. tricorpus* Isaac, *V. alfalfae* Inderb., Platt, Bostock, Davis & Subbarao, *V. nonalfalfae* Inderb., Platt, Bostock, Davis & Subbarao, and *V. zaregamsianum* Inderb., Usami, Kanto, Bostock, Davis & Subbarao (Inderbitzin et al., 2013). Those that are generally recognized as endophytic or weakly pathogenic include *V. isaacii*, *V. klebahnii*, and *V. nubilum* Pethybr. (Inderbitzin et al., 2011).

Verticillium spp. are identified by their white to cream coloured mycelium which can lie dormant in soils for many years in either the form of microsclerotia or dark-coloured mycelium, depending on which clade they belong to (Inderbitzin et al., 2011). They bear ovoid, hyaline conidia on phialides arranged in verticillate whorls along conidiophores (Inderbitzin et al., 2011).

1.4.2. *Verticillium* spp. as plant pathogens

Of the *Verticillium* spp. that cause wilt, *V. dahliae* is the most economically important due to its extremely wide host range that includes many crop species (Inderbitzin et al., 2013). Additionally, of increasing economic importance is *V. longisporum*, a hybrid of *V. dahliae* that is adapted to infect crucifers (Heale and Karapapa, 1999; Depotter et al., 2016)

The disease cycle of *Verticillium* sp. starts with microsclerotia which germinate in the presence of root exudates, and the resulting mycelium colonizes the cortical cells of the plant root, initiating the biotrophic stage of infection (Karapapa et al., 1997). Often the biotrophic phase is asymptomatic, and asymptomatic plants can be infected for several months without showing symptoms (Karapapa et al., 1997). In a successful infection, the mycelia enter the xylem where the hyphae proliferate, conidia are formed and travel via the transpiration stream to infect systemically (Karapapa et al., 1997). At this point, symptoms of chlorosis and necrosis appear in the plant (Heale and Karapapa, 1999). The fungus then switches to a necrotrophic stage, colonizes the dead tissue, and produces microsclerotia (Heale and Karapapa, 1999; Karapapa et al., 1997). As the plant tissue decays, the microsclerotia are released back into the soil where they can remain viable for over a decade (Karapapa et al., 1997; Pegg and Brady, 2001).

Verticillium wilt is found primarily on crops grown in temperate climates with warm, dry summers and cool winters and are especially problematic in irrigated regions (Pegg and Brady, 2001). Wilt conditions are exacerbated by moist soil conditions, and optimal temperatures for infection are between 21-27 °C (Pegg and Brady, 2001). However, it is thought that stresses brought on by drought and high temperatures are likely to increase disease severity and yield loss in infected plants (Heale and Karapapa, 1999).

In laboratory studies, it was demonstrated that *Verticillium dahliae* cultures can be killed in 4 minutes at 55 °C and microsclerotia can be killed in 10 minutes at 50 °C or 40 minutes at 47 °C (Miller and Stoddard, 1956; Nelson and Wilheim, 1958). The optimal

soil pH for *V. dahliae* growth was determined to be 5.5, and the disease severity on ornamentals (*Antirrhinum* sp.) was greater in alkaline soil than acidic soil (Dutta, 1981).

1.4.3. Verticillium isaacii

Verticillium isaacii was first described in 2011, by Inderbitzin *et al*. It was previously considered as part of *Verticillium tricorpus* but was found to be genetically distinct (Inderbitzin *et al.*, 2011). It has been isolated from spinach, artichoke, and lettuce, but it is sometimes endophytic in these plants rather than pathogenic (Gurung *et al*., 2015). Isolates of *V. isaacii* were found to weakly infect sunflower, potato, strawberry, artichoke, and lettuce (Gurung *et al.*, 2015; Wheeler and Johnson, 2019).

1.5. Powdery mildew

1.5.1. Taxonomy and identification

Pathogens of powdery mildew are obligately biotrophic, filamentous, phytopathogens in the Order Erysiphales and Phylum Ascomycota (Glawe, 2008). Most species grow epiphytically on plant surfaces, including leaves, stems, flowers, and fruits (Heffer *et al*., 2006; Glawe, 2008). Powdery mildew pathogens are pleomorphic and have both sexual and asexual stages; however, either of these stages may be absent depending on the species (Glawe, 2008). The asexual conidia are borne on the terminal end of condiophores, while the sexual ascospores are enclosed in asci which are encapsulated in enclosed hyphal structures known as chasmothecia (Heffer *et al.*, 2006).

Powdery mildew pathogens are identified by their white mycelia and the abundant amounts of conidia they produce, which gives them a ‘powdery’ appearance (Glawe,

2008). The taxonomy of these pathogens is complex and still under review, but, broadly, there are 5 holomorphic tribes – Phyllactinieae, Erysipheae, Blumerieae, Golvinomyceteae, and Cystotheceae (Glawe, 2008).

Erysiphe spp. belong to the tribe Erysipheae (Heffer *et al*., 2006). The genus *Erysiphe* is split into three sections – section *Erysiphe*, section *Microsphaera*, and section *Uncinula* (Heffer *et al*., 2006). The anamorphs are all *Oidium* species. *Erysiphe* spp. can be distinguished from other powdery mildew species by their conidia which form singly or in pseudochains, rather than true chains (Heffer *et al*., 2006). The sections of the *Erysiphe* genus can be distinguished by the appendages on their chasmothecia (if present); section *Erysiphe* has simple appendages, section *Microsphaera* has dictomously branched appendages, and section *Uncinula* has coiled or hooked appendages (Heffer *et al*., 2006).

1.5.2. *Erysiphe* spp. as plant pathogens

Individual *Erysiphe* species tend to be highly specialized plant pathogens and typically have small host ranges (Glawe, 2008). Haustoria are specialized hyphal cells which are formed in the space between the plant cell wall and cell membrane, and they function to absorb nutrients from plant epidermal cells (Heffer *et al*., 2006).

1.5.3. Erysiphe cruciferarum

Erysiphe cruciferarum is a pathogen responsible for powdery mildew on brassica species including oilseed rape, broccoli, and cabbage (Koike *et al.*, 2007; Alkooranee *et al.*, 2015). Colonies may be grey in colour, due to host responses produce black speckling

underneath the colony (Koike *et al.*, 2007). Heavily diseased plants have symptoms of chlorosis, necrosis, and early defoliation (Koike *et al.*, 2007).

E. cruciferarum has singly borne, ovoid conidia typical of *Erysiphe* spp. Appressoria (cells responsible for penetrating into host tissues) are variable, ranging from simple to lobed, and haustoria are multilobed (Koch and Slusarenko, 1990). Observations of chasmothecia in *E. cruciferarum* are rare. The sexual stage has not been observed in the Pacific Northwest, and how this species overwinters here has not yet been determined (Glawe, 2006).

1.6. Research objectives

Wasabi growers in the Pacific Northwest region of B.C. are at a disadvantage when it comes to managing diseases – partially due to the lack of information on wasabi pathogens present in the region, and partially due to the lack of available management options, including pesticides, that are available to them. Additionally, much of the current literature on wasabi pathogens is on field grown wasabi, not greenhouse grown wasabi. In order to assist growers, improved knowledge of current wasabi pathogens, their occurrence, and their severity is needed. Additionally, commercial fungicide products must be tested for efficacy on wasabi diseases. Growers are increasingly interested in organic production and are requesting management solutions, including biological fungicides and other reduced risk products, for controlling diseases.

The objectives of this research were to:

1) Survey B.C. wasabi greenhouses for diseases caused by pathogens.

2) Determine the causal agent of wilt and rhizome blackening symptoms on wasabi grown in B.C. greenhouses.

3) Confirm the causal agent of powdery mildew on wasabi in B.C.

4) Identify commercially available reduced risk fungicide products that are efficacious at reducing disease severity of powdery mildew on greenhouse grown wasabi.

Chapter 2. Surveys of microbes associated with wasabi diseases in British Columbia greenhouses

2.1. Introduction

As wasabi is a relatively new crop to North America, there is a lack of knowledge of what pathogens are present on B.C. grown wasabi. Additionally, growers are reporting symptoms of previously unknown wasabi diseases and they have no way to combat these pathogens (S. Sabaratnam, Plant Pathologist, B.C. Ministry of Agriculture, pers. comm.). The severity of these pathogens in the Pacific Northwest region is also unknown.

We conducted 3 surveys of fungal diseases on wasabi (*Wasabia japonica*) from May to August each year from 2016 to 2018. In 2016, thirty-one plant samples were taken from 5 greenhouses in B.C. – 3 in the Lower Mainland (Abbotsford and Surrey) and 2 on Vancouver Island (Sooke and Nanoose Bay). In 2017 and 2018, 42 and 44 plant samples, respectively, were collected from six greenhouses in B.C. – four in the Lower Mainland (Abbotsford, Burnaby, and Surrey) and two on Vancouver Island (Sooke and Sointula). We strived to obtain samples from as many different greenhouses as possible every year. In some years, certain greenhouses were unavailable, but we managed to get samples from three greenhouses on a consistent yearly basis.

2.2. Materials and methods

2.2.1. Sample collection

Plants with visible disease symptoms including foliar blight and discolouration, leaf spots, wilt, and root and rhizome rot, were documented, photographed, collected, and brought to Simon Fraser University (SFU) for pathogen isolation and identification.

2.2.2. Microbe isolation

Samples were taken from infected areas (leaves, petioles, rhizomes, roots). Tissues were assessed for disease symptoms, and areas that were blackened or discoloured were excised, cleaned in 0.37% sodium hypochlorite (5% commercial bleach solution) for 20 seconds, then 70% EtOH for 20 seconds, before thoroughly rinsing in autoclaved distilled water. Samples cut into ~1 cm^2 pieces and four such pieces were placed onto potato dextrose agar (PDA) and vegetable juice (V8) agar containing 100 mg/L streptomycin. Bacterial samples were isolated using nutrient agar (NA). Plates were left to incubate for up to three weeks at room temperature under 8 hours light/16 hours dark conditions. Plates were checked weekly for microbe growth, and microbes of interest were sub-cultured. Fungi were sub-cultured by taking a ~0.5 cm^2 agar plug from the edge of the fungal colony and plating on fresh media every 3-4 weeks, and bacteria were sub-cultured by streaking onto fresh media every week.

Since obligate biotrophs cannot be cultured, approximately a 1 cm^2 piece of infected leaf tissue was placed in a 2 mL Eppendorf tube and frozen at -20 °C and saved for future identification using molecular methods.

2.2.3. Morphological Identification

We examined cultures grown on PDA and V8A for characteristic features of the pathogens of interest including colour, growth pattern, and presence or absence of microsclerotia. Fungal tissue was mounted in water onto a glass slide and observed using a Zeiss compound microscope. Conidial morphology was examined for defining characteristics such as pigmentation, septation, ornamentation, shape, size, and presence or absence of spores produced in chains. Conidiophore morphology was also considered based on conidial development (thallic or blastic), shape, septation, and arrangement of conidia.

2.2.4. Molecular Identification

DNA was extracted using a CTAB extraction protocol. Briefly, fungal tissue (0.01 grams) was ground in liquid nitrogen using a mortar and pestle, then added to 600 µL CTAB buffer (2% w/v cetyl trimethylammonium bromide, 1% w/v polyvinyl pyrrolidone, 1.4 M NaCl, 100 mM Tris HCl, 20 mM EDTA) and incubated for 15 min at 65 °C. Samples were centrifuged at 20000 x *g* for 5 min and the supernatant transferred to a new Eppendorf tube. A 1:1 Phenol:Chloroform mix (according to sample volume) was mixed into the supernatant and the tubes centrifuged at 20000 x *g* for 1 min. The upper phase was transferred to a new tube and a 1/10 volume ratio of 7M ammonium acetate was added, followed by 1 volume of ice-cold 100% ethanol. The sample were stored for a minimum of 1 hour at -20 °C before being centrifuged at 20000 x *g* for 10 min and the supernatant removed. The resulting pellet was washed with 500 µL ice-cold 70% ethanol and centrifuged for 2 min at 20000 x *g*. Samples were left in a laminar flow

hood for 20 minutes to evaporate excess ethanol, then re-suspended in 100uL TE and kept at -20 °C.

Fungal species were further identified by sequencing the ITS1-5.8S-ITS2 rDNA region using universal primers UN-UP18S42 (5'-CGTAACAAGGTTTCCGTAGGTGAAC-3') and UN-LO28S22 (5'-GTTTCTTTTCCTCCGCTTATTGATATG-3'). PCR was performed for 35 cycles with an annealing temperature of 55 °C. PCR amplicons were sent to Eurofins (Toronto, ON) for Sanger sequencing. The resulting fasta files were returned to Simon Fraser University where we blasted the files against sequences in GenBank for identification.

Isolated bacteria were identified as gram-positive or gram-negative using the Ryu non-staining KOH Technique (Powers, 1995). Gram-negative bacterial species were further identified using MicroLog® by the Plant Health Laboratory of the British Columbia Ministry of Agriculture. Suspected virus infected tissues were sent to the Summerland Research Centre (Agriculture and Agri-Food Canada) for confirmation based on whole genome sequencing.

2.3. Results and discussion

In 2016, thirty-one plant samples were collected from which 8 potential fungal pathogens, as well as two oomycetes and one bacterial pathogen were identified (Table 2.1). In 2017, 42 plant samples were collected from which nine potential fungal pathogens, as well as one oomycete, two bacterial pathogens, and one virus were identified (Table 2.1). In 2018, 44 plant samples were collected from which 12 potential fungal pathogens, as well as one oomycete, two bacterial pathogens, and one virus were

identified (Table 2.1). Plant samples collected included samples that were from greenhouses that were only visited in a particular year, as well as a few greenhouses that were sampled subsequently across 2016 through 2018. In total, seven different greenhouses were sampled. Several pathogens previously reported on wasabi were isolated over the three survey years and confirmed, including *A. candida*, *B. cinerea*, *C. higginsianum*, *E. cruciferarum*, *L. biglobosa*, *P. carotovorum*, and *P. intermedium* (Rodríguez and Punja, 2007; 2009; MacDonald and Punja, 2016; Park et al., 2016; Punja et al., 2016). Moderate to severe levels of powdery mildew infection were recorded at three of the locations surveyed (Betz and Punja, 2018) (Fig. 2.1a). Phoma leaf spot was recorded in moderate levels in three of seven greenhouses (Fig. 2.1b). Rhizome rot and leaf blight (Fig. 2.1c) caused by *B. cinerea* was also found in three of seven greenhouses, but with more severe symptoms than previously reported (MacDonald and Punja, 2016). Vascular blackening caused by *V. isaacii* was also found at two greenhouses (Betz and Punja, 2018). Root rot, thought to be caused in part by *Fusarium* and *Pythium* species, was also found at low levels across locations. Bacterial soft rot was also present in low levels across multiple greenhouses. Multiple species that have phytopathogenic capability were also isolated (Table 2.2). PDA and V8 agars were used to maximize the number of fungal pathogens isolated from samples. Other isolates found on wasabi plants include *Rhizopus* spp., *Mucor* spp., *Penicillium* spp., *Cladosporium* spp., and *Aspergillus* spp. These species are commonly believed to be saprophytic or endophytic in plant tissues.

In summary, disease surveys are important to track, and ultimately manage, wasabi pathogens. This is the first set of surveys done in British Columbia, and multiple isolates were recovered that have not been previously reported on wasabi. Future surveys

should be continually conducted to keep track of the spread of prevalent and problematic diseases.

Table 2.1 Summary of known wasabi diseases identified during surveys of BC greenhouses in the summers of 2016, 2017, and 2018. Each sample denotes one affected plant.

Disease/ Symptom	Causal organism	Number of samples (2016)	Number of samples (2017)	Number of samples (2018)
Crown rot, leaf blight	*Botrytis cinerea*	3	4	2
Anthracnose	*Colletotrichum higginsianum*	1	-	2
Powdery mildew	*Erysiphe cruciferarum*	5	4	4
Leaf spot, vascular blackening	*Leptosphaeria biglobosa* (*Phoma wasabiae*)	5	6	1
White rust	*Albugo candida*	2	1	1
Root rot	*Pythium intermedium*	1	-	-
Soft rot	*Pectobacterium carotovorum*	2	1	1
Ringspots, leaf mottle	Wasabi mottle virus	-	6	4

Table 2.2 Summary of potential wasabi diseases identified during surveys of BC greenhouses in the summers of 2016, 2017, and 2018. Each sample denotes one affected plant.

Disease/ Symptom	Possible associated organism	Number of samples (2016)	Number of samples (2017)	Number of samples (2018)
Root rot, wilt	*Acremonium sclerotigenum*	-	1	2
Leaf spot	*Alternaria tenuissima*	-	-	2
Root rot, blight	*Drechslera dematioidea*	-	-	1
Root rot, wilt	*Fusarium avenaceum*	2	1	2
Root rot, wilt	*Fusarium oxysporum*	-	1	1
Root rot, wilt	*Fusarium solani*	1	-	-
Root rot, wilt	*Plectosphaerella cucumerina*	-	3	3
Root rot	*Pythium irregulare*	1	-	-
Wilt, vascular blackening	*Verticillium isaacii*	6	5	1
Leaf spot	*Stemphylium vesicarium*		1	1
Soft rot	*Pseudomonas marginalis*		5	1

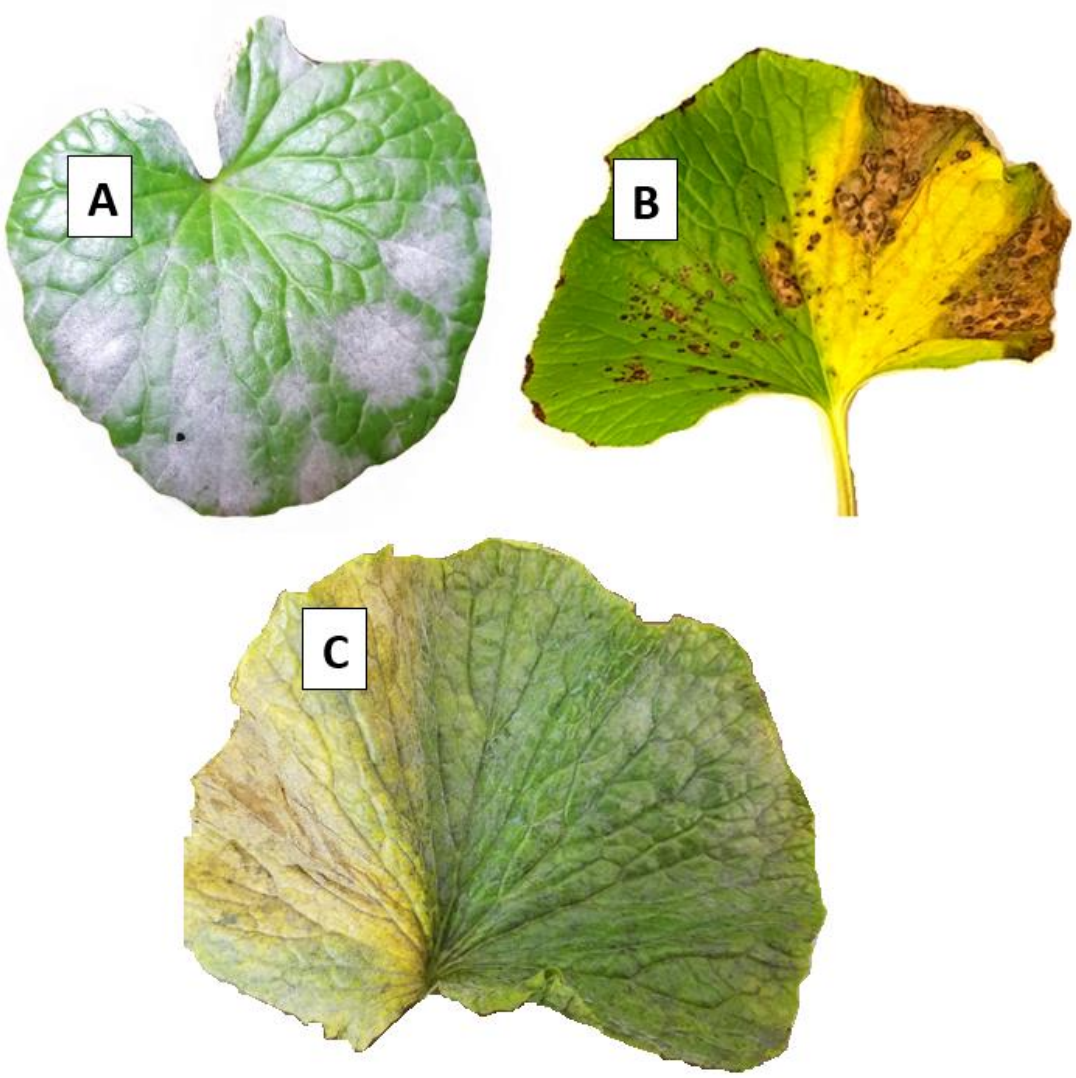


Figure 2.1 Common leaf diseases of wasabi in British Columbia. A) powdery mildew caused by *Erysiphe cruciferarum*, B) leaf spot caused by *Leptosphaeria biglobosa*, and C) leaf blight caused by *Botrytis cinerea*.

Chapter 3. First report of *Verticillium isaacii* causing wilt and vascular blackening on wasabi (*Wasabia japonica*) plants in Canada

3.1. Introduction

Wasabi (*Wasabia japonica* (Miq.) Matsumura, syn. *Eutrema japonicum* Matsum.) is a plant in the Brassicaceae family primarily grown for its valuable rhizome, which is traditionally ground into a paste and used as a condiment (Chadwick et al. 1993). Wasabi was traditionally grown in Japan (Adachi, 1987), but it is now cultivated in several countries including Taiwan (Lo and Wang, 2000), China (Weng et al., 2010), South Korea (Choi et al., 2014), New Zealand (Palmer, 1990), the United States (Chadwick et al., 1993), and Canada (Rodríguez and Punja, 2007; Macdonald and Punja, 2017). The natural habitat of wasabi is that of shaded mountain streambeds or riverbanks in Japan (Adachi, 1987). Wasabi prefers cool, moist environments with ideal growing temperatures from 15-18 °C (Chadwick et al., 1993).

Wasabi in British Columbia (BC) is commercially grown in either double layer polyethylene or glass greenhouses, most often using semi-hydroponic systems with river rock as a growth substrate and overhead misting systems to provide fertilizer (Macdonald and Punja, 2017). As this method creates high moisture conditions ideal for pathogen growth, more growers are experimenting with other systems, such as drip irrigation or hand watering, and various growing substrates including gravel, peat, and hydroton are being tested. The three main cultivars grown in BC are ‘Daruma’, ‘Mazuma’, and ‘Green Thumb’ (Macdonald and Punja, 2017). The slow-growing rhizomes are harvested after

12-18 months, and new plants are obtained by either vegetative cuttings or through tissue culture (Rodríguez and Punja, 2007; Macdonald and Punja, 2017).

Wasabi plants showing wilting symptoms and vascular blackening were observed in one greenhouse in the Fraser Valley of B.C. in 2016. Initial isolations made from symptomatic tissues suggested that a *Verticillium* species may be involved. Previously, vascular blackening in wasabi was reported to be due to a physiological defense response in which the xylem tracheid cells in the rhizome accumulated phenolic compounds within damaged tissues (Rodríguez and Punja, 2009). This symptom was primarily associated with infection by the pathogens *Phoma wasabiae* (*Leptosphaeria biglobosa*) and *Pectobacterium carotovorum* (Lo et al., 2002; Rodríguez and Punja, 2009).

The objectives of this study were to isolate the organism causing the disease symptoms on wasabi, identify the pathogen to species level, and complete Koch's postulates.

3.2. Materials and methods

3.2.1. Sample collection and pathogen isolation

Surveys were conducted for diseases of wasabi in seven B.C. greenhouses during the summers of 2016-2018 (Betz and Punja, 2017a; Betz et al., 2019). Wasabi plants with symptoms of wilting (Fig. 3.1a) were observed in one location in Abbotsford. Additionally, in 2016, plants with leaf necrosis were recovered from a different greenhouse on Vancouver Island (Fig. 3.1b). Infection by a *Verticillium* species was confirmed (Betz and Punja, 2017b). Tissues from symptomatic plants were collected and

used for the isolation of the causal agent. Leaves, roots, and rhizomes were cut into 1 cm^2 pieces and surface-sterilized in 0.37% sodium hypochlorite (5% commercial bleach) solution for 20 s, followed by 70% ethanol for 20 s, and rinsed in sterile distilled water (SDW) for 1 min. The tissues were then cut into smaller chunks (~25 mm^2) and plated onto potato dextrose agar (PDA) with 100 mg/ mL streptomycin sulphate. Plates were incubated at 20 °C ± 2 °C for seven days under ambient light, and the resulting fungal colonies were transferred to fresh PDA.

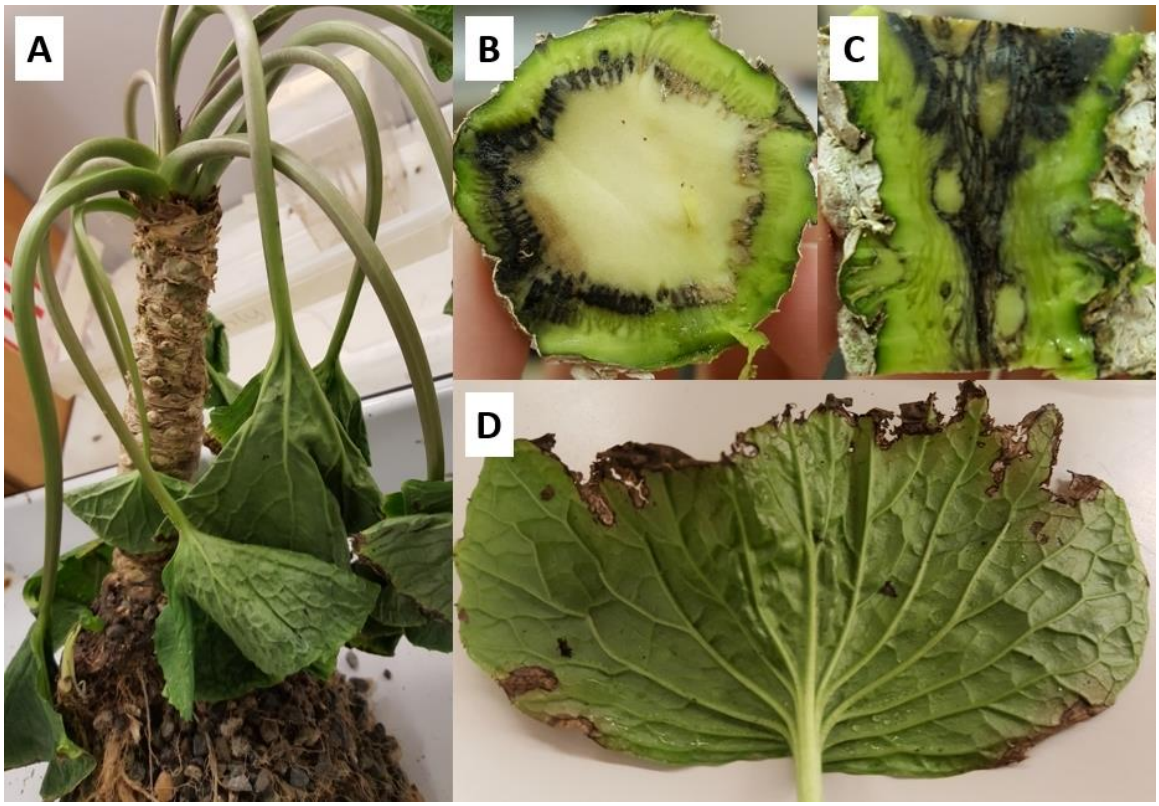


Figure 3.1 Natural progression of disease symptoms on greenhouse grown wasabi plants includes wilt (A) and blackening of the vascular tissue (B, C). In one instance, necrotic lesions were observed on leaves (D).

3.2.2. Identification of *Verticillium* isolates

Morphological criteria

A representative isolate NFIS4923 (National Fungal Identification Service isolate 4923, isolated at Simon Fraser University from a 3-month-old 'Green Thumb' wasabi plant in 2016) was grown on potato dextrose agar at ambient room temperature (20 °C ± 2 °C) for 30 days. The isolate was identified as *Verticillium* sp. by examining colony morphology, as well as characteristics of the conidia and conidiophores (Fig. 3.2). The length of 15 phialides, and the length and width of 25 conidia were measured from a 14-day old culture of isolate ECB1 (isolate information in Table 3.1).

Table 3.1 Isolates of *V. isaacii* used in pathogenicity studies

Isolate	Age of Plant (Months)	Cultivar	Location	Date Collected
ECB1	15	Green Thumb	Abbotsford	April 6th, 2017
ECB2	15	Daruma	Nanoose Bay	August 23rd, 2016
ECB3	16	Green Thumb	Abbotsford	August 16th, 2016
ECB4	15	Green Thumb	Abbotsford	April 6th, 2017
NFIS4923	3	Green Thumb	Abbotsford	May 6th, 2016

Molecular identification

Two isolates of *Verticillium* sp. (NFIS4923 and ECB2), were sent to the University of Guelph Microbial Identification Services, Guelph, ON for identification to species using the internal transcribed spacer (ITS) region and the primers ITS1F and ITS4. Additionally, isolate NFIS4923 was sent to the National Fungal Identification Service (NFIS), Ottawa, to confirm the ITS analysis and to use the actin gene for further species characterization. The DNA sequences were compared with the National Center for Biotechnology Information (NCBI) database using the BLAST program. The NFIS created phylogenetic trees comparing isolate NFIS4923 to other *Verticillium* sp. in order to determine species identification.

3.2.3. Effect of temperature on pathogen growth

A 14-day old culture of isolate NFIS4923 was cut into 1 cm^2 plugs and plated onto fresh PDA plates with 100 mg/L streptomycin sulphate. Five plates each were placed in growth chambers set at 5, 10, 15, 20, 25, 30, and 35 °C and left to incubate for 7 days. After 7 days, the diameter of the colony was measured. This experiment was repeated using isolate ECB1.

3.2.4. Pathogenicity tests

Detached leaf, petiole, and rhizome inoculations

To determine pathogenicity of recovered isolates identified as *V. isaacii* (isolates NFIS4923, ECB2, and ECB3), they were grown in 50 mL of potato dextrose broth (PDB) for 3 weeks, and then homogenized (to a total volume of 150 mL) using a blender. This suspension contained a mixture of mycelium, microsclerotia, and conidia. Five leaves, five petioles, and two rhizomes were excised from healthy greenhouse-grown 'Green Thumb' plants, surface-sterilized with 95% ethanol, wounded using a sterilized scalpel, and inoculated with 20 mL of the suspension. The inoculated tissues were placed in plastic containers lined with moist paper towels, and symptom development was assessed after 7 days. Equal numbers of control plant tissues were wounded and received 20 mL of sterile distilled water instead of a mycelial suspension.

Whole plant inoculations

In addition to detached tissue assays, pathogenicity tests were also conducted on plants grown under greenhouse conditions. Five 6-month-old 'Green Thumb' plantlets grown in 3.8 L pots containing PRO-MIX HP peat/perlite mix (Premier Horticulture,

Rivière du Loup, QC) were wounded by inserting a scalpel at several locations through the substrate to sever the roots. A mycelial suspension was prepared as above, and 50 mL was poured into the substrate. Five control plants received sterile water. Plants were grown in a greenhouse maintained at 20 °C ± 5 °C with a 16/8-hour day/night cycle, and watered with a specialized fertilizer solution containing nitrogen, phosphate, potassium, and micronutrients optimized for wasabi growth (Your Wasabi Farms Ltd, BC). Symptoms were assessed approximately 6 months post infection by uprooting the plants, rinsing the roots with tap water, and measuring the root lengths. The roots and rhizomes were also cut open to examine for symptoms of vascular blackening.

To speed up the infection process, dip treatments were also used to inoculate wasabi plants. Three 1-year-old 'Green Thumb' were uprooted from PRO-MIX HP substrate and their roots were rinsed with tap water. The roots were trimmed to ~10 cm in length using sterile scissors. The wounded roots were soaked in a mycelial suspension (prepared as above, then diluted 5 times with sterile water) for 10 min and then repotted in 3.8 L pots filled with coco coir (CANNA Canada Corp, ON) saturated with fertilizer solution. Two control plants had their roots soaked in 5x-diluted PDB instead of mycelial suspension. The plants were returned to the greenhouse and were assessed after approximately 3 months for symptoms as above.

To assess if different *V. isaacii* isolates varied in pathogenic capability, twelve 15-month-old 'Green Thumb' plants were inoculated as above and immersed in either a mycelial suspension of each of 3 different isolates (NFIS4923, ECB1, and ECB2) or diluted PDB (control). There were 3 plants per treatment. Plants were left to soak in inoculum for 1 hour, followed by repotting in coco coir. Plants were placed in a Conviron

growth chamber set at 15 °C with a 16/8-hour day/night cycle and 80% RH for approximately 4 months.

3.3. Results

3.3.1. Disease symptoms

Symptoms on naturally infected wasabi plants generally included wilting of the leaves and petioles (Fig.1). Blackening of the vascular tissues of the rhizome occurred with disease progression and in some cases, extended into the petiole (Fig. 1 B,C). Often, plants also had symptoms of a secondary bacterial soft rot infection (Supplemental Fig. 1) which was identified through MicroLog™ (BioLog Inc., Hayward, CA) as *Pseudomonas marginalis*.

3.3.2. Species identification

Morphological criteria

Isolate NFIS4923 developed velvety, slow-growing, white-coloured colonies on PDA (Fig. 3.2a). After 30 days, cultures darkened due to production of resting mycelium and microsclerotia (Fig. 3.2b). Abundant amounts of colourless, oval-shaped conidia were produced on phialides arranged in a verticillate pattern along conidiophores (Fig. 3.2d). These characteristics were used to identify the isolates as *Verticillium* sp.. Further narrowing down the identification, some isolates (ECB4) secreted a yellow pigment in culture (Fig. 3.2c) which is consistent with *Verticillium* sp. placed in clade Flavexudans (Inderbitzin et al. 2011). The average length of lateral phialides was 23.6 µm (17.5 µm – 28 µm), and the average length and width of conidia were 7.4 µm (5 µm – 10 µm) and 6.14 µm (4µm – 10µm), respectively.

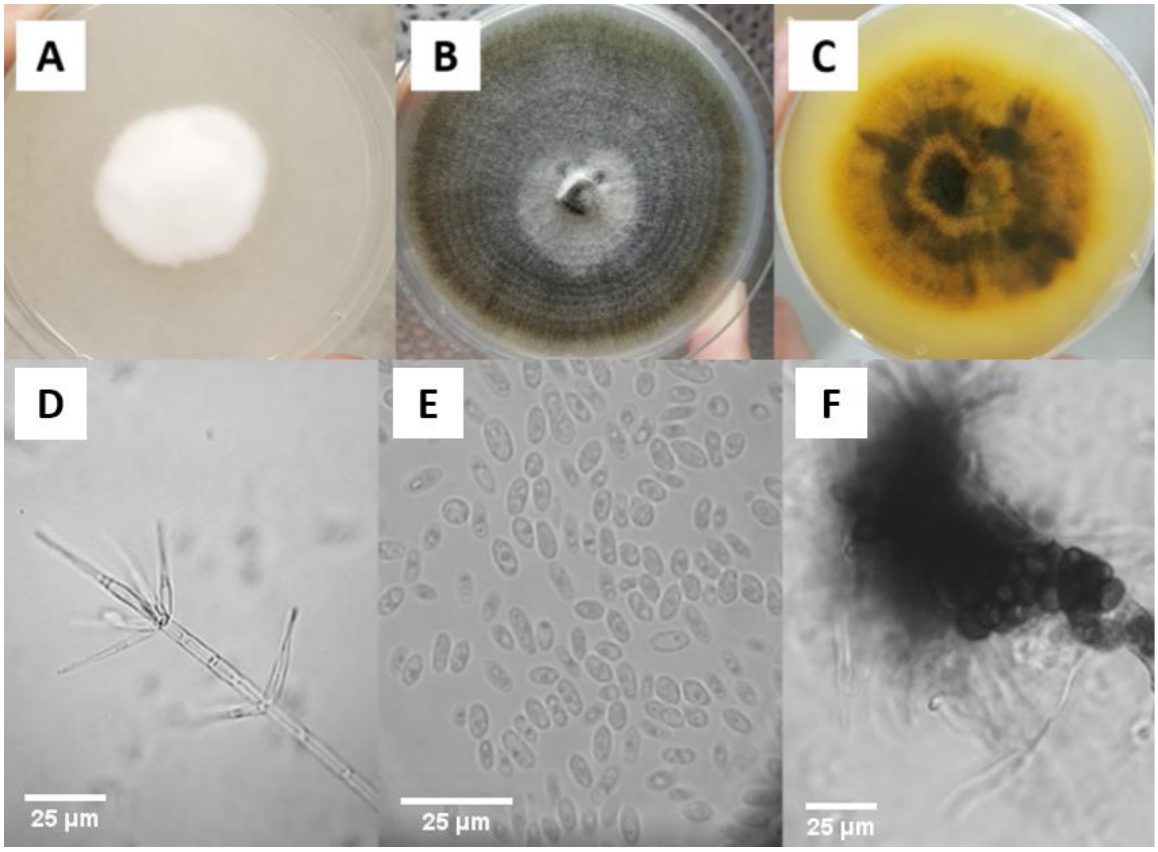
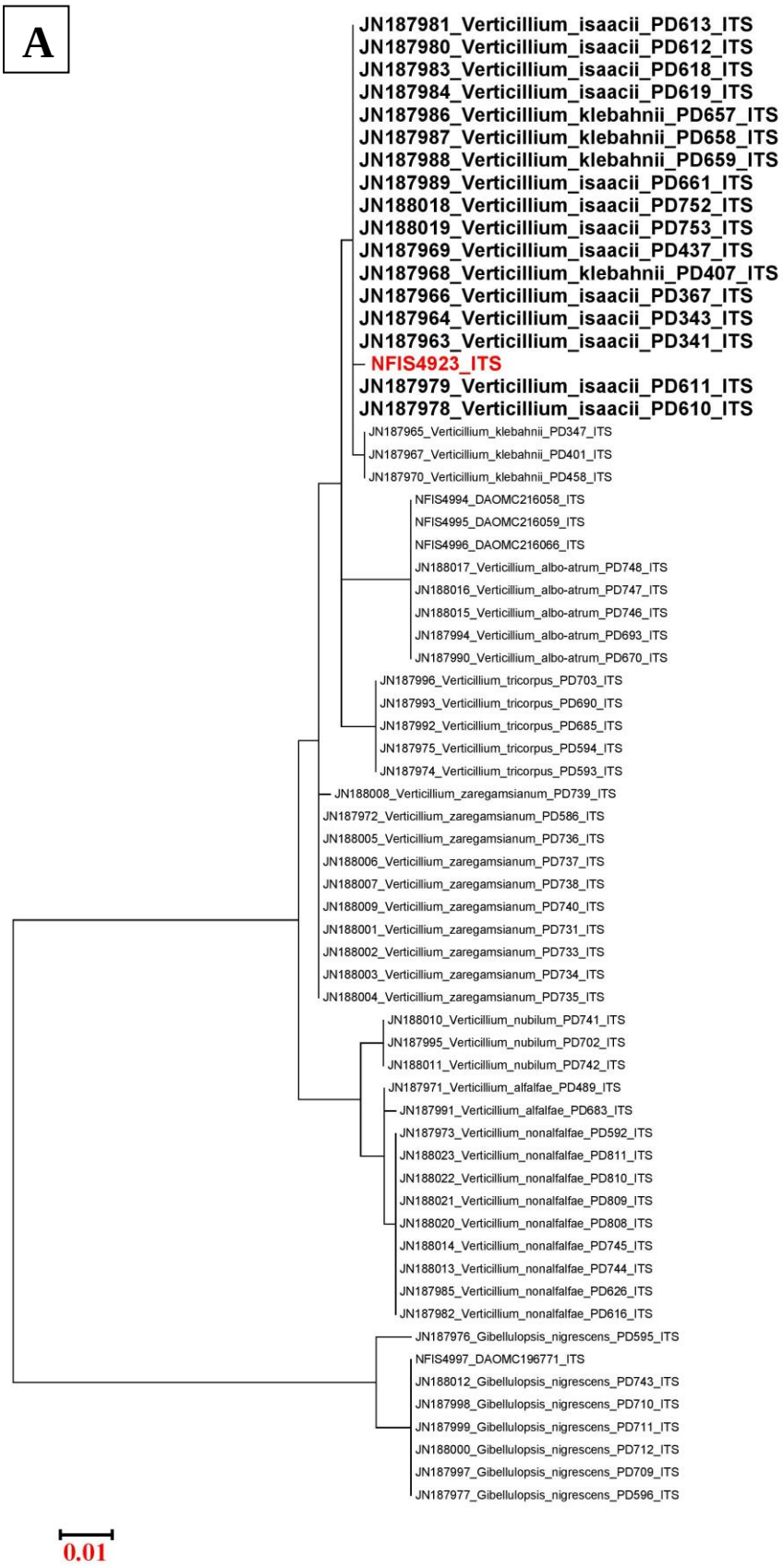


Figure 3.2 Colonies of *Verticillium isaacii* grown on PDA. A) Isolates form velvety, white colonies after 7 days. B) Colonies darken to black as time progresses to 14 days. C) Some isolates, such as ECB4, produce yellow pigments in culture after 7 days. (D-F) Morphology of conidiophores and conidia of *Verticillium isaacii*. D) Verticilliate conidiophores with 3 to 5 phialides. E) Abundant hyaline conidia. F) Dark microsclerotia about 1 mm in diameter. Scale bars = 25 µm.

Molecular identification

The ITS rDNA region of isolate NFIS4923 from wasabi was grouped with a cluster of isolates comprised of 3 species: *V. isaacii* Inderb., Bostock, Davis & Subbarao, *V. klebahnii* Inderb., Bostock, Davis & Subbarao, and *V. tricorpus* (Fig. 3.3a). It was not possible to identify isolate NFIS4923 by PCR of the ITS region alone, but PCR with a *Verticillium*-specific primer set targeting a portion of the actin gene shared a >99%

sequence identity with *V. isaacii* and the isolate clustered with a group of *V. isaacii* isolates from other hosts (Fig. 3.3b).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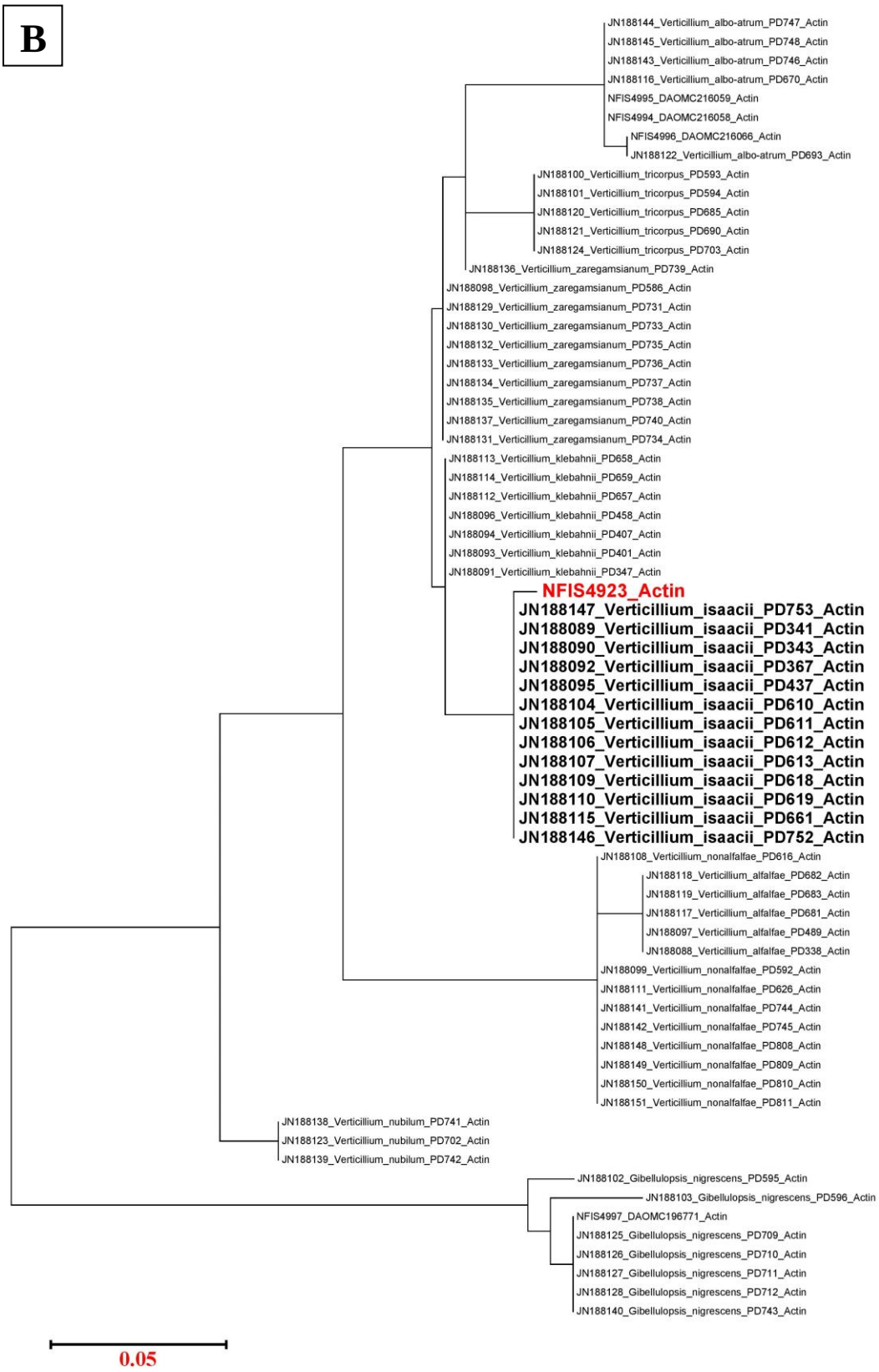

Figure 3.3 Phylogenetic analysis performed by the NFIS of isolate NFIS4923 identified it as *Verticillium isaacii* based on analyses using ITS (A) and actin (B) barcodes.

3.3.3. Effect of temperature on pathogen growth

Verticillium isaacii isolate NFIS4923 grew best at 20 °C with an average diameter of 27 mm colony growth on PDA while isolate ECB1 grew best at 25 °C with an average colony diameter of 21.6 mm. Both isolates have an optimal growth range of 15 – 25 °C (Fig. 3.4).

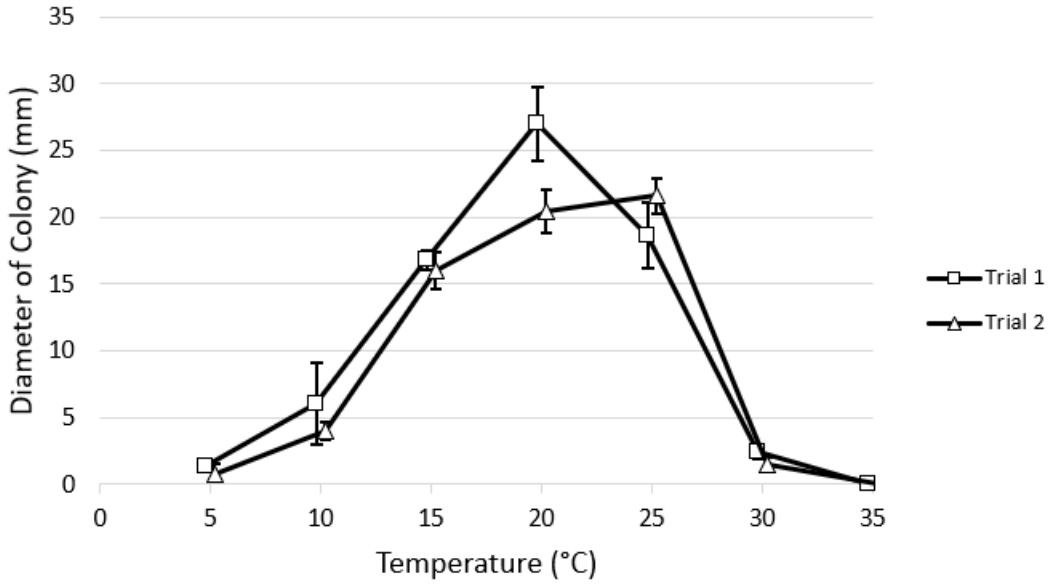

Figure 3.4 *V. isaacii* grown for 7 days at temperatures from 5° to 35° C. Graph displays average diameter of colonies based on two trials. Error bars = 95% CI.

3.3.4. Pathogenicity tests

Detached leaf, petiole, and rhizome inoculations

Inoculations of *V. isaacii* on detached leaves placed in moistened containers resulted in chlorosis and necrosis of tissues around wound sites (Fig. 3.5a), while inoculations of detached petioles resulted in a blackening of tissues around the wound sites with an extension of blackening several millimeters into the vascular tissue (Fig. 3.5c). Inoculation of detached rhizomes resulted in blackening of internal tissues (Fig. 3.5e). The leaves and rhizomes of the controls were free of chlorosis and blackening,

respectively (Fig. 3.5b,d). The petioles of the controls had slight blackening around the wound edges, but this blackening did not extend into the vascular tissue (Fig. 3.5f).

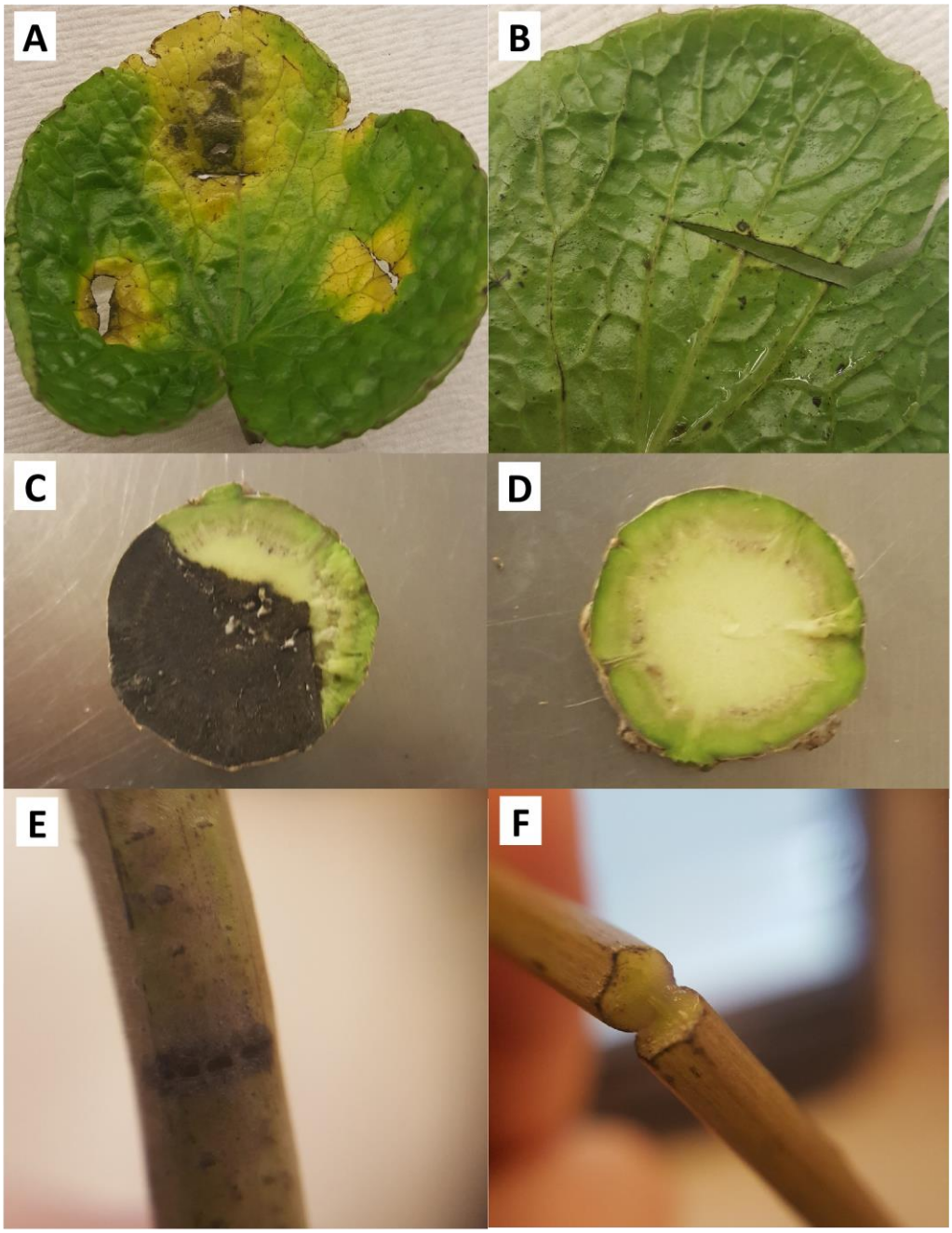

Figure 3.5 Detached and wounded tissues inoculated with a mixed-isolate suspension of *V. isaacii* resulted in chlorotic lesions on leaves (A), blackening of rhizome tissue (C), and vascular blackening of petiole tissue (E). Control tissues (B,D,F) were asymptomatic.

Whole plant inoculations

Inoculations made by pouring a mycelial suspension of *V. isaacii* into the growing substrate failed to cause wilting or any visible symptoms, even after 6 months. Inoculated plants, however, had visibly shorter roots than the control plants (Fig. 3.6a), and the roots were visibly sparser on inoculated plants (Fig. 3.6b). The inoculated treatments also showed blackening of the rhizome vascular tissues which was absent from the control plants (Fig. 3.6c).

Inoculations using the dip method resulted in more severe symptoms with one of three inoculated plants showing wilt symptoms after 3 months (Fig. 3.6e), and all inoculated plants showed vascular blackening symptoms. Control plants were asymptomatic (Fig. 3.6d,f).

In plants inoculated with three different isolates, NFIS4923 caused vascular blackening in the rhizome of all 3 plants. Isolates ECB1 and ECB2 each caused vascular blackening in 2 out of 3 plants. Control plants were asymptomatic.

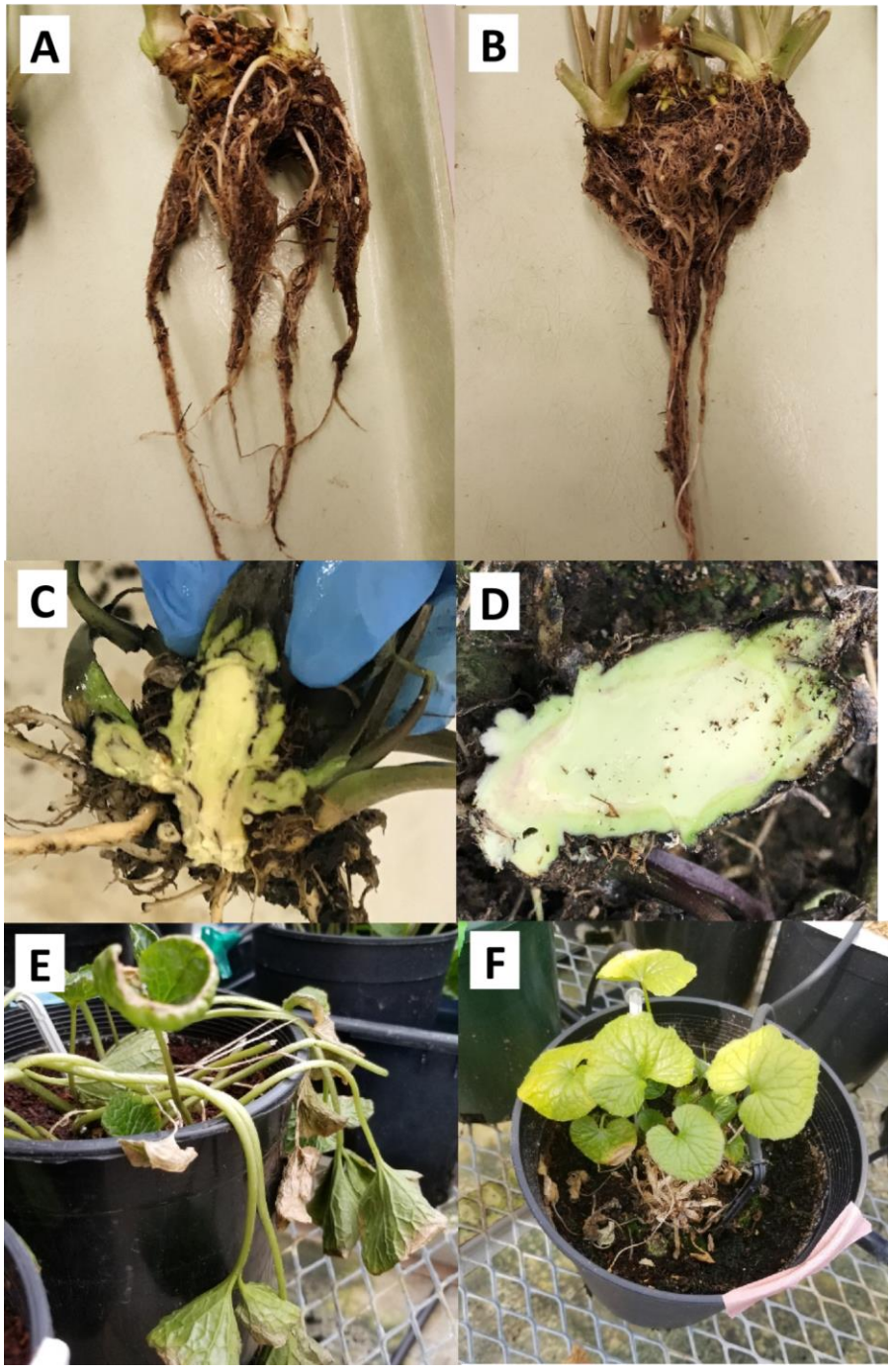

Figure 3.6 After 3 months, plants inoculated with a mixed-isolate mycelial suspension of *V. isaacii* show visibly sparser roots (A) compared to a control plant (B). All inoculated plants had some degree of vascular blackening in their rhizome tissues (C), while controls were asymptomatic (D). Wilting occurred in one of the three inoculated plants (E) while the controls remained unwilted (F).

3.4. Discussion

There is much controversy surrounding species identification of *Verticillium* sp. that infect brassica hosts (Hwang et al., 2017). For the clade of microsclerotia-producing *Verticillium* sp., verticillium wilt on plants in the Brassicaceae family is attributed to *V. longisporum* whereas wilt on plants outside the Brassicaceae family is attributed primarily to *V. dahliae* (Karapapa et al., 1997). However, some short-spore (4-8 µm) isolates of *Verticillium* can infect brassicas, and some long-spore (6-11 µm) isolates can attack plants outside of brassicas (Fahleson et al., 2004; Inderbitzin et al., 2011; Novakazi et al., 2015). Thus, some researchers have argued that *V. longisporum* should not be a separate species from *V. dahliae* but be a subspecies (Barbara and Clewes, 2003; Pegg and Brady 2001). This creates major confusion when diseases caused by *Verticillium* are reported as different researchers will use different identities for the same species (Inderbitzin and Subbarao, 2014).

Verticillium isaacii was first described by Inderbitzin et al. (2011) from a previously indistinguishable clade of *V. isaacii*, *V. klebahnii*, and *V. tricorpus*. These species cannot be differentiated morphologically and furthermore cannot be identified using ITS primers alone, as their ITS sequences are identical (Inderbitzin et. al, 2011). Therefore, we used the actin region to distinguish between species in order to identify our isolate. There are previous reports of *V. tricorpus* infecting wasabi in B.C. (Vippen Joshi, Plant Diagnostic Pathologist, BC Ministry of Agriculture, pers. comm.) but this was based on ITS sequences and the pathogen may in fact be *V. isaacii* as shown in the present study.

Previous pathogenicity tests conducted with *V. isaacii* on various crops have shown the isolates to be weakly pathogenic, with only 1 out of 4 of isolates causing disease symptoms on artichoke and 'Salinas' lettuce after 10 weeks, but all isolates caused wilt on strawberry plants (Gurung et al., 2015). Isolates tested on cauliflower were asymptomatic (Gurung et al., 2015). In fact, a strain of *V. isaacii* has been shown to be a preventative biocontrol agent in cauliflower against verticillium wilt (Tyvaert et al., 2014). It endophytically colonized the plant and prevented *V. longisporum* from colonizing through competition for space (Tyvaert et al., 2014). In sunflower and potato plants, four out of five *V. isaacii* isolates tested were pathogenic and endophytic strains were also reported (Wheeler and Johnson, 2019). These reports suggest that *V. isaacii* can have both an endophytic and pathogenic lifestyle and is generally a weak pathogen.

Gurung et al. (2015) poured a conidial suspension onto the growing media in order to inoculate artichoke, lettuce, strawberry and cauliflower plants, whilst Wheeler and Johnson (2019) mixed microsclerotia suspended in sand into the growing medium to inoculate sunflower and potato. On wasabi plants grown in the greenhouse, we observed that conidial suspensions were not effective at producing symptoms (unpublished data). The suspensions used in our experiments that produced symptoms contained a blend of mycelium, conidia, and microsclerotia. Using these suspensions was faster than using microsclerotia alone, as inoculum as conducted by other authors (Wheeler and Johnson, 2019).

On wasabi plants grown under commercial conditions, symptoms generally appeared on 12-18 month-old plants, suggesting the pathogen could have infected at any time during the preceding period and symptoms developed depending on the prevailing

moisture, temperature, and incubation times. Symptoms were most apparent during the summer season, when ambient temperatures were over 30 °C, and were accompanied by wilting and soft rot symptoms, believed to be caused by *P. marginalis* (unpublished data). We believe that *V. isaacii* infects plants during colder months, but due to heat stress and the prevalence of *P. marginalis* in the summer months, disease incidence was higher. The pronounced symptoms seen in commercially grown plants were difficult to replicate experimentally, as the temperature and moisture conditions, and microbe complex were undetermined. In earlier studies, presence of *P. carotovorum* together with *Pythium* spp. caused soft rot symptoms to develop (Rodríguez and Punja, 2007). The initial source of inoculum of *V. isaccii* is unknown but could be from other Brassicaceae plants or from the growing substrate.

Verticillium wilt is of increasing importance to wasabi growers, as it can be difficult to eradicate, likely due to the presence of microsclerotia in the growing substrate. During surveys, infected plants were found growing in hydroton, gravel, and river rock substrates. Both the cultivars ‘Green Thumb’ and ‘Daruma’ were found to be susceptible to verticillium wilt, but it is yet unknown if other B.C. cultivars are susceptible. The wasabi industry has no registration to traditional pesticides, including the soil fumigants traditionally used to control verticillium wilt. However, as *V. isaacii* is a weak pathogen, some growers have observed that using organic substrates as a growth substrate reduced disease severity, perhaps due to the resident microbial community suppressing the pathogen. Since most growers do not use organic soils to grow wasabi, verticillium wilt will remain problematic until adequate control options are identified.

Chapter 4. Management of powdery mildew, caused by *Erysiphe cruciferarum,* on wasabi (*Wasabia japonica*) plants in British Columbia

4.1. Introduction

Wasabi (*Wasabia japonica* (Miq.) Matsumura, syn. *Eutrema japonicum* Matsum.) is a slow growing crop in the Brassicaceae family. It is grown for its valuable stem (generally referred to as a rhizome), which is traditionally ground into a paste and used as a condiment in Japanese cuisine (Chadwick et al., 1993). In B.C., wasabi is grown in glass or double-polyethylene greenhouses using semi-hydroponic systems. The most popular production systems use beds of river rock as a growing substrate and overhead misters to provide high humidity and deliver fertilizers (Chadwick et al., 1993). Recently, however, some growers are experimenting with drip irrigation and growing plants in other substrates including hydroton, gravel, peat, and soil. The wasabi rhizome takes 12 to 18 months to reach a harvestable size, and due to the long periods between rhizome harvests, growers are increasingly interested in marketing other parts of the wasabi plant, including leaves. Wasabi leaves are edible and can be eaten as salad greens (Chadwick et al., 1993). Additionally, some growers have been processing and selling the leaves for nutraceutical supplements.

Notable root and rhizome-infecting pathogens in B.C. wasabi production facilities include *Leptosphaeria biglobosa* Shoemaker & H. Brun, *Botrytis cinerea* Pers., *Pectobacterium carotovorum* (Jones) Waldee subsp. *carotovorum* (Jones) Bengey et al., as well as *Pythium intermedium* de Bary and *Pythium dissotocum* Dreschler (Macdonald and Punja, 2017; Punja et al., 2017; Rodríguez and Punja, 2007, 2009). However, with

the increasing demand for high quality leaves, foliar pathogens are becoming of increasing importance for wasabi producers. Previously reported foliar pathogens of wasabi in B.C. include white blister rust caused by *Albugo candida* (Pers. ex Lev) Kuntze, leaf spots caused by *Colletotrichum higginsianum* Sacc. and *Leptosphaeria biglobosa*, leaf blight caused by *Botrytis cinerea*, and powdery mildew caused by *Erysiphe cruciferarum* Opiz ex L. Junell (Joshi et al., 2014; Macdonald and Punja, 2017; Punja et al., 2017).

Powdery mildew of wasabi has been previously reported from Japan, Taiwan, and Korea, indicating *E. cruciferarum* to be the causal agent (Lo and Wang, 2000; Oku et al., 1993; Park et al., 2016). The first report of *E. cruciferarum* causing powdery mildew on wasabi in B.C. was in 2013, and powdery mildew has been consistently observed since then (Joshi et al., 2014; Joshi and Jefferies, 2016; Betz and Punja, 2017; Betz et al., 2019). In severe cases of powdery mildew infection, the leaf surfaces can be completely covered by the fungal mycelium and chlorosis and defoliation may occur (Fig. 4.1).

Diseases continue to be a major barrier to successful wasabi production in Canada, as the optimal growing environment for wasabi requires around 80% ambient relative humidity (Chadwick et al., 1993). For disease management, there are four biopesticides registered by the Pesticide Management Regulatory Agency (PMRA) for use on wasabi in Canada (Health Canada, 2016). These include Rootshield® Plus WP (BioWorks Inc.,Victor, NY) and BW240 WP (BioWorks Inc.,Victor, NY), both of which contain a blend of *Trichoderma harzianum* Rifai strain KRL-AG2 and *T. virens* (J.H. Mill., Giddens, & A.A. Foster) Arx strain G-4 as the active ingredients. Also registered are Rhapsody® ASO™ (Bayer Cropscience Inc., Calgary, AB) and Cease® (BioWorks

Inc.,Victor, NY), which contain *Bacillus subtilis* (Ehrenberg) Cohn strain QST 713 as the active ingredient. The published literature lacks any data on the comparative efficacy of these biopesticides for management of wasabi diseases. There is increased demand for disease management options that are both environmentally sustainable and allowed for organic production, and producers are requesting efficacy data for additional products (S. Sabaratnam, Plant Pathologist, BC Ministry of Agriculture, pers. comm.). Furthermore, available fungicide products with varying active ingredients are necessary to help prevent loss of product efficacy due to the pathogen developing resistance.

In this study, four commercially available products, with differing modes of action, were tested for their ability to suppress powdery mildew development on wasabi. The products tested were Actinovate® SP (*Streptomyces lydicus* strain WYEC 108; Novozymes BioAg Inc., Brookfield, WI), Rhapsody® ASO™ (*Bacillus subtilis* strain QST 713), Cueva® (Copper Octanoate Soap; Certis USA L.L.C., Columbia, MD), and Regalia® Maxx (*Reynoutria sachalinensis* extract; Marrone Bio Innovations Inc., Davis, CA). Actinovate® and Rhapsody® are formulated with microbes that produce an array of antimicrobial compounds involved as the primary mode of action (Yuan and Crawford, 1995; Ongena and Jacques, 2008). Cueva® is a copper soap product which causes copper toxicity in bacterial and fungal spores, inhibiting germination. Regalia® is a plant extract of giant knotweed that induces systemic acquired resistance (Su et al., 2012). Induction of polyphenolics involved in plant defense, including fungitoxic flavonoids, in plants sprayed with this extract has been demonstrated (Daayf et al., 2000; Fofana et al., 2002). These four products are approved for use in commercial crop production systems and appear on the Organic Materials Review Institute (OMRI) Canada Products List© (2019).

Of these, only Rhapsody® is currently approved for use on wasabi. The objectives of this study were to confirm the causal agent of powdery mildew on wasabi and demonstrate the utility of selected reduced-risk products as disease management approaches for powdery mildew.

4.2. Materials and methods

4.2.1. Sample collection and pathogen identification

Disease surveys were conducted during the summers of 2016 and 2017 of three wasabi production facilities located in Abbotsford, Surrey and Burnaby, B.C. where the cultivar 'Green Thumb' was showing symptoms of powdery mildew infection (Fig. 1). Diseased leaves were collected and ~1 cm^2 pieces of infected tissues were placed inside Eppendorf tubes and sent to the University of Guelph Laboratory Services, Agriculture and Food Laboratory, Guelph, ON, for PCR identification using ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers. The resulting sequence was compared to entries in the National Center for Biotechnology Information (NCBI) database using the BLAST program. Entries from NCBI were aligned in MEGA X using ClustalW alignment and compared in a Neighbour-Joining phylogenetic tree (Saitou and Nei, 1987; Kumar et. al, 2018).

In addition to the molecular identification, infected tissue was cryofixed in a graphite-water colloidal mixture (G303 Colloidal Graphite, Agar Scientific, UK) and Tissue-Tek O.C.T. (O.C.T. Compound, Sakura Finetek, NL) by submergence into a nitrogen slush for 120-230 s, and the conidial morphology of the pathogen was observed using scanning electron microscopy (FEI Helios NanoLab 650 SEM/FIB equipped with

the Quorum PP3010T cryo system; 4D LABS, SFU, Burnaby, BC. Conducted by Darren Sutton). Conidia were also observed using light microscopy in a Zeiss compound microscope and the length and width of 25 conidia were measured.

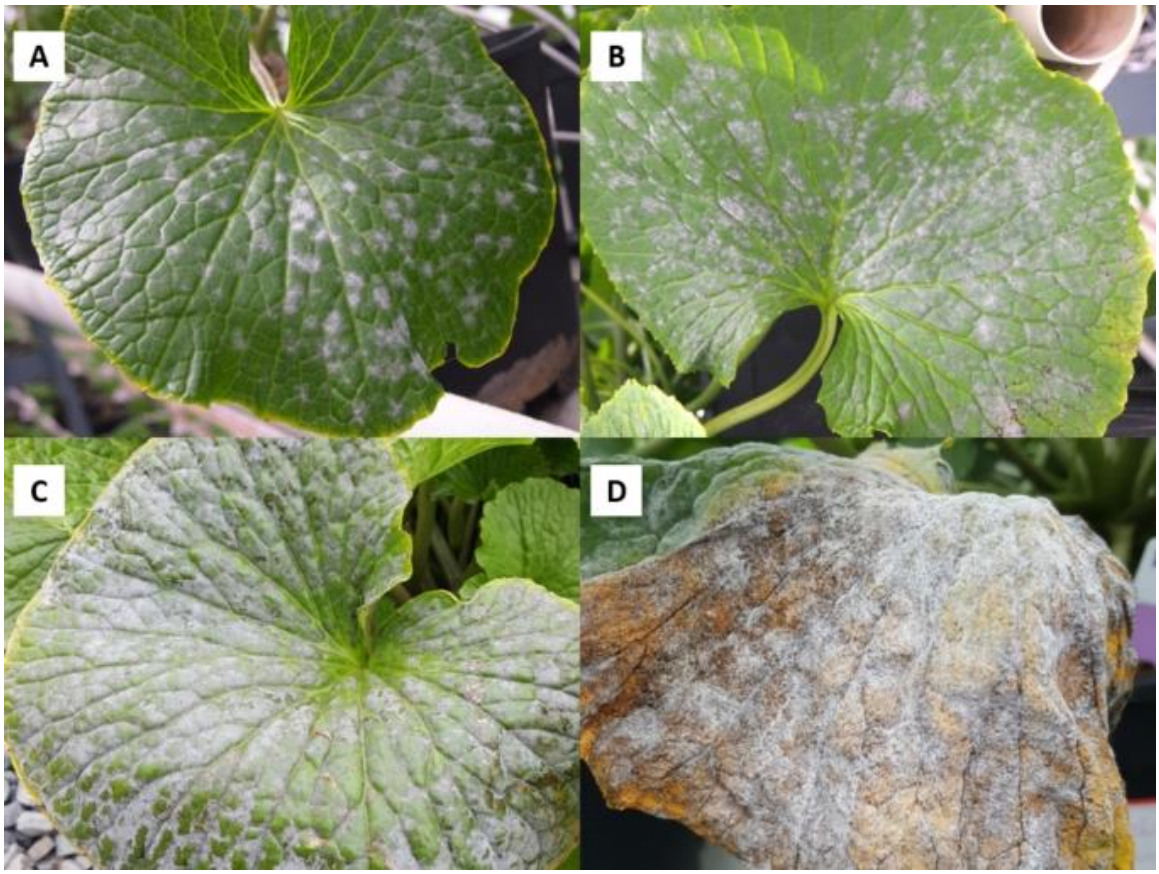

Figure 4.1 Powdery mildew development on wasabi leaves caused by *Erysiphe cruciferarum*. (A) Early development of disease, showing white colonies. (B) Advanced stages of infection. (C, D) Severe powdery mildew infection that can cause yellowing.

4.2.2. Applications of environmentally sustainable products for disease management

Mature wasabi 'Green Thumb' plants (between 14-16 months of age) growing in a commercial greenhouse were treated with either Actinovate®, Rhapsody®, Cueva®, Regalia®, or a water control using a backpack sprayer. The greenhouse was glass with natural light, and the plants were grown in a hydroton substrate in individuals pots in 18 rows with approximately 200 plants per row. As the greenhouse is a controlled

environment, drift was assumed to be minimal. Sprays were made to run-off (with the exception of Actinovate which was sprayed to wetness, according to label directions) every two weeks over a total of 10 (in 2017) or 12 (in 2018) weeks. Applications were made according to label rates of 1% (v/v) for Cueva® and Rhapsody®, 0.5% (v/v) for Actinovate®, and 0.25% (v/v) for Regalia®. These rates were selected to represent the middle of the recommended label rates, except for Regalia®, which was at the upper range of recommended rates (Bayer CropScience Inc., 2017; Monsanto Canada ULC, 2018; Marrone Bio Innovations Inc., 2019; W. Neudorff GmbH KG, 2019). The trials were conducted three times in the same greenhouse as described below.

Trial 1. May – August 2017

The trial was located within a row in the center of the greenhouse, with plants sprayed with Actinovate® (n=20), Rhapsody® (n=20), or water (n=40) for a total of 80 plants. Plants within each treatment were divided into groups of five and were randomly assigned positions along the greenhouse row. Each group had a minimum of four untreated buffer plants between them. This made it practical to measure disease development and to minimize differences in disease progression due to location. Grouping plants together also helped to avoid drift and cross-contamination.

Trial 2. September – December 2017

The trial was located within a row in the centre of the greenhouse, with 20 plants each sprayed with either Actinovate®, Cueva®, Rhapsody®, Regalia®, or water. Each treatment had four groups of 5 (n=20/treatment), and each group was placed randomly along the row, ensuring a buffer of at least four plants between treatment groups (Fig. 4.2). This trial was repeated once.

Trial 3. March – May 2018

The trial was located within a row that was towards the edge of the greenhouse, with 20 plants each sprayed with Cueva®, Rhapsody®, Regalia®, or water. Actinovate® was omitted from this trial. Each treatment had four groups of 5 (n=20/treatment), and each group was placed randomly along the row, ensuring a buffer of at least four plants between treatment groups. This trial was repeated once.

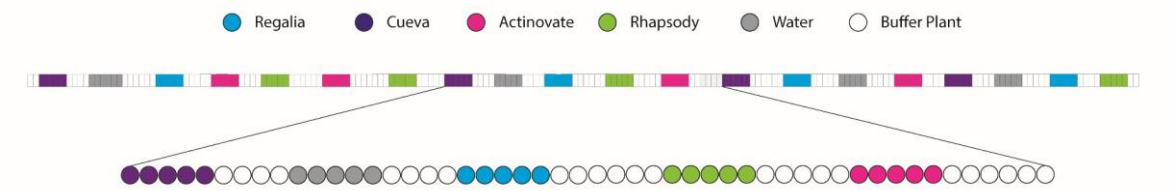

Figure 4.2 Experimental design for Trial 2. Treatments were divided into 4 groups/ treatment with each group containing 5 plants. At least 4 untreated buffer plants were placed between treated plants.

4.2.3. Assessment of disease development

Powdery mildew developed naturally from pre-existing inoculum in the greenhouse onto wasabi plants. Once symptoms started to appear, plants were randomized (via random number generation) into trials. Since the trials were situated within an existing commercial operation, all plant pruning, management and other practices were conducted according to industry recommendations. Plants in the trial were not sprayed with any products other than the experimental treatments. Disease severity was assessed every two weeks by using a custom scale (Trial 1) or the Horsfall-Barratt scale (Trials 2 and 3) to measure the mean percentage of leaf surface area infected by mildew (Horsfall and Barratt, 1945). The custom scale evaluated coverage on a 5-point scale, 1 = 1-10%, 2 = 11-25%, 3 = 26-50%, 4=51-75%, and 5=76-100%. The Horsfall-Barratt scale evaluated

coverage on a 12-point scale, 1=0%, 2=1-3%, 3=4-6%, 4=7-12%, 5=13-25%, 6=26-50%, 7=51-75%, 8=76-87%, 9=88-94%, 10=95-97%, 11=98-99%, and 12=100%. The 10 most basal leaves of each plant were used to calculate the means, seeing as they develop the most mildew. The scores from the scales were converted to percent leaf coverage which was then used to calculate an area under the disease progress curve (AUDPC) value for each treatment according to the following formula (Shaner and Finney, 1977):

$$AUDPC = \sum_{i=1}^{n-1} \left[\frac{Y_i + Y_{i+1}}{2}\right] [X_{i+1} - X_i]$$

Where Y_i is disease severity in percent at the i^{th} observation, X_i is time (weeks) at the i^{th} observation and n is the total number of observations. Statistical analysis of AUDPC values was completed in R 3.5.1 using a one-way ANOVA (Tukey's HSD, α=0.05).

4.3. Results

4.3.1. Pathogen identification

The sequence of the ITS1-5.8S- ITS2 rDNA region from a sample of powdery mildew infected leaf tissue placed the B.C. isolate within a cluster with *Erysiphe cruciferarum* isolates from a range of cruciferous hosts and geographic regions with 100% similarity, including a powdery mildew isolate found on wasabi in Korea (Fig. 4.3). Scanning electron micrographs showed oblong to cylindrical conidia borne singly on conidiophores (Fig. 4a,b) which is consistent with the morphology of members of the genus *Erysiphe* section *Erysiphe* (Heffer et al., 2006). The spore measurements showed that conidia had an average length of 39.4 µm (32.5 µm – 45 µm) and an average width

of 16.3 µm (12.5 µm – 20 µm) (Fig. 4.4c) which is consistent with other reports of *E. cruciferarum* on wasabi (Oku et al., 1993; Park et al., 2016).

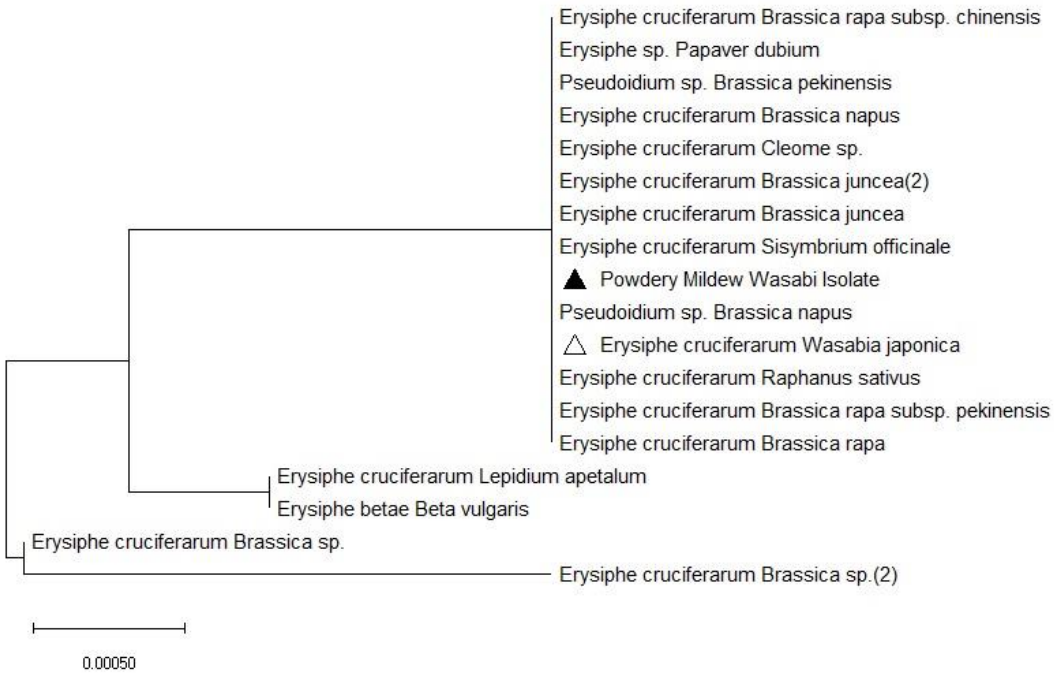

Figure 4.3 Phylogenetic analysis of powdery mildew isolates identified as *E. cruciferarum* from several cruciferous hosts, including the wasabi powdery mildew from Korea (Δ). The sequence from the BC wasabi isolate (▲) was subjected to NCBI Blast, and aligned in MEGA X using ClustalW, and compared using a Neighbour-Joining tree.

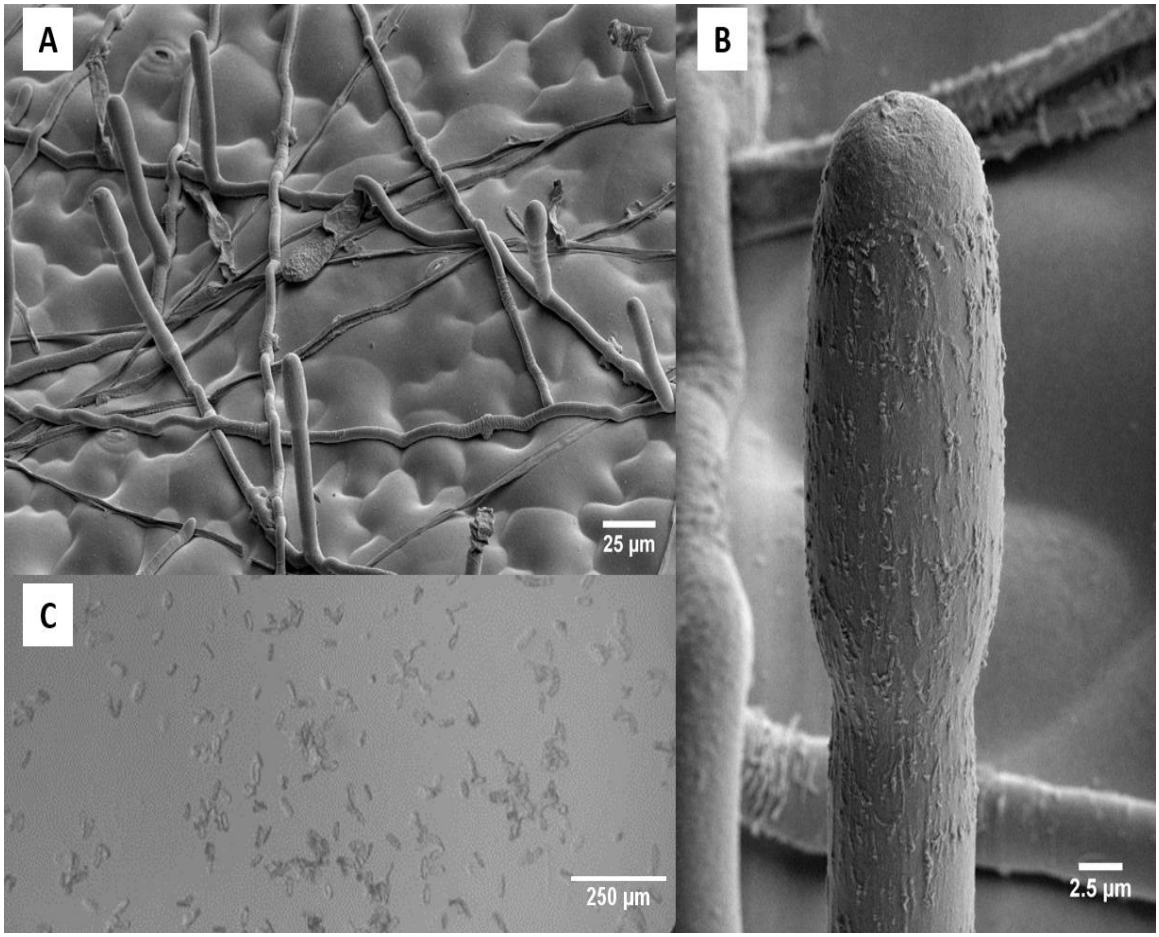

Figure 4.4 Morphology of conidiophores and conidia of *E. cruciferarum*. A) Conidiophores and hyphae growing across a wasabi leaf surface (scale bar = 25 µm). B) A close-up of a developing conidium (scale bar = 2.5 µm). Note the lack of conidial chains. C) Conidia observed under light microscopy (scale bar = 250 µm).

4.3.2. Assessment of disease development

Powdery mildew disease severity increased over time in all treatments in each of the three trials (Fig. 4.5). After 10 weeks, plants treated with Cueva®, Regalia®, and Rhapsody® had visibly lower development of disease. (Fig. 4.6). Actinovate® treated plants had comparable mildew development to the water sprayed plants (Fig. 4.6). AUDPC values calculated after 10 weeks showed that Actinovate® did not significantly decrease the progression of powdery mildew in either of Trial 1 or Trial 2 (Fig. 4.7 a,b). In contrast, both Regalia® and Cueva® significantly ($p<0.05$) reduced progression of

powdery mildew in all trials. Rhapsody treatments also reduced disease progression in Trials 1 and 3 (Fig. 4.7b). Applications of Regalia® and Cueva® significantly reduced disease progression compared to Rhapsody® but did not differ significantly from each other.

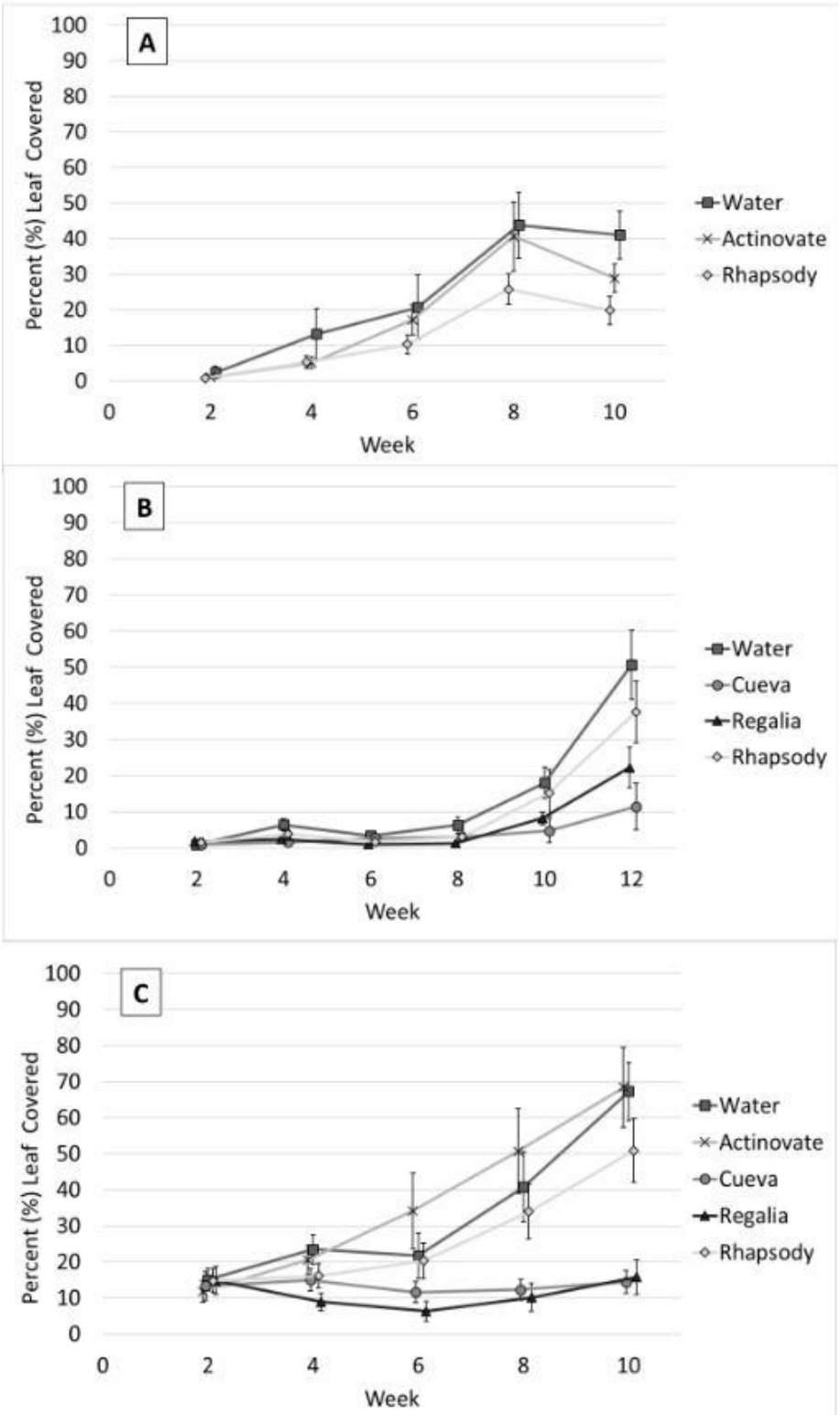

Figure 4.5 Progression of powdery mildew infection on wasabi plants following treatments with Actinovate, Cueva, Regalia, Rhapsody, and a water control. A) Trial 1 (10 weeks), B) Trial 2 (10 weeks), and C) Trial 3 (12 weeks). Bars represent 95% confidence intervals.

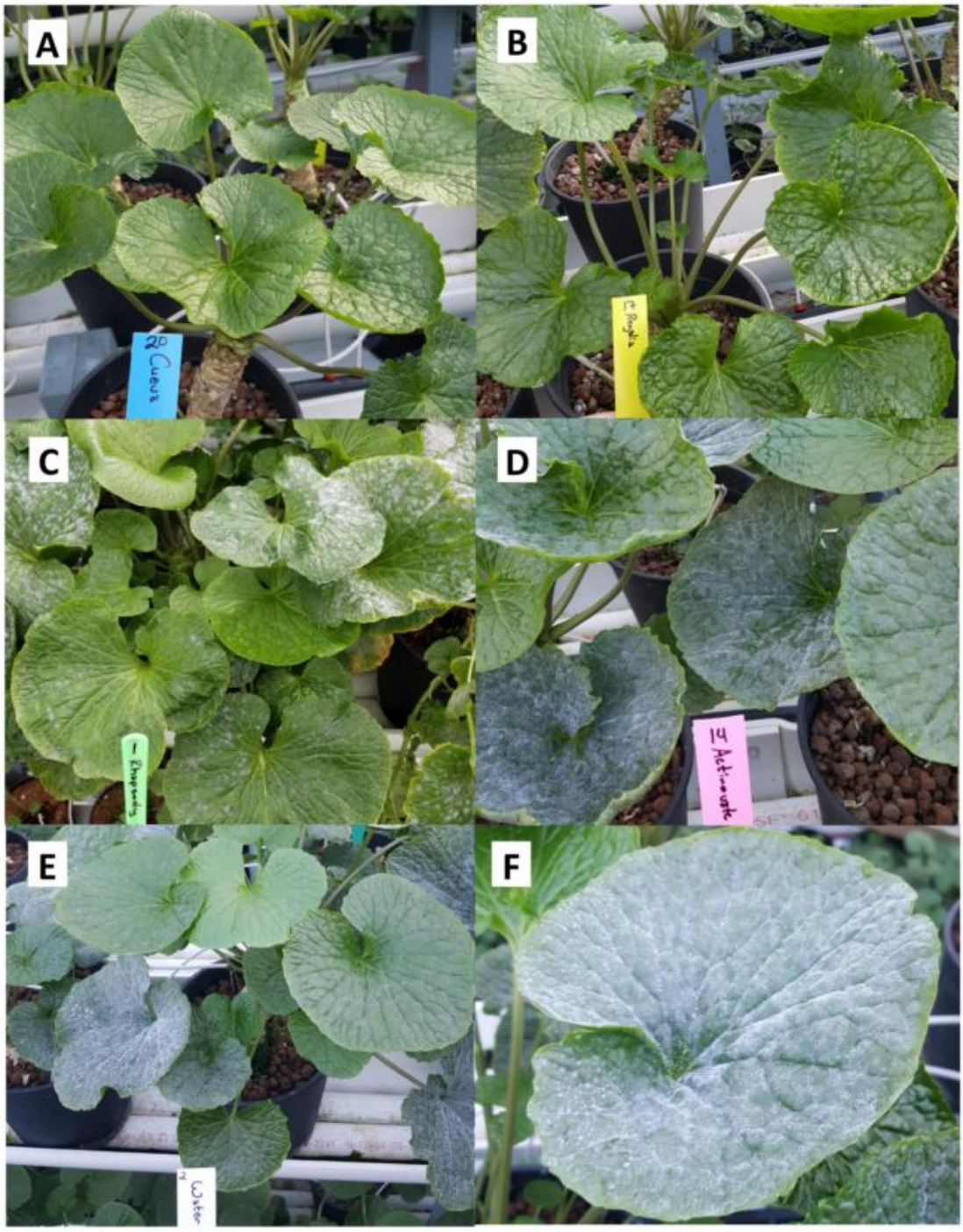

Figure 4.6 Development of powdery mildew on wasabi plants, 10 weeks after treatments were initiated. (A) Cueva and (B) Regalia applications reduced powdery mildew development compared to a water control (E). (C) Rhapsody showed slight-to-moderate disease suppressive capability. (D) Actinovate had no disease suppression compared to the water control (E,F).

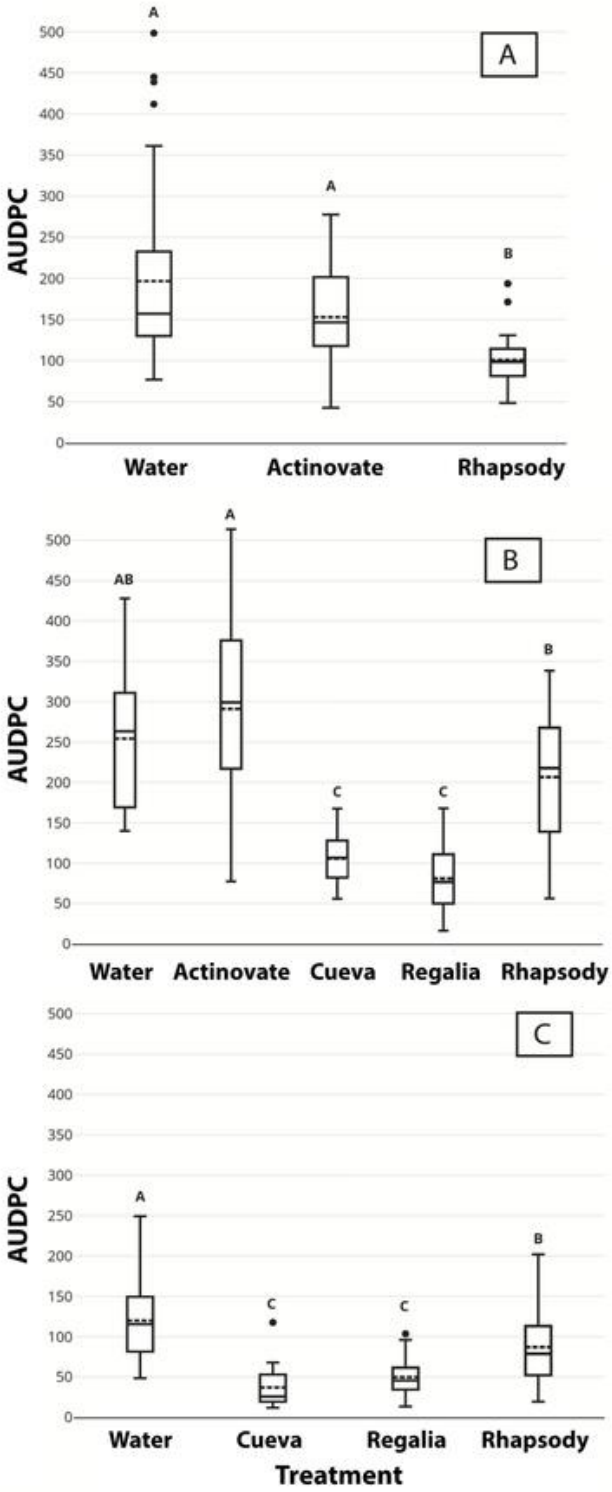

Figure 4.7 AUDPC assessments to evaluate disease severity over time on wasabi plants treated with reduced risk products. A) Trial 1, B) Trial 2, C) Trial 3. Disease progression was similar in all trials. The boxes indicate the interquartile range, while the whiskers denote the largest and smallest sample points. The solid line represents the median, where the dotted line represents the mean. Outliers are represented by dots outside the box plot. The letters above the plot indicate which groups are statistically different from one another.

4.4. Discussion

Previous reports have shown that the pathogens affecting wasabi in B.C. have wide host ranges that include other cruciferous plants (Joshi et al. 2014; Betz and Punja, 2017; Betz et al., 2019). The powdery mildew pathogen, *Erysiphe cruciferarum*, can also infect other members of the Brassicaceae family, including weed species (Koch and Slusarenko, 1990; Enright and Cipollini, 2007), which may provide an additional inoculum source. Powdery mildew can spread rapidly through a wasabi greenhouse by production of large numbers of air-disseminated conidia, resulting in foliar infections that can render diseased leaves unmarketable. The production system used to grow wasabi may play a role in severity of powdery mildew. Plants receiving drip irrigation tended to have higher levels of powdery mildew infection than those that receiving overhead misting. This is likely due to the leaves remaining wetter for longer periods with misting, limiting infection as powdery mildew fungi are favoured by drier conditions (Schnathorst, 1960). However, higher moisture levels can enhance the incidence of other diseases, including those caused by *Botrytis*, *Colletotrichum*, and *Leptosphaeria* (Punja et al., 2017; MacDonald, 2018). The current strategies used to manage powdery mildew development on wasabi include regular pruning of infected leaves and weekly sprays of Rhapsody® during heavy infection (L. Benkrima, Lead Scientist, Your Wasabi Farms Ltd., pers. comm.). While removal of diseased leaves is effective when infection levels are low, it is less effective under high disease pressure and increases cost of production. Mechanical removal of leaves can also provide entry points for other pathogens, including *B. cinerea*, *L. biglobosa* and *Wasabi mottle virus* (Punja et al. 2017; Macdonald et al., 2019).

Rootshield® and BW240 are registered on wasabi for control of root rot and damping off due to *Pythium* sp., *Rhizoctonia* sp., *Fusarium* sp., and *Phytophthora* sp. (BioWorks Inc., 2019a; 2019b); Rhapsody® and Cease® are registered for pythium root rot (*Pythium dissotocum* and *Pythium intermedium*), phytophthora crown and root rot (*Phytophthora cryptogea* Pethybr. & Laff.), and powdery mildew (*E. cruciferarum*) (Bayer CropScience Inc., 2017; BioWorks Inc., 2016). Rhapsody® and Cease® are the only products currently approved for use for management of powdery mildew on wasabi in Canada and they have a common active ingredient, *B. subtilis.* The identification of additional disease management products would provide wasabi growers with alternatives for rotation programs. Both Cueva® and Regalia® Maxx were shown to suppress *E. cruciferarum* development on wasabi in the present study. Copper-containing products are known for their fungitoxic properties and inactivate enzymes and other proteins in microbes (Lamichhane et al., 2018). They are widely used in organic agriculture and are effective against multiple diseases on a range of crops, including powdery mildew on cruciferous crops (Lamichhane et al., 2018). Cueva®'s formulation consists of copper bound to octanoic acid (copper octanoate) (W. Neudorff GmbH KG, 2019), which renders it more persistent on the leaf surface and reduces its uptake by the plant, thereby reducing phytotoxicity (Marine et al., 2016; W. Neudorff GmbH KG, 2019). Applications of copper octanoate were found to reduce powdery mildew on tomato and cucurbits (Baysal-Gurel and Miller, 2015; Marine et al., 2016). Applications of Cueva® to wasabi leaves resulted in higher levels of copper in leaf tissues (195 ppm in treated leaves compared to 18 ppm in control leaves) based on tissue analysis, but the levels in the rhizomes were unaffected (unpublished data). The majority of the copper adhered to the

leaf surface as washing the leaves prior to tissue analysis significantly reduced copper levels (unpublished data).

Regalia®, a product formulated with extracts from the giant knotweed plant, *Reynoutria sachalinensis*, was previously reported to induce chalcone synthase and chalcone isomerase pathways in cucumber plants, boosting flavonoid production (Fofana et al., 2002). Phytoalexins and phenolic compounds were also shown to be enhanced in plants treated with *R. sachalinensis* extract (Daayf et al, 1997; Daayf et al., 2000). The mode of action of Regalia® on cruciferous crops, including wasabi, has not been reported, but is likely to be similar to that in previous studies. Total phenolic content assays using the Folin-Ciocalteu method showed a trend of increased phenolic content in wasabi leaves treated with Regalia® (unpublished data), which suggests a similar mode of action on wasabi plants. Some symptoms of phytotoxicity were observed in this study following five applications of Regalia® (Fig. 4.8). This could be alleviated by reducing the concentration and frequency of spray applications; however, this may also reduce the efficacy for disease management.

Figure 4.8 Regalia applications may cause phytotoxic effects on wasabi leaves including chlorosis and blackening of the leaf.

Both Rhapsody® and Actinovate® were less effective for powdery mildew suppression on wasabi plants. Both formulations contain microbes that produce a spectrum of antimicrobial compounds and hydrolytic enzymes (Yuan and Crawford, 1995; Ongena and Jacques, 2008), and Rhapsody was shown to reduce powdery mildew development on cucumber plants (Ni and Punja, 2019; Punja et al., 2019). However, biocontrol efficacy can be affected by environmental factors such as ultraviolet radiation, low humidity, nutrient availability, and competition from other microorganisms present in the phyllosphere (Mnif and Ghribi, 2015). On the surfaces of tomato fruits, populations of *B. subtilis* declined steadily following an application of Rhapsody in a greenhouse

environment, requiring multiple applications to maintain population levels (Punja et al., 2016). Additionally, volatile compounds released from wasabi leaves can inactivate entomopathogenic fungi such as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Atsumi and Saito, 2015) and may similarly influence survival of biopesticides, but this requires further research.

In conclusion, Cueva® and Regalia® show potential for powdery mildew disease management on wasabi but are not currently registered for use in Canada.

Chapter 5. General discussion and conclusions

5.1. Disease surveys

The identification of *Verticillium isaacii* as a new pathogen of wasabi brings the current worldwide total of known wasabi diseases to 24. Along with the confirmed presence of *Erysiphe cruciferarum*, these identifications bring the total of confirmed pathogens in B.C. up to 11. The three years of surveys also identified 8 previously unreported disease-associated microbes found on wasabi, including *Fusarium* sp. Additionally, the surveys found *Pseudomonas marginalis* associated with soft rot, and *Pythium irregulare* associated with root rot – both of which have been reported in literature (Goto and Matsumoto, 1986; Joshi *et al.*, 2014; Joshi *et al.*, 2015) but lack the inoculation studies required to confirm pathogenicity. This brings the total of disease-associated microbes found on wasabi worldwide from nine to eighteen and from four to twelve in North America.

5.2. Control of wasabi diseases

As most wasabi diseases found in B.C. have broad host ranges that include other brassica species, most of the primary inoculum sources are assumed to be other cruciferous crops and weed species. Other possible inoculum sources include the growing substrate, watering systems, and mechanical transmission from infected wasabi plants. In the case of wasabi mottle virus, the inoculum source is suspected to be imported wasabi plants. It is also possible that insect vectors play a role in the transmission of certain diseases, especially viruses.

5.3. Future research

The disease surveys we conducted identified a multitude of microorganisms with the potential to be pathogenic on wasabi. These microbes should be confirmed for phytopathogenic capability and virulence on wasabi. This involves plant inoculation experiments, and well as subsequent recovery of microbes from symptomatic plants. This would help to further identify pathogens found on wasabi in the Pacific Northwest region of B.C.

During the years surveying wasabi plants, cultivar was found to greatly influence the presence of disease. ‘Green Thumb’ plants were observed to be more susceptible to a variety of diseases than the other common grown cultivars. In fact, ‘Green Thumb’ plants in the laboratory all developed a serious white rust infection while the ‘Daruma’ plants sitting beside them on the bench were unaffected. These suspected cultivar differences should be further investigated in a controlled setting.

As Regalia® and Cueva® were effective at slowing the progression of powdery mildew on wasabi, it is possible that they would also be efficacious on other wasabi pathogens. Therefore, experiments should be conducted on the ability of these, and other reduced risk products, to supress commonly found wasabi diseases such as phoma leaf spot and botrytis rhizome rot. It would also be useful to have data on the effects of chemical pesticides on wasabi diseases, in case of severe disease outbreaks.

The lack of current disease control methods, as well as information on what diseases are present, limit options for disease management. Without more management

options (such as products registered for use on wasabi) growers will continue to suffer losses.

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Appendix A.

Dual culture assays to assess *in vitro* pathogen inhibition by biofungicides

A.1 Methods

Biocontrols were assessed in vitro (on solid media) for efficacy against several wasabi pathogens including *Leptosphaeria biglobosa*, *Verticillium* sp., *Fusarium avenaceum*, *Fusarium oxysporum*, *Pythium intermedium*, and *Pythium irregulare*.

We chose 5 commercially available biological control agents to test – Actinovate® SP (*Streptomyces lydicus* strain WYEC 108), Rhapsody ® ASO (*Bacillus subtilis* strain QST 713), Rootshield ® WP (*Trichoderma harzianum* strain T-22), Cueva® (Copper octanoate soap), and Regalia® (*Reynoutria sachalinensis* extract).

For Actinovate, Rootshield, and Rhapsody, the active organism was isolated onto either LB or V8 Agar plates. For Cueva and Regalia, the solutions were diluted to the maximum rate recommended on the label (2% and 0.25%, respectively). For the microbial controls, the organism was streaked (Actinovate and Rhapsody) or added as a 7mm plug (Rootshield) onto LB and PDA Agars. For Cueva and Regalia, 100µL was pipetted into 7mm wells made on the plate. For each plate, a pathogen was added 24 hours later. The plates were assessed either 5 days (both *Pythium* sp.) or 3 weeks (*Fusarium*, *Phoma*, and *Verticillium*) depending on rate of pathogen growth.

A.2 Results

There were obvious inhibition zones in all Rhapsody and Actinovate plates. Rootshield also inhibited pathogen growth, but without the inhibition zones (Fig. A.1 shows an example). Both Cueva and Regalia showed little to no inhibition on plates. When grown on PDA, all pathogens had significant growth reduction on Actinovate, Rhapsody, and Rootshield plates (Fig. A.2).

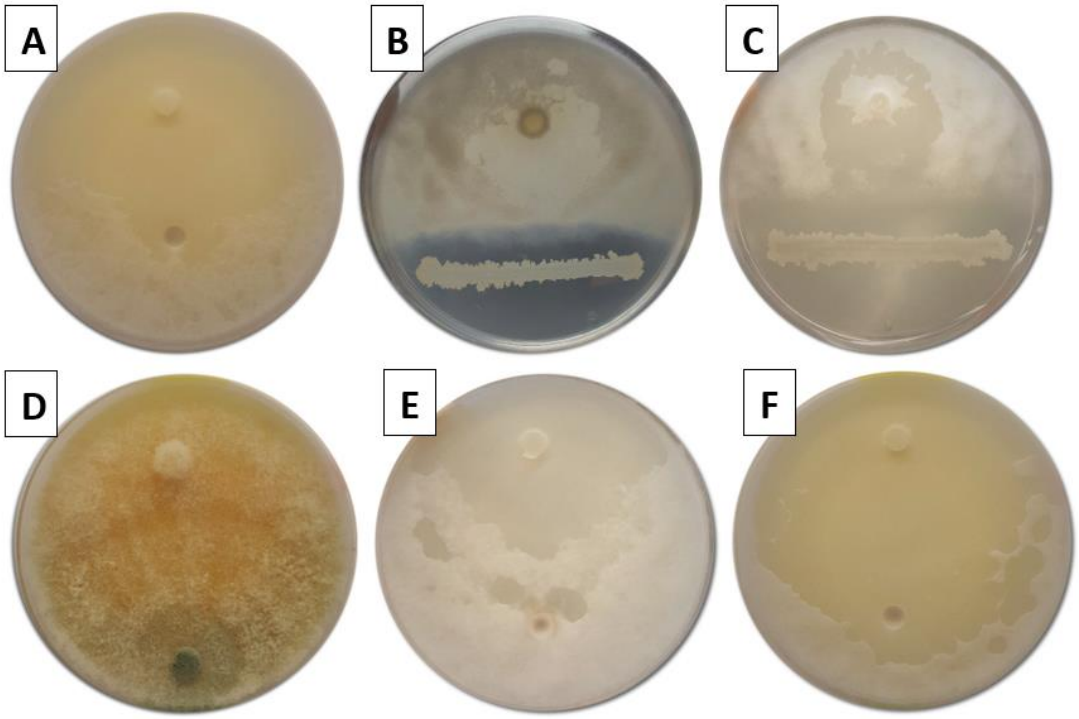

Figure A.1 *Pythium intermedium* dual culture assay plates showing pathogen inhibition due to biocontrols. The pathogen was plated at the top of the plate, and after 24h, the following treatments were added: A) Water, B) Rhapsody, C) Actinovate, D) Rootshield, E) Cueva, F) Regalia.

The inhibition zones found in the Actinovate and Rhapsody plates are likely due to the antimicrobials including antibiotics and chitinases. Rootshield works primarily through competitive exclusion, so the reduction of pathogen growth without inhibition zones is to be expected. Regalia works by inducing systemic immunity in plants, so it is

unsurprising it did little to inhibit growth in this assay. Likewise, Cueva works by inducing copper toxicity in spores preventing germination, and hyphae have already been established in this assay.

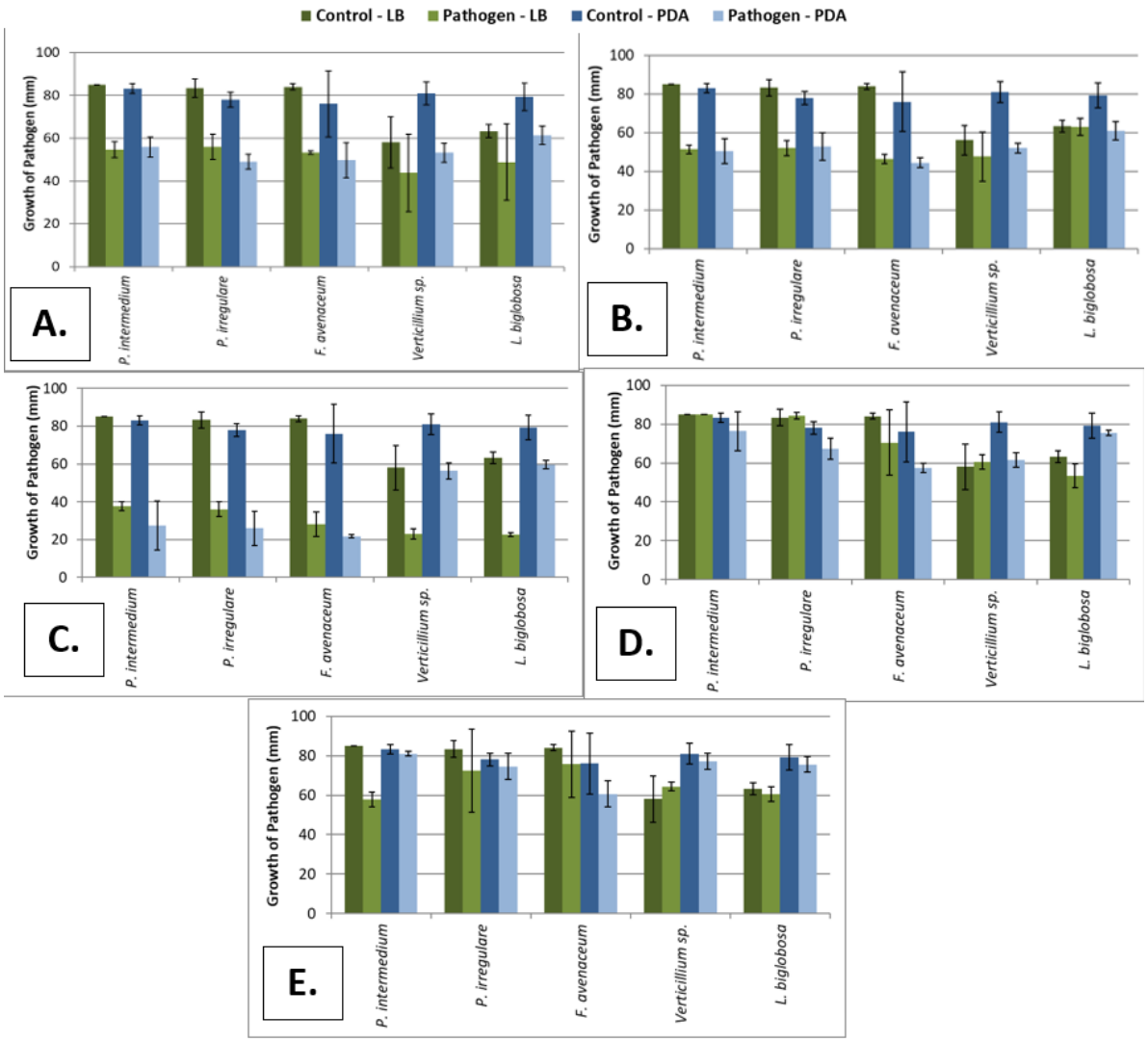

Figure A.2 Pathogen growth on dual culture plates against different biocontrols. A) Rhapsody, B) Actinovate, C) Rootshield, D) Cueva, and E) Regalia. Error bars show 95% confidence intervals.

Appendix B.

Effect of temperature on pathogen growth *in vitro*

B.1 Methods

The growth of certain pathogens – *Pythium intermedium*, *Fusarium avenaceum*, *Fusarium oxysporum*, *Leptosphaeria biglobosa*, and *Verticillium* sp. – were evaluated under different temperature conditions. Five plates of each pathogen were sub-cultured and placed in incubators at 5 °C, 10 °C, 15 °C, 20 °C, 30 °C, and 35 °C. Growth across the plate was assessed by measuring the colony diameter after 7 days.

B.2 Results

Most pathogens grew best between 20 °C and 25 °C with growth declining on both sides of this range (Figure B.1). The *P. intermedium* isolates reached the edge of the petri dish before 1 week in all treatments from 10 °C to 25 °C. The *F. oxysporum* isolates reached the edge of the petri dish before 1 week in the 25 °C treatments. For all pathogens, except *F. oxysporum*, growth was completely inhibited at 35 °C. Most of these pathogens grow best at the upper temperature range of wasabi, and therefore, heat stress of the plants may play a role in pathogen outbreaks.

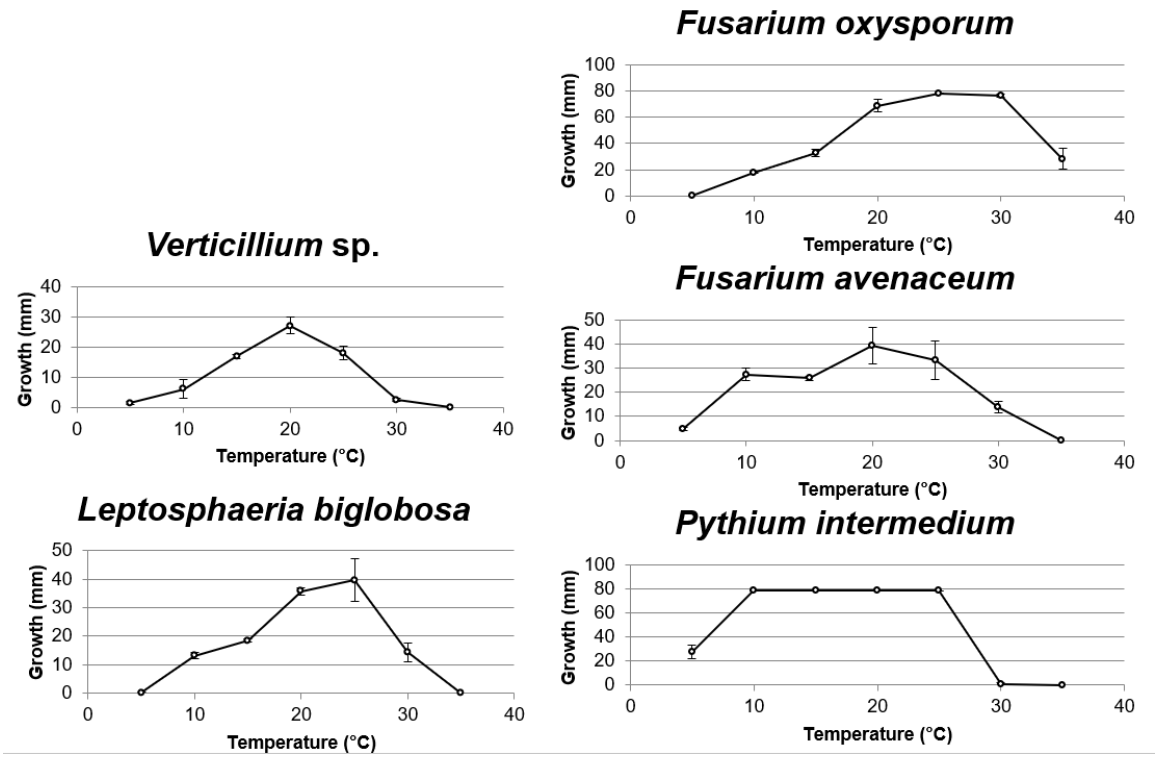

Figure B.1 Fungal growth across PDA plates kept in incubators at various temperatures for 1 week. Error bars represent 95% CI.

Appendix C.

Fungitoxic effect of Cueva® on *Verticillium isaacii*

C.1 Methods

Flasks containing 30 mL of Potato Dextrose Agar with varying concentrations of Cueva Copper Fungicide (n=5 for each treatment) were inoculated with plugs of *Verticillium isaacii*. The cultures were left to incubate on a shaker for 7 days. After a week, mycelium was filtered out and dried in an oven at 50 °C for 48 hours. The resulting dry weights were measured and used to assess fungal growth. Statistical analysis was performed using One-Way ANOVA and Tukey's HSD Test.

C.2 Results

V. isaacii growth was significantly ($p>0.05$) reduced by about 50% in concentrations of Cueva from 100 ppm – 300 ppm (Fig. C.1). At concentrations of 150 ppm and above¸ microsclerotial production was very reduced (Fig. C.2). No growth occurred at concentrations of 500 ppm. As microsclerotia are the main source of inoculum for *Verticillium* infection, Cueva may be efficacious in reducing spread of *V. isaacii*.

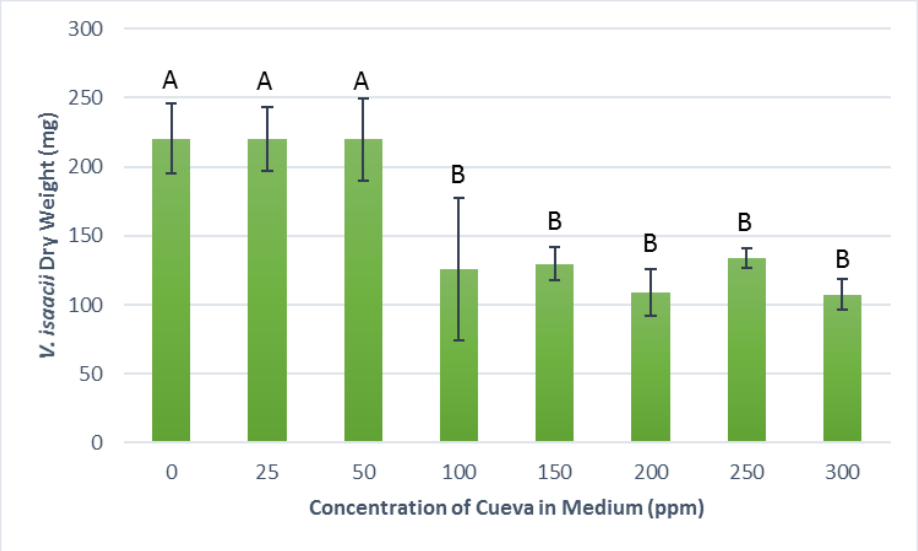

Figure C.1 Growth of *V. isaacii* in PDB with varying concentrations of Cueva Fungicide. Growth is measured in milligrams of dry weight. Error bars are 95% confidence intervals. Letters denote which bars are statistically different from one another.

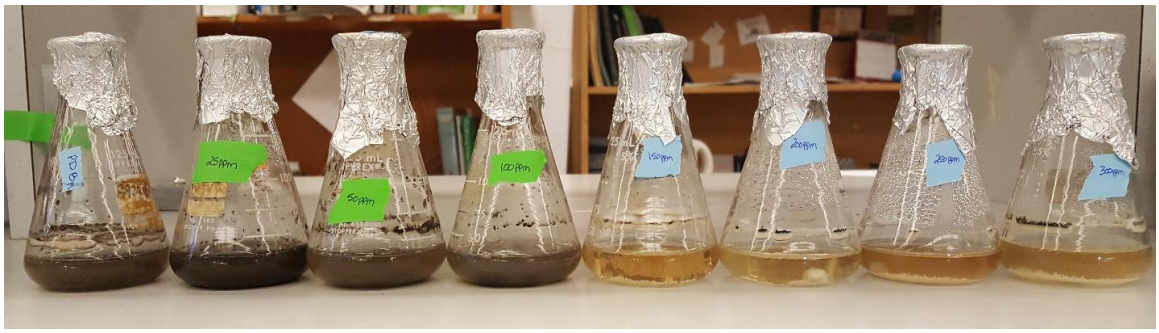

Figure C.2 Growth of *V. isaacii* in PDB with varying concentrations of Cueva Fungicide. Concentrations of Cueva from left to right are 0 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, and 300 ppm. Note the reduction of microsclerotial production at concentrations of 150 ppm and above.