

FUNGAL PATHOGENS OF WASABI IN BRITISH COLUMBIA

by

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ABSTRACT

Wasabi (*Wasabia japonica* L.) is a high-value crop in British Columbia and is cultivated in greenhouses where diseases cause economic losses and insect pest issues are emerging. A review of the current literature on wasabi reveals a lack of information on wasabi pest and disease management, especially in North America. The objective of this research was to identify current diseases affecting wasabi in BC isolates from plants showing symptoms of leaf blight, leaf spot, and white blister rust were identified by molecular and morphological methods. Results revealed that *Botrytis cinerea*, *Collectotrichum higginsianum*, and *Albugo candida* were present. Inoculation studies showed *B. cinerea* was weakly pathogenic, while *C. higginsianum* caused lesions on wasabi and *Brassica juncea*, but not on alfalfa (*Medicago sativa*). In culture, fastest growth of *C. higginsianum* occurred at 25 and 30°C, and the highest conidial production occurred under continuous darkness. Isolates of *A. candida* from shepherd's purse (*Capsella bursa-pastoris*) plants were identical to those from wasabi, suggesting a source of inoculum. Disease control in an integrated pest management system will remain an important aspect of mitigating economic losses.

Keywords: anthracnose, aphids, *Albugo candida*, *Botrytis cinerea*, *Colletotrichum higginsianum*, inoculation, isolation, leaf blight, *Wasabia japonica*, white blister rust

DEDICATION

To my parents, Richard and Denise, who have always nudged me in the right direction, but still allowed me the opportunity to create my own successes and make my own mistakes, and to Corin, who pushed me to start this in the first place.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 *Wasabia japonica* (Miq.) Matsum.: Production in North America and Modern Utilization

1.1.1 Introduction to wasabi

Wasabi (*Wasabia japonica* (Miq.) Matsumara syn. *Eutrema japonicum*) is a member of the Brassicaceae (Cruciferae) family which is native to the Japanese islands. There, it is found growing naturally on the shaded, wet banks of cool streams and springs, at temperatures between 6 – 20 °C (Chadwick et al. 1993). It is a perennial plant that produces a central cylindrical rhizome (stem) from which its root mass grows (Fig. 1-1a); older plants often produce numerous offshoot rhizomes. From the crown of the rhizome emerge many petioles, up to 50 cm in length, each of which terminates with a single glabrous and globular leaf which can be up to 25 cm in diameter (Chadwick et al. 1993) (Fig. 1-1b). In spring, peduncles up to 200 cm in length may emerge from the mature plants, each with a terminal single inflorescence (Adachi 1987; Chadwick et al. 1993). The white flowers are bracteate, raceme, and cruciform in shape (Fig. 1-1c). Seed production is variable, and cross-pollination is usually required due to self-incompatibility (Chadwick 1993; Palmer 1990).

Wasabi is a traditionally staple condiment in the Japanese diet (Hodge 1974). The first known cultivation of wasabi was recorded in “Honzo-wamyō” (Japanese Names of Medicinal Plants by Sukahito Fukae) in the 10th century (Hodge 1974), and the first utilization for food consumption was by Zen Buddhist monks in soup during the Kamakura Era (1198-1333 A.D.) (Chadwick et al. 1993). It was not until sometime after the Keicho Era (1596-1615 A.D.),

Figure 1-1. Rhizome, leaf, and inflorescence of *W. japonica*. (a) Rhizome. (b) Leaf. (c) Inflorescence.



and shortly after it became a popular ground condiment reserved for the ruling class, that wasabi became desirable to the rest of the Japanese population (Kojima 1981 as cited by Chadwick et al. 1993).

Traditionally, a fresh rhizome is grated against a rough surface such as shark skin (Morgan 2005) or a ginger grater (Chadwick et al. 1993). Chadwick et al. (1993) describe how the application of the grated material is then utilized differently depending on the dish: for sashimi, it is presented next to the main dish in a mound, for noodles it is served on a separate dish accompanying it, and for sushi, it is spread directly on the raw fish portion during preparation. The leaves and petioles were traditionally used to flavour sake brine, soy sauce, white rice (Haruki et al. 1987 as cited by Chadwick et al. 1993), and more recently in the production of ice cream, wine, cheese, salad dressings, and crackers (Chadwick et al. 1993) or to flavour soups and salads.

1.1.2 Expanding commercial cultivation

Wasabi cultivation continued exclusively in Japan until it was introduced to Taiwan in 1915, where it was cultivated with the intent of shipping back to Japanese markets (Lo et al. 2002). Since its introduction, wasabi has also become a popular spice ingredient in Taiwan (Wu et al. 2015). Market demand in Japan was successfully met by these two countries until the 1970's, when prices began to climb. By 1984, the fresh market prices had risen from US\$15/kg (Chadwick et al. 1993) to nearly US\$50/kg (Adachi 1987), and continued to climb to at least US\$75 by 1992 (Chadwick et al. 1993). In the early 90's production in Japan was limited to approximately 880 ha, and less than half that in Taiwan (Chadwick et al. 1993). The increasing

price and the limited production generated an interest by other foreign nations, including Korea, China, New Zealand (NZ), and the United States of America (USA).

The first recorded interest by a western nation in cultivating wasabi was by researchers in the USA. In 1903, a US Department of Agriculture (USDA.) employee by the name of David Fairchild returned from a trip to Japan, and expressed potential interest in wasabi (Fairchild 1903). Fairchild secured a number of plants for propagation trials in the USA, but the results of those could not be found and the trials were likely not successful (author, pers. obs.). Shortly after the initial increase in market price, interest returned. Hodge (1974) provided much needed literature on the production in Japan; however, he was not convinced that wasabi would be accepted as a condiment by Americans. The potential of this crop was not lost on NZ agricultural specialists, however; in 1982 wasabi was introduced, and research soon intensified (Douglas and Follett 1992).

By the mid-1980's, production reports from Japan were published for maximizing cultivation capabilities in NZ (Follett 1986a; 1986b), and by 1987, a full research planting was established in a decommissioned fish hatchery (Douglas and Follett 1992). Five years later, the first NZ production was being assessed for value, and specific production requirements for NZ had been established (Douglas and Follett 1992). Douglas and Follett (1992) found that NZ could produce wasabi similar in quality to the popular wasabi-producing region of Japan, Shizuoka, and recognized both the potential for it become a significant export crop and the potential to significantly increase yield and quality with better disease and pest control.

Around this time, wasabi production in North America also blossomed after Chadwick et al. (1993) published their paper, "The Botany, Uses and Production of *Wasabia japonica* (Miq.)

(Cruciferae) Matsum,” which was the first publication to reference Japanese literature on wasabi, and is still referred to by modern wasabi growers. As growers in the USA and Canada began to experiment with best practices and were harvesting their first successful crops, the need for research into non-traditional production methods became apparent. By the 2000’s, Agriculture and Agri-Food Canada (AAFC) was actively researching alternative production methods and uses for wasabi, concluding with the potential for a niche greenhouse industry in Canada, and the possibility of intercropping within these systems to maximize consumable yield in limited space (Ehret et al. 2004). At this time, wasabi was also being assessed as a biofilter for land-based salmon farms, which would remove soluble phosphorus and nitrogen from the effluent and at the same time produce a secondary commercial crop with inland fish farms (Ehret and Swift 2005).

In 2015, retail prices for wasabi rhizomes reached over US\$220/kg (B. Oates, Pacific Coast Wasabi, British Columbia (BC), Canada, pers. comm.). Currently, online retail prices for small quantities of rhizome tend to be approximately US\$70 per ½lb, or over US\$300/kg (Frogeye Wasabi 2017; Half-Moon Bay 2017; Pacific Coast Wasabi 2017; The Wasabi Company 2017). Globally, wasabi is now produced commercially in the following countries: Japan, Taiwan, USA, Canada, Australia, New Zealand, Britain, Malaysia, and China. Although the total acreage in North America is small, there has been increasing interest as growers of other crops attempt to diversify. Simultaneously, the total acreage in Japan may be decreasing; there are anecdotal reports that only 604 ha are remaining in production in Japan (Macalpine 2013) of the 880 ha from the beginning of the 1990’s (Chadwick et al. 1993). Chadwick et al. (1993) noted that wasabi production areas were already then being degraded due to agricultural malpractice and urbanization. Along with the increasing acreage domestically, diversification of cultivation methods and an increased demand for pest control products has begun.

1.1.3 Current production and propagation

Commercial production in Canada and the USA has been primarily in Oregon, Washington, and British Columbia, with at least one other farm currently in California, and one having tested and abandoned the crop in North Carolina (D. Groenendale, Western Wasabi, Washington, USA, pers. comm.). Growers tend to be secretive about their production capacity and methods; however, it is estimated that there are 10 – 20 acres currently cultivated in the Pacific Northwest.

In Japan, there are two traditional cultivation methods (Chadwick et al. 1993). The valuable rhizome produced using semi-aquatic field methods is referred to as ‘mizu’ wasabi, and upland field cultivation is known as ‘hatake’, ‘oka’, or riku’ cultivation (Chadwick et al. 1993). In North America, however, the most widely used production system is an enclosed river-rock growing substrate covered by polyethelene tunnels (polyhouses) (author, pers. obs.). These polyhouses are heated in the winter months, and cooled in the summer months via venting and rolling-up sides (author, pers. obs.). In order to reduce expose to UV radiation, which damages wasabi plants and reduces yield and quality, 70% shade cloth covers the entire tunnel from approximately April until November, when it is again removed (author, pers. obs.) (Fig. 1-2a). This growing system employs overhead misters set for regular, short intervals or based off of light accumulation – sometimes triggered less than 5 minutes apart - which supply water, nutrients, and cooling (author, pers. obs.). The roll-up sides expose the crop to potential pests and pathogens, as does the venting system which draws air in one side and forces it out the other.

Figure 1-2. Typical wasabi production system in British Columbia. (a) Polyethylene greenhouse (polyhouse) in Agassiz, BC, covered in 70% shadecloth. (b) Vegetatively propagated wasabi in a polyhouse with overhead misters.



This, combined with the overhead misters which can increase humidity to 100% (author, pers. obs.; Kestral 3000, Loftopia, LLC, Birmingham MI, USA), provide an excellent environment for introduction and establishment of fungal pathogens.

Globally, pathogens cause substantial crop losses on wasabi. Leaf spot and black rot disease caused by *Leptosphaeria biglobosa* (*Phoma wasabiae*) in Japan (Adachi 1987; Chadwick 1993), Taiwan (Lo and Wang 2000; 2001; 2002), NZ (Douglas and Follet 1992; Martin & Deo 2000), and most recently in Canada (Punja et al. 2017) can affect 30-70% of production annually (Lo and Wang 2002). Another major economically important pathogen is *Pectobacterium* [*Erwinia*] *carotovora* subsp. *carotovora*, which has been identified in NZ (Douglas and Follet 1992) and Canada (Rodríguez and Punja 2009) as the causal agent of internal blackening of the rhizome and reduced plant growth. Blackening of the rhizome can render a rhizome unmarketable. Other potentially destructive pathogens identified include: *Erysiphe cruciferarum* causing powdery mildew (Park et al. 2016), *Pythium dissoticum* and *P. intermedium* causing root rot (Rodríguez and Punja 2007), and *Albugo candida* or *A. wasabiae* causing white blister rust (Lo and Wang 2000; Joshi and Jeffries 2010; Choi et al. 2014). Besides fungal and bacterial pathogens, Douglas and Follett (1992) reported infection on wasabi from tobacco (TMV), turnip (TuMV), and cucumber mosaic viruses (CMV). Although these viruses have yet to be reported on wasabi in North America, CMV has also been found infecting wasabi in Australia (Wilson 1998).

Lo et al. (2002) demonstrated control of black rot disease by treating plantlets and tillers at the start of a crop, but currently there are limited registered fungicidal control options for growers in North America (Table 1). Despite the suggestion of replacing overhead irrigation with drip irrigation to reduce optimal pathogen environmental conditions and dispersal

Table 1. List of registered pesticides on wasabi in the United States and Canada.

Product trade name	Active Ingredient/microorganism	Pest/Disease	Manufacturer/distributor
United States			
Quadris F	azoxystrobin	Fusarium root rot, pythium root rot	Syngenta Crop Protection, LLC
Canada			
Ambush 500EC	permethrin	Cabbage looper, imported cabbage looper, diamondback moth larvae, crucifer flea beetle	AMVAC Chemical Corp.
Bioprotec CAF	<i>Bacillus thuringiensis kurstaki</i>	Cabbage looper, alfalfa looper	AEF Global, Inc.
Bioprotec PLUS	<i>Bacillus thuringiensis kurstaki</i>	Cabbage looper, alfalfa looper	AEF Global, Inc.
Botanigard 22WP	<i>Beauveria bassiana</i> GHA	Aphids	LAM International Corp.
Botanigard ES	<i>Beauveria bassiana</i> GHA	Aphids	LAM International Corp.
Cease®	<i>Bacillus subtilis</i>	Powdery mildew, pythium root rot, phytophthora crown rot and root rot	BioWorks, Inc.
Dipel® 2X DF	<i>Bacillus thuringiensis kurstaki</i>	Cabbage looper, alfalfa looper	Valent BioSciences Corp.
Rhapsody® ASO™	<i>Bacillus subtilis</i>	Powdery mildew, pythium root rot, phytophthora crown rot and root rot	Bayer CropScience Inc.

probability (Lo et al. 2002), few growers have yet altered their growing methods in Canada and the USA (author, pers. obs.).

Little research has been conducted on the arthropod pests of wasabi. Currently, even their presence is limited to anecdotal references. Regardless, there is a common knowledge among growers that leaf aphids, root aphids, diamondback moth, and slugs are recurring pests that need to be managed (B. Oates, Pacific Coast Wasabi, BC., Canada, pers. comm.; D. Groenendale, Western Wasabi, Washington, USA, pers. comm.).

In NZ, Douglas and Follett (1992) recognized the lettuce root aphid, *Pemphigus bursarius*, to be a potential problem, and noted it as a difficult pest to detect and with few options for control. They did not, however, describe their identification methods or provide any other information. *Plutella xylostella* – the diamondback or cigarette moth – has long been known to be damaging to wasabi leaves in its larval form (Adachi 1987; Chadwick 1993; D. Groenendale, Western Wasabi, Washington, USA, pers. comm.). Despite the damage caused by these pests, there are few control options available to growers in both Canada and the USA (Table 1). In addition, Atsumi & Saito (2015) demonstrated that germination of *Beauveria bassiana* – the active ingredient in a number of the registered bioinsecticides – can be inhibited by exposure to wasabi volatiles, making them ineffective for control.

The spread of disease, and perhaps even some insect pests, may be attributed to propagation practices. Replant of wasabi has traditionally been accomplished through seedlings or vegetative propagation via axillary buds (Chadwick 1993)(Fig. 1-2b). Vegetative propagation has repeatedly been implicated as a pathway for disease transmission and persistence in wasabi polyhouses, and it has been suggested that replant with offshoots should be limited to 2 – 3 crop

cycles (Adachi 1987; Chadwick 1993; Punja et al. 2017). Seed production does not produce true-to-type plants, and therefore desirable characteristics of the parent plants may not be expressed (Adachi 1987; Chadwick 1993). Ideally, crops would be replanted with disease-free material (Lo & Wang 2001; Lo et al. 2002). In an effort to provide an avenue for this, a methodology for successful tissue culture of wasabi was published by Rodríguez (2007). Tissue culture has become more common since this publication due to dedicated propagation facilities being available, and the realization of the importance of clean plant material. It is now possible to purchase tissue-cultured plants for propagation from local suppliers in BC

1.1.4 Modern utilization of wasabi and wasabi components

In recent years, there has been considerable interest in properties of wasabi outside the traditional cuisine uses. The most recognizable components of wasabi are the volatile allyl isothiocyanates – organosulfur compounds responsible for the pungent taste (Kumagai et al. 1994). However, besides producing a unique flavour, the isothiocyanates have been linked to health benefits in a number of studies. These include the inhibition of platelet aggregation (blood clotting) (Kumagai et al. 1994), anti-cancerous and chemopreventative properties (Chen et al. 2014; Wu et al. 2015; Hsuan et al. 2016), anti-oxidant properties (Lee et al. 2008; Sekiguchi et al. 2010), anti-hypercholesterolemic properties (Lee et al. 2008), anti-allergic effects (Yamada-Kato et al. 2012), as well as reducing dermatitis-like symptoms (Nagai & Okunishi 2009). Although there are five known isothiocyanates reported to be medicinally active in wasabi, Hao et al. (2016) found that two closely related *Eutrema* species may possess higher concentrations when compared to the commercially cultivated *W. japonica*, and could provide a better source of isothiocyanates for study and medicinal use. Regardless, Martin et al. (2002) demonstrated that isothiocyanate concentrations in wasabi can be manipulated by adjusting

fertilizer ingredients (specifically, sulphur). It's also been confirmed that wasabi rhizomes harvested from water-grown plants produce higher concentrations of isothiocyanates than soil-grown plants (Sultana et al. 2003), which means the demand for the higher-priced, water-grown wasabi could come from both food and nutraceutical companies.

Wasabi has also been studied for some interesting pest control properties. Li et al. (2014) demonstrated that fresh wasabi residue alone or combined with wasabi's natural endophytic bacteria has nematicidal properties in the management of the plant parasitic root-knot nematodes. Interestingly, in 1988, Kasuya et al. also showed that wasabi extract causes mortality *in vitro* of *Anisakis simplex*, the anisakiasis causing nematode of accidental human hosts whom have consumed infected fish (Valero et al. 2015).

Following the discovery of the anti-microbial activity of wasabi extracts, investigations into utilizing these properties for disease resistance for other crops followed. Transgenic crops expressing a wasabi defensin gene have successfully shown enhanced resistance to a number of pathogens: *Fusarium oxysporum* resistance in tobacco (Ntui et al. 2011; Kong et al. 2014), potato (Khan et al. 2014), melon (Ntui et al. 2010), and tomato (Kong et al. 2014), *Botrytis cinerea* resistance in potato (Khan et al. 2006), tobacco (Kiba et al. 2007), and petunia flowers (Kahn et al. 2011), and *Alternaria solani* resistance in potato (Kahn et al. 2014) and melon (Ntui et al. 2010). The anti-microbial activity of wasabi is not unique, however, as shown by Hoshikawa et al. (2012) who enhanced *B. cinerea* resistance in transgenic potatoes by expressing defensin genes isolated from a number of other Brassicaceae species. Regardless, this recent research hints at the future potential for wasabi.

1.1.5 Discussion

Wasabi production in BC is a valuable and expanding industry. Currently, there are approximately 10 acres of wasabi grown in BC (authors, pers. obs.; D. Groenendale, Western Wasabi, Washington, USA, pers. comm.), but there has been expressed interest that could double or triple that (Brian Oates in Gittleson 2014; Findlay 2015). With the interest in wasabi increasing beyond that of culinary use, the additional demand for nutraceutical utilization has pushed the demand well beyond current supply (D. Groenendale, Western Wasabi, Washington, USA, pers. comm.). These new opportunities present a novel market for wasabi growers, who will still have to manage emerging pest issues regardless of their target market.

The current lack of registered Pest Control Products in Canada and the USA, as well as any sort of integrated pest management literature for local wasabi growers could be a major limitation to wasabi production. Although the biggest problems growers face in Canada are diseases, only one biofungicidal active ingredient is registered (Table 1). In the USA, growers also only have access to a single synthetic fungicide (Table 1). This may lead to resistance problems caused by a lack of chemical rotation options. Similarly, there are insecticides registered in Canada; however, they are primarily bioinsecticides that may not offer the level of control required (Table 1). It is likely that disease and pest issues will continue to expand with time and increased acreage of wasabi. Pest management research should remain a top priority for the wasabi industry in order to remain viable, especially because it can take years to register a pesticide for use on a crop. The best answers for the time being will be integrated pest management based on pest and pathogen biology, and to continue exploring biopesticide and biocontrol options; although, the first step is to identify local problems on wasabi.

1.2 *Botrytis* grey mold

1.2.1 Taxonomy and identification

The imperfect genus *Botrytis* is more appropriately classified by its teleomorph, *Botryotinia* (Whetzel, 1945). *Botryotinia* is in the kingdom Fungi, phylum Ascomycota, a group known as sac fungi (ascomycetes) due to their defining feature, the ascus. This is the sexual structure that produces the nonmotile sexual spores called ascospores (Jarvis, 1977). Although it is now generally accepted to refer to fungi by their teleomorphic name, *Botrytis* is such a well-known, common, and wide-spread fungus that the anamorphic genus name is often used instead.

Botryotinia species are in the family Sclerotiniaceae, created by Whetzel (1945) to describe the fungi that form a sclerotium, or food storage organ, for extended survival in less-than-optimal conditions. Plano-convexoid sclerotia are characteristic of all *Botryotinia* species; they are black, flat or concave, and present on or just below the surface of necrotized plant material and made of tightly bound hyphae (Whetzel, 1945; Horst, 2013). *Botrytis*, the conidial stage, produces single-celled, hyaline, and egg-shaped conidia (Horst, 2013) on branching conidiophores that are visible on the surface of plant material. These look similar to clusters of grapes, which is where it gets its name from – “*botrys*” is Greek for cluster of grapes (Horst, 2013).

Botrytis was first described in 1729 (Micheli), and was included in Persoon’s work to adapt to Linneaus’ binomial system in 1801 (Jarvis, 1977). Within the genus, Persoon also included *B. cinerea* – one of the oldest named fungal species, named by von Haller (1771) (Jarvis, 1977). It was not until de Bary indicated a connection that it was accepted that *Botryotinia* and *Botrytis* were related (Jarvis, 1977). Even today, there are few definite cases

where the connection between the conidial stage and the ascospore stage have been shown (Horst, 2013).

Botrytis – specifically *B. cinerea* – is one of the best characterized fungi. Morphology based on conidia and conidiophores, coupled with the host and cultural characteristics, have been the primary means of identification for the *Botrytis* genus (Jarvis, 1977). However, the historically accepted single species *B. cinerea* has been found to actually be a number of cryptic species, divided into Group I and Group II (Giraud et al., 1997; Fournier et al., 2005; Isenegger et al., 2008; Fekete et al., 2012; Asadollahi et al., 2013; Saito et al., 2016). Although all are classified as being a part of the *Botrytis cinerea* species complex, the most accurate identification method within this complex is comparing polymorphisms in nuclear genes such as glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*), heat shock protein 60 (*HSP60*), *MS547* (encoding an ATP-dependent RNA helicase), or the noncoding region 63R (Walker et al., 2011).

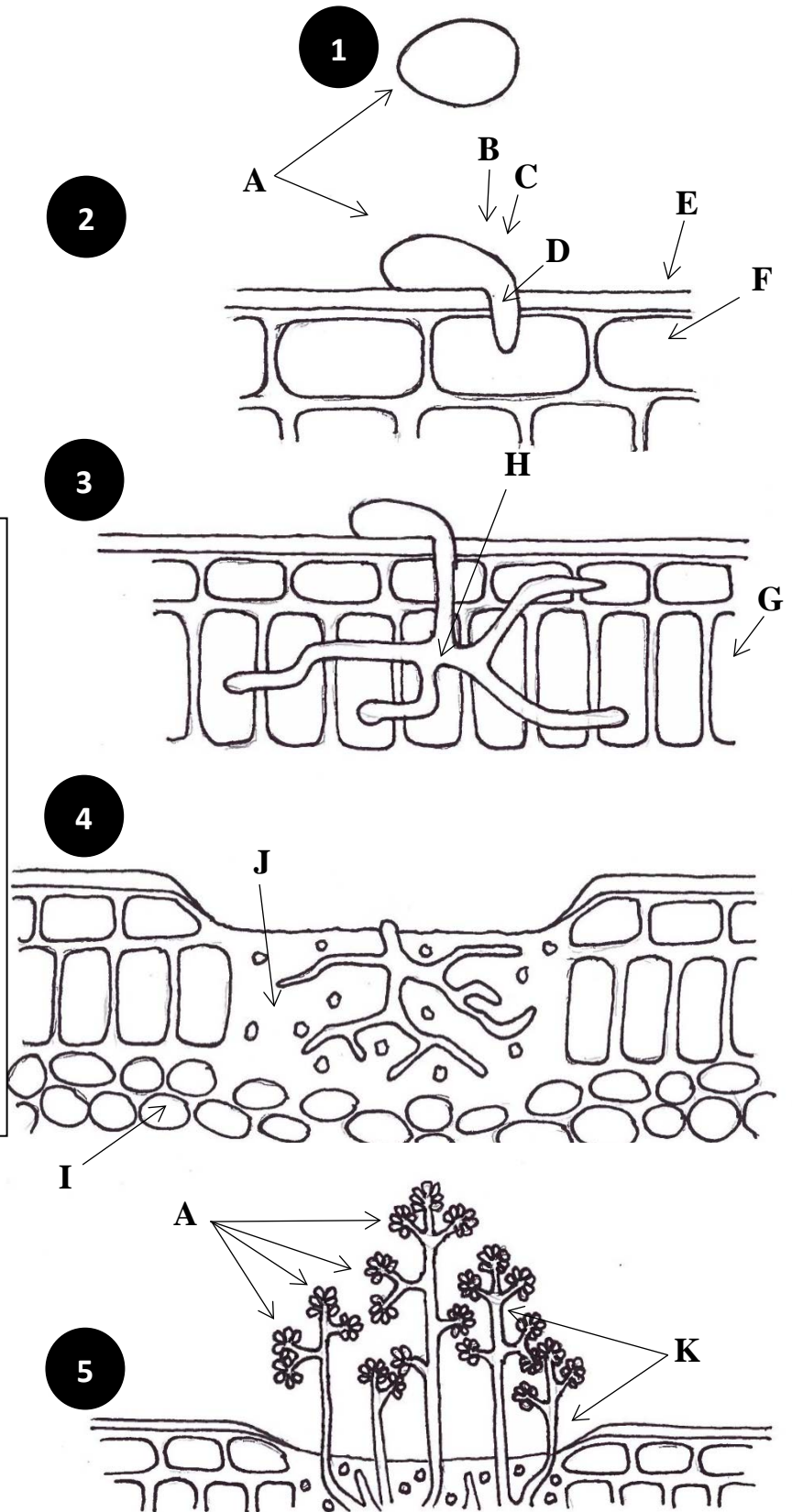
1.2.2 *Botrytis* spp. as plant pathogens

Botrytis, commonly known as “grey mould” (gray mold), primarily causes leaf blights and fruit rots and is one of the first genera of fungi described (Jarvis, 1977; Rosslenbroich & Stuebler, 2000). The branching conidiophores produce clusters of grey conidia on plant material, which to the unaided eye appear as a grey fuzzy mass. There are approximated 30 different species in the genus *Botrytis*, and they can be necrotrophic or saprotrophic (Horst, 2013).

Botrytis requires 18 – 23°C temperatures and damp weather or high humidity for best establishment. It survives as mycelium in plant material or sclerotia over winter; in spring conidia are produced which enter dicotyledenous plants through wounds, flowers, or otherwise weak or damaged material, and quickly produce mycelium which penetrate host tissue. As the

Figure 1-3. Asexual infection process of *Botrytis cinerea* on *W. japonica*. (1) Conidium lands on leaf surface. (2) Conidium germinates and penetrates the host cuticle, invading the cellular epidermis. (3) Primary hyphae form and penetrate surrounding host cells. (4) Host tissue dies and rots surrounding the affected site. (5) Conidiophores emerge on the surface of the host tissue from the rotted site, producing many conidia which can be dispersed to begin the infection process again. Adapted from Agrios (2005).

Legend	
A	– conidium/conidia
B	– germ tube
C	– appressoria
D	– penetration peg
E	– host cuticle
F	– host epidermis cell
G	– host palisade mesophyll cell
H	– primary hyphae
I	– host spongy mesophyll
J	– rotted host tissue
K	– conidiophore



infection progresses – the mycelium spreading under the cuticle – host cells collapse, disintegrate, become soft, and rot. Conidiophores loaded with conidia form and are visible as grey mould on the surface of the infected tissue (Agrios, 2005; van Kan, 2006). Rarely is the perfect fungus *Botryotinia* produced for the most common *Botrytis*, *B. cinerea* (Agrios, 2005; Horst, 2013). Fig. 1-3 demonstrates the infection process of *Botrytis* on *W. japonica*.

Infection is common at blossoming time by light-grey to light-brown web-like mould covering rotted buds and flowers, which can later become a blossom end rot of fruit. Leaf spots, when produced, are typically small yellow spots that later become gray or tan, and eventually coalesce (Agrios, 2005). van Kan (2006) in *Botrytis: Biology, Pathology and Control* (Elad et al. eds, 2004) suggest there is evidence that in order to penetrate leaf cuticles, *B. cinerea* produces an appressorium and penetration peg. Under wet or humid conditions, the conidial masses on conidiophores are visible as grey to brown fuzzy moulds on the plant surface.

1.2.3 *Botrytis cinerea*

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a serious pathogen of crops. It is prevalent globally and can cause significant decreases in yield and/or economic damage on most plants or plant parts, including more than 200 crops (Rosslénbroich & Stuebler, 2000; Williamson et al., 2007). Because of its importance, it is the most studied necrotrophic fungal pathogen. Often, *B. cinerea* enters a host at an early stage but remains latent and does not produce visible symptoms of infection until much later – even after harvest and well after shipping has occurred (Rosslénbroich & Stuebler, 2000; Williamson et al., 2007). This is problematic for producers who expect to have quality yield, only for their shipments to become rejected due to rot. *B. cinerea* infection is especially important for post-

harvest kiwifruit (Wurms et al., 1999; Michailides & Elmer, 2000), and for wine grapes where it has earned the name “bunch rot” (Latorre et al., 2015; Steel et al., 2016). Williamson et al. (2007) note that while *Botrytis* spp. have caused economic damage to crops for many generations, it is still a difficult group of pathogens to control.

In the latter part of the 1990's, Giraud et al. (1997) first suggested that *B. cinerea* may actually be a complex of cryptic, sympatric species. In subsequent investigations, *B. cinerea* became accepted as the *Botrytis cinerea* species complex, which was divided into Group I and Group II (Giraud et al., 1997; Fournier et al., 2005; Isenegger et al., 2008). Currently, Group I consists of *B. pseudocinerea* (Walker et al., 2011), *B. sinoviticola* (Zhou et al. 2014), and *B. californica* (Saito et al., 2016), while Group II is recognized as the *B. cinerea* type species.

1.3 *Colletotrichum* leaf spot

1.3.1 Taxonomy and identification

Colletotrichum is an ascomycete within the family Glomerellaceae, and is the anamorph of *Glomerella* (Hyde 2009b). The genus is comprised of both symbionts and plant pathogens (Hyde et al. 2009b).

Hyde et al. (2009a) state the number of species within *Colletotrichum* ranges from 22 – 60 in the literature (Sutton 1980; 1992; Kirk et al. 2008). *Colletotrichum* spp. are strongly influenced by photoperiod and light quality, especially regarding conidia production and UV tolerance when grown on PDA (Soltani et al. 2014; de Menezes et al. 2015). Zakaria & Bailey (2000) suggested that morphological identification has limitations for *Colletotrichum*, which was supported by Hyde et al. (2009a) in their note that conidial morphological identification is not

always possible, such as in the case of *C. destructivum* O’Gara, *C. higginsianum* Sacc., and *C. linicola* Pethybr. & Laff., as all have a characteristic slight curve and taper, with rounded ends, and they all produce a distinguishing multi-lobed infection structure inside the initially infected epidermal cells (Latunde-Dada and Lucas 2007; O’Connell et al. 2004).

Although molecular identification using the ITS1-5.8S-ITS4 regions of ribosomal DNA is typically adequate for fungal species identification, in *Colletotrichum* it is not so simple due to a high degree of genetic similarity within that region. In some studies, use of only the ITS regions has led to the assumption that closely related species are instead a single species (O’Connell et al., 2004; Sun & Zhang, 2009). Crouch et al. (2009) found that 86% of 14 species tested were incorrectly identified using this method. It was proposed by Cannon et al. (2012) that a robust multi-locus identification tool is required for the genus. In an attempt to disentangle the *C. destructivum* species complex of 24 species, Damm et al. (2014) performed a multi-locus analysis, which also confirmed the ambiguous use of only the ITS region. Neither ITS studies nor host-range studies alone should be used for species identification (Crouch et al. 2009; Damm et al. 2014); however, a combination of both of these and morphological characteristics may be useful for identification.

1.3.2 *Colletotrichum* spp. as plant pathogens

Colletotrichum spp. are a widely studied genus of fungi that cause anthracnose on over 900 plants worldwide (Crouch et al. 2009), appearing as lesions on any part of the plant, but primarily leaves or fruit (Agrios 2005).

Although *Colletotrichum* as a genus is considered one of the most important pathogen groups, special attention is given to the *C. destructivum* species complex which consists of

hemibiotrophic pathogenic species (Damm et al. 2014). Conidia initiate infection by germinating on the surface of the plant and producing a series of penetration structures: germ tubes, appressoria, and penetration pegs (Perfect et al. 1999; Perfect & Green 2001). Some important *Colletotrichum* spp. are distinguished by their interesting hemibiotrophic hyphal stages: primary biotrophic stage is intracellular within living host cells, followed by a secondary intercellular, necrotrophic stage (Perfect et al. 1999; Perfect & Green 2001; Wharton et al. 2001; O’Connell et al. 2012). For most (but not all) species, the entirety of the biotrophic stage occurs within a single epidermal cell, while the necrotrophic stage occurs in surrounding tissue and is associated with the visible disease symptoms (Damm et al. 2014).

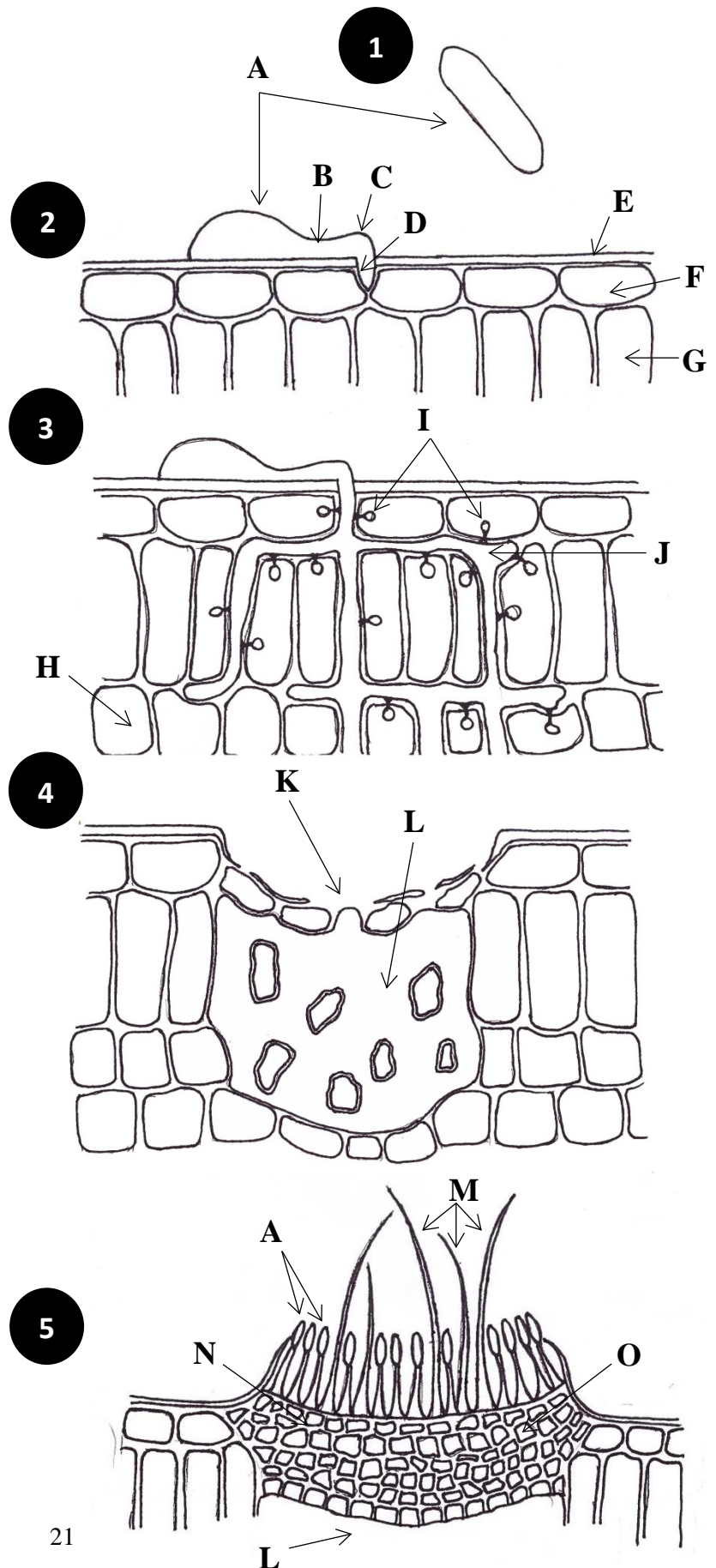
After the early infection and initiation of the necrotrophic stage, when mycelia spread throughout the tissue and associated cells, the invaded cells collapse and form visible sunken areas on the plant surface (Agrios 2005). From these regions, acervuli with masses of conidia and sometimes black setae develop. The conidia are eventually released, usually by wind or water dispersal, or overwintering perithecia will release ascospores to begin the infection process over again (Agrios 2005). The infection process on *W. japonica* is shown in Fig. 1-4.

1.3.3 *Colletotrichum destructivum*

C. destructivum was described by O’Gara (1915), after it was isolated from clover, and is now known to be pathogenic to plants in at least 11 different families (Damm et al. 2014). It causes anthracnose of *Brassicaceae*, *Cuscutaceae*, *Leguminosae*, *Solanum*, and *Perilla* (Hyde et al. 2009a); however, the extent of its Brassica host-range is so far limited to laboratory infections of *Arabidopsis thaliana*, and it is unclear whether the pathogen in question was actually *C. destructivum* or instead misidentified as *C. higginsianum* (O’Connell et al. 2004; Sun & Zhang

Figure 1-4. Asexual infection process of *Colletotrichum higginsianum* on *W. japonica*. (1) Conidium lands on leaf surface. (2) Conidium germinates, forms an appressoria and penetration peg, and penetrates intercellular space at the beginning of a biotrophic stage. (3) Primary hyphae form and spread, and develop haustoria which infect intracellularly. (4) A mycelial mass is formed during a necrotrophic stage, which results in collapsing host tissue at the infection site. (5) An ascervulus forms at the infection site, which can begin the infection process again. Adapted from Agrios (2005).

Legend	
A	– conidium/conidia
B	– germ tube
C	– appressoria
D	– penetration peg
E	– host cuticle
F	– host epidermis cell
G	– host palisade mesophyll cell
H	– host spongy mesophyll
I	– haustorium
J	– primary hyphae
K	– collapsed host tissue
L	– mycelial mass
M	– setae
N	– conidiophore
O	– stroma



2009). Some of the most important crops affected in Canada are lucerne/alfalfa (Boland & Brochu 1989) and soybean (Manandhar et al. 1986).

C. destructivum was identified as the anamorph of *Glomerella glycines* Lehman and Wolf (Manandhar et al. 1986); however, a recent multi-locus analysis did not support that, and therefore the status of the teleomorph is currently unknown (Damm et al. 2014). It is closely related to other hemibiotrophic *Colletotrichum* species including *C. linicola* and *C. higginsianum* (Hyde et al. 2009a).

1.3.4 *Colletotrichum higginsianum*

Originally referred to as *C. brassicae* Higgins, *C. higginsianum* is the most common pathogen causing anthracnose of Brassicas. It causes significant damage on many crops, including Chinese cabbage, turnip, mustard, and radish (Scheffer 1950), and is typically associated with the southern USA, the Caribbean, southeast Asia, and Japan (Higgins 1917; Scheffer 1950; Sutton 1980; Moriwaki et al. 2002)

Currently, the teleomorph of *C. higginsianum* is unknown (Hyde et al. 2009a). It has been considered a synonym of *C. destructivum* based on morphology of conidia and appressoria (Hyde et al. 2009a) and similarity in infection process as well as rDNA sequence (O'Connell et al. 2004; Sun & Zhang 2009). Although common molecular identification using the ITS1, 5.8S, and ITS2 regions of ribosomal DNA is ambiguous when delineating the two species, host range studies have been used to clarify identification (Sun & Zhang 2009).

Martin et al. (2002) noted *C. higginsianum* causing anthracnose on a research crop of wasabi in New Zealand; however, no background information was published on its identification

or symptoms. The Brassicaceae host-range and its anecdotal pathogenicity on wasabi in New Zealand suggest that *C. higginsianum* may be the causal agent of anthracnose leaf spot on wasabi in British Columbia.

1.4 *Albugo* white rust

1.4.1 Taxonomy and identification

Albugo spp. are oomycetes, members of the kingdom Straminipila, part of the stramenopile-alveolate-Rhizaria supergroup (Burki 2014). Thines & Spring (2005) revised the taxonomy of *Albugo*, dividing it into three clades at the genus level; it now is differentiated into *Albugo*, *Pustula*, and *Wilsoniana*. They also removed it from the class Oomycota and placed it in class Peronosporales, within the order Albuginales; however, the taxonomy Chromista: Oomycota: Oomycete: Peronosporales is still widely regarded (Agrios, 2005; Saharan et al., 2014). Members of Albuginaceae are differentiated from related families by the development of basipetal chains of asexual sporangia (Saharan et al., 2014).

The most important morphological trait for *Albugo* species differentiation is the walls of the sexual-stage oospores (Saharan et al., 2014); however, without access to oospores, morphological identification is extremely difficult (Choi et al., 2006). In most cases, genetic sequencing and comparing ITS regions is considered a powerful identification method for Oomycota, and the most reliable method of species identification, although as obligate parasites the use of these tools has been limited for logistical reasons (Choi et al., 2006).

1.4.2 *Albugo* spp. as plant pathogens

Albugo is a clade responsible for white rusts on economically important *Brassica* and *Raphanus* species (Choi et al., 2006). As obligate parasites, they require living plant material in order to survive (Horst, 2013). Typically *Albugo* spp. infection results in visible pustules of white sporangia in the epidermal layer of leaves, while intercellular mycelia cause host cell death (Horst, 2013).

Typically oospores are the cause of initial infection after a resting period, even one as long as 20 years (Verma & Petrie, 1975; Verma, 2012). Germination is either by direct development of a germ tube or the release of zoospores, which then encyst and germinate by germ tubes (Petrie & Verma, 1974; Verma & Petrie, 1975). Zoospore germ tubes penetrate a susceptible host through the stomata on leaves, develop an intercellular mycelia, which then penetrate cell walls with haustoria to feed on intracellular nutrients (Saharan et al., 2014). Eventually, all intracellular space is filled by branched mycelia, and subepidermal sporangiophores develop (Verma et al., 1975). These sporangiophores produce linked sporangia in succession, with the oldest at the top of the chain and the youngest at the bottom (Saharan et al., 2014). The pressure exerted outwards from increasing mycelial growth and continuous sporangia development eventually cracks the epidermis of the host, releasing sporangia onto the surface, where wind or water disseminate them (Saharan et al., 2014). When germinating, each sporangium releases up to 12 zoospores to begin the cycle again (Saharan et al., 2014). Although zoospores can produce secondary infection, it is the oospores that allow the pathogen to survive in the absence of the host, and to enable primary infection (Saharan et al., 2014). Fig. 1-5 shows the complete infection process on *W. japonica*.

1.4.3 *Albugo wasabiae*

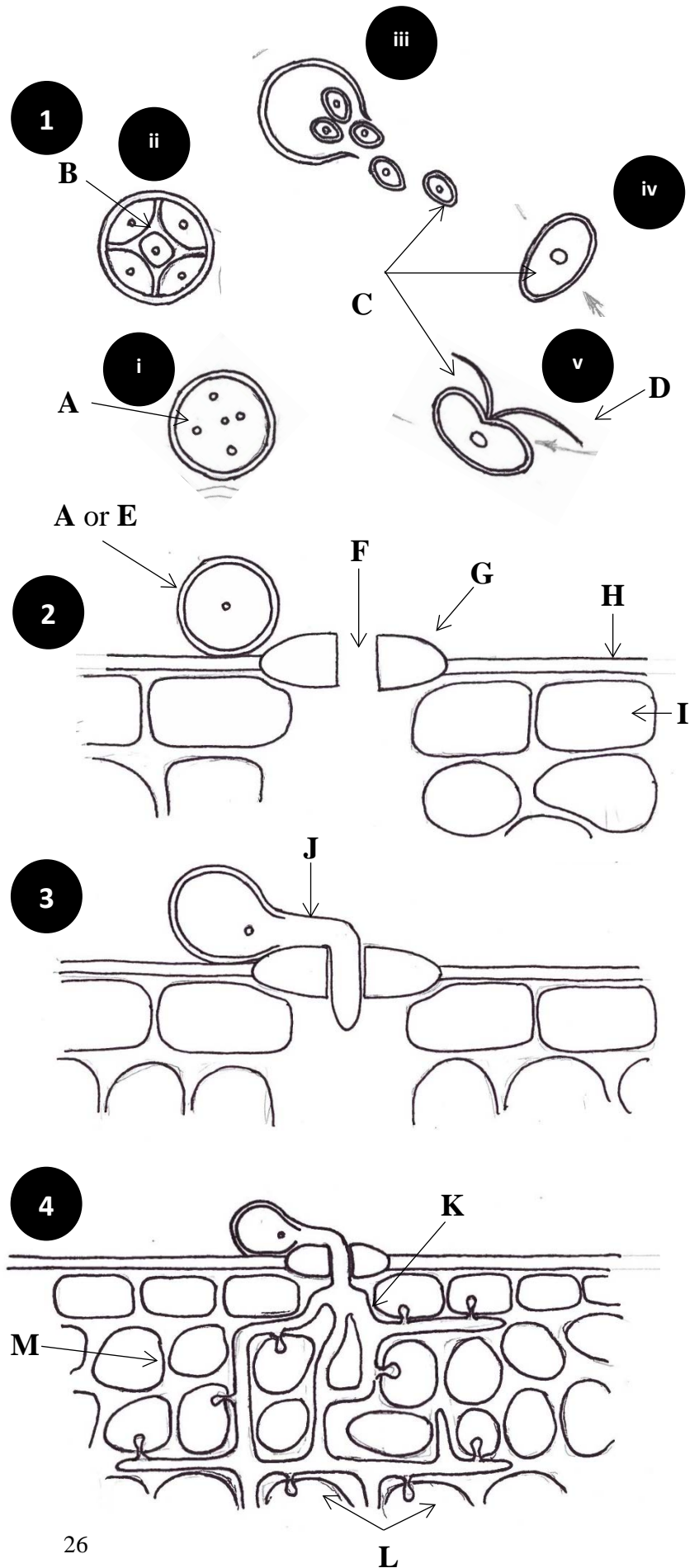
Albugo wasabiae Hara was identified after being isolated from wasabi in Japan (Saharan et al., 2014), and since then, it has been identified on wasabi in New Zealand (Martin & Deo, 2000), Taiwan (Lo & Wang, 2000), and British Columbia (Joshi & Jeffries, 2010). Despite these reports, it is generally accepted that *A. wasabiae* is a synonym for *A. candida* (Choi et al., 2014; Saharan et al., 2014).

1.4.4 *Albugo candida*

First described as a species by Persoon in Gmelin (1792, as referenced by Saharan et al., 2014), *A. candida* (Pers.) Kuntze causes white rusts of crucifers (Horst, 2013); it is noted to infect plants in at least the families Brassicaceae, Cleomaceae, and Capparidaceae (Choi et al., 2009; Saharan et al., 2014). *A. candida* is prevalent worldwide on cultivated oilseed Brassicas and non-cultivated species (Saharan et al., 2014), but its hosts have been reported to include over 60 genera and over 240 species (Biga, 1955; Saharan & Verma, 1992). Infection can be localized and limited to an exposed plant part, or systemic (Horst, 2013).

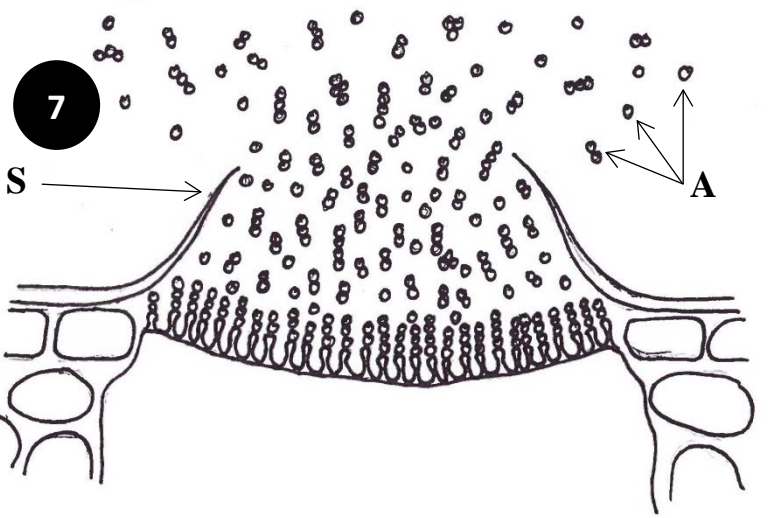
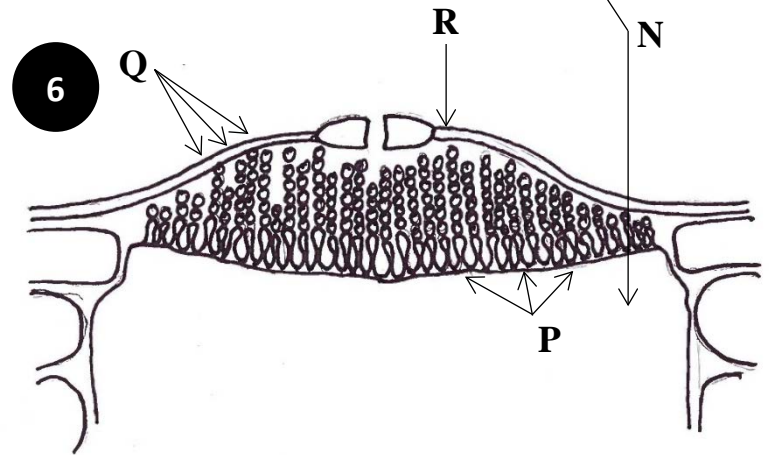
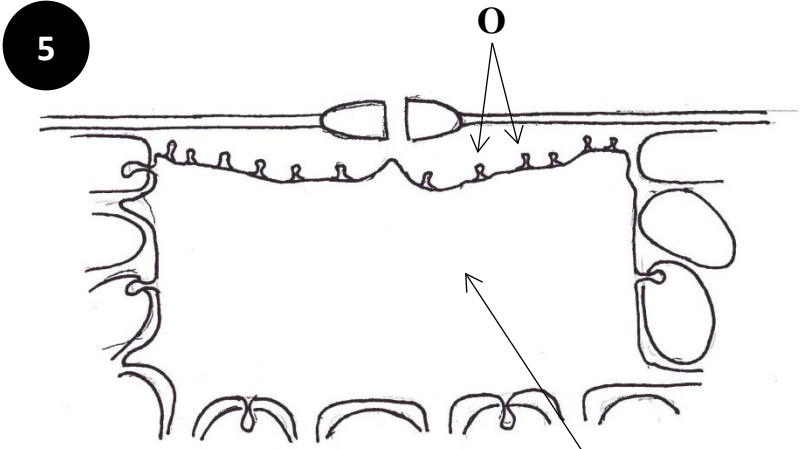
Sporangia have been demonstrated to survive for 4-5 days at 15°C on host leaves (18 hours without host material), but up to 105 days if dried and stored at 40 °C (Lakra & Saharan, 1989). Regardless of survival, high humidity (>70%) and cool temperatures (10 – 20 °C) are required for spore germination and infection (Horst, 2013; Saharan et al., 2014). Germination ceases at temperatures above 20 – 25°C and below 0°C (Melhus, 1911 as cited by Saharan et al., 2014; Napper, 1933), and an aqueous environment is essential (Lakra & Saharan, 1988; Lakra et al., 1989). *Albugo candida* has been identified on wasabi in parts of Asia, including Korea (Choi

Figure 1-5. Asexual infection process of *Albugo candida* on *W. japonica*. (1) i. Sporangium lands on leaf surface or begins to differentiate. ii. Sporangium begins to develop zoospores. iii. Sporangium ruptures and releases zoospores. iv. Zoospore is independent. v. Zoospore develops flagella and searches for penetration site. (2) Zoospore encysts on leaf surface. (3) Germ tube forms and penetrates through host stoma. (4) Primary hyphae develop and spread through host tissue, while developing haustoria within host cells. (5) A dense mycelial plate forms within host tissue; sporangiophores begin to develop under the epidermal layer. (6) Chains of basipetal sporangia form on sporangiophores, exerting an outwards force on the epidermal layer that is visible as a leaf gall. (7) The outwards force ruptures the host tissue, releasing sporangia which can begin the infection process again. Adapted from Agrios (2005) and pers. obs. (author).



See following page for legend.

Legend	
A	– sporangium/sporangia
B	– developing zoospores
C	– zoospore
D	– flagellum
E	– encysted zoospore
F	– host stoma
G	– host guard cell
H	– host (lower) cuticle
I	– host (lower) epidermal cell
J	– germ tube
K	– primary hyphae
L	– haustorium
M	– host spongy mesophyll
N	– dense mycelial plate
O	– young sporangiophore
P	– sporangiophore
Q	– basipetal chain of sporangia
R	– gall from upwards pressure
S	– ruptured host tissue



See previous page for descriptions.

et al. 2014). In Canada, most attention has been devoted to *Brassica* crops of economic importance (Pidskalny & Rimmer 1985; Rimmer et al. 2000).

1.5 Pathogen Management

1.5.1 General remarks

Currently, there are few resources for wasabi growers to assist them in disease and pest management. Historically, chemical controls have been regarded as the “first line of defense;” however, the lack of registered products and the apparent desire for wasabi growers to avoid using synthetic chemicals in order to maintain a strong niche market (author, pers. obs.) suggests that cultural and biological controls will remain the most important management strategies. This underscores the need for continued research into disease and pest biology in order to be effective.

1.5.2 Management of *Botrytis*

Rosslenbroich & Stuebler (2000) suggest that *B. cinerea* is one of the first fungal pathogens to have chemical agents applied for management purposes. They conclude that it was for *B. cinerea* that the Romans used sulphur in their vineyards in the 1300's, and the Germans used sulphur and potassium from 1793.

B. cinerea is considered a high-risk pathogen for resistance management (Brent & Hollomon 2007; Williamson et al. 2007). Cross-resistance within fungicide groups and development of initial resistance is common and often surprisingly quick (Rosslenbroich & Stuebler 2000), credited to the genetic plasticity of *B. cinerea* (Williamson et al. 2007). Starting in the mid-1990's, highly efficacious fungicides for the control of *B. cinerea* became available: cyprodinil, pyrimethanil, mepanipyrim, fludioxinil, and fenhexamid. These reduced the potential

for development of resistance by adding three new chemical groups to what historically had been a single option, dicarboximides, and was putting modern intensive agricultural practices at risk (Rosslénbroich & Stuebler 2000). Currently, there are over 150 fungicidal formulations, including at least 40 active ingredients and approximately 13 fungicidal groups – plus a number of biopesticides – registered for management of *Botrytis* on horticultural crops (PMRA 2016). These chemical groups range from those that have systemic properties and inhibit germ-tube elongation, initial mycelial growth (Rosslénbroich & Stuebler 2000), and the ability of the fungus to secrete extracellular proteins which affect pathogenicity (Milling & Richardson 1995; Miura et al. 1994), such as cyprodinil, pyrimethanil, and mepanipyrim, to those, like fenhexamid, which cause granular structures to form in the cytoplasm then collapses spores and/or germ tubes prior to host penetration (Rosslénbroich & Stuebler 2000).

In no small part due to the resistance potential of *Botrytis* pathogens, cultural efforts to minimize infection are important. Improving airflow within horticultural plots, removing infected dead and senescing plant material, and preventing infection in early infection stages are all valuable management options (Agrios 2005). Reducing humidity reduces *Botrytis* development and spread potential, and maintaining a dry environment is especially important for vulnerable foodstuff in storage (Agrios 2005).

1.5.3 Management of *Colletotrichum*

Colletotrichum anthracnose can be managed by regularly monitoring nurseries and testing for its presence in stock material to prevent spread, as maintaining disease free propagation material is essential (Freeman 2008). Because *Colletotrichum* spores are typically spread by splashing (eg. rainfall or irrigation), reducing overhead irrigation, changing irrigation

methods, and/or using plastic to cover plants can all help prevent the spread of disease (Freeman 2008). There is evidence of cultivar tolerance and resistance traits in peppers (Lewis Ivey et al. 2004), indicating a precedent for cultivar response studies of wasabi. Preliminary leaf disc bioassay data suggests that ‘Daruma’ is more tolerant to *C. higginsianum* infection than ‘Mazuma’ (Appendix A).

Fungicidal treatment is an effective management strategy. Strobilurins are particularly effective at managing *Colletotrichum*; however, they must be used in a rotation in order to reduce resistance potential (Lewis Ivey et al. 2004). Bravo® Ultrex, Cabrio® EG Fungicide, and Pristine® WG Fungicide are effective fungicides among those registered for use in anthracnose management (PMRA 2016).

1.5.4 Management of *Albugo*

White rust in crops can be managed through the use of fungicides, of which there are a number registered for commercial use in Canada (PMRA 2016). Preventative management of white rust infection typically utilizes inhibition of oospore germination in order to prevent initial infection (Verma & Petrie 1979). It is generally regarded as futile to attempt chemical management after infection has established; however, Stone et al. (1987) showed that metalaxyl has limited curative as well as preventative qualities. Meena et al. (2014) suggest that metalaxyl is still currently the most effective fungicide available for management of *A. candida*. The rapid rate of disease symptom development after initial onset, however, underscores the importance of preventative management. Some authors, such as Horst (2013), claim that spray applications are impractical for *Albugo*. He instead suggests that removal of infected plant parts as they are

discovered and clean-up of any plant waste at the end of the season is the best management method.

Awareness that weedy Brassicaceae, such as *C. bursa-pastoris*, may harbour the source inoculum for white blister rust infection on wasabi suggests that managing these weed species might be important. Removing these sources might reduce disease incidence on commercial wasabi crops.

1.6 Biological control of wasabi pathogens

Some endophytic soil microbes, such as *Bacillus velezensis* Ruiz-García et al. ZSY-1, have been found to produce volatile organic compounds that have antifungal activity (Gao et al. 2017). Among the pathogens that were inhibited were *B. cinerea* and *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara (Gao et al. 2017), suggesting there could be a biocontrol application in wasabi production. Similarly, the entomopathogenic fungi *Ophiocordyceps sobolifera* was shown to inhibit mycelial growth and conidial germination of plant pathogenic *Colletotrichum* spp. in vitro, and on chili fruits (Jaihan et al. 2016).

Many *Brassica* species produce allyl isothiocyanates, which are known to have fungicidal properties (Mayton et al. 1996; Oliver et al. 1999), and are often released due to mechanical or insect damage (Atsumi & Saito 2015). Taylor et al. (2014) showed that various isothiocyanates (including allyl isothiocyanate) were able to suppress the growth of the potato pathogens *Rhizoctonia solani* Kühn and *Helminthosporium solani* Durieu & Mont. It should be noted that in this study, although some isothiocyanates were able to reduce growth in vitro of *Colletotrichum coccodes* (Wallr.) Hughes, allyl isothiocyanate did not (Taylor et al. 2014).

Interestingly, some insect pest biocontrol agents, such as *Beauveria bassiana sensu lato* Balsamo, may actually be inhibited by the very property that makes wasabi desirable; the presence of wasabi volatiles such as allyl isothiocyanate was found to reduce conidial germination of entomopathogenic fungi used to control insect pests (Atsumi & Saito 2015). This may lead to some interesting interactions within wasabi integrated pest management programs if fungal biological agents are utilized. In order to develop an effective biocontrol strategy for wasabi, it will be important to understand the dynamics between the wasabi volatiles, the biocontrol agents, the plant pathogens, and to a lesser extent, insect pests that may activate volatile releases. Increasing the concentrations of wasabi volatiles such as allyl isothiocyanate through breeding may not only result in a more desirable culinary product, but may also result in cultivars that are more resistant to disease.

1.7 Breeding for resistance

In commercially important *Brassicaceae* crops, such as cabbage, broccoli, and cauliflower, classical breeding for disease resistance has been identified to be the most economically realistic method and effective tool for disease management (Diederichsen et al. 2009; Nowicki et al. 2012). In the case of Canadian canola (*Brassica napus* L.), resistance breeding for clubroot (*Plasmodiophora brassicae* Woronin) has taken a high priority (Diederichsen et al. 2009). Race-specific resistance has been developed, usually through molecularly assisted identification of host R-genes that enable gene-for-gene resistance (Feng et al. 2014); however, the need for broad genetic resistance via polygenes has been noted in order to maintain the feasibility of disease management for longer term use (Diederichsen et al. 2009).

Nowicki et al. (2012) noted that –for a number of *Alternaria* spp. – it has been difficult to find resistant cultivars to provide genetic material, and that the best source of resistant genotypes has been demonstrated to be from wild-type, closely related species to the *Brassicaceae* crops in question. This will likely be the same for *W. japonica*; uncultivated plant material will need to be harvested from natural stands in Japan, which will likely include identifying resistance traits in the closely related *W. tenuis* (Miq.) Matsum. (Chadwick et al. 1993). However, other sources may be considered, such as horseradish (*Armoracia rusticana* Gaertn., Mey., & Scherb.), which Ohi et al. (1994) successfully crossed with *W. japonica*. Somatic hybridization has also been demonstrated to enable gene transfer between other *Brassica* species to confer disease resistance (Wang et al. 2011), and may be possible for *W. japonica* should classical breeding not be feasible. Ultimately, the limiting factor for breeding disease resistance into wasabi will be identifying sources of genetic material. It is not likely for resistance breeding to be a complete solution, but as Diederichsen et al. (2009) state, it will be a central role in an integrated management program for *Brassicaceae* crops.

1.8 Integrated pest management in wasabi

Much of the literature on wasabi has been focussed on production methods and increasing the culinary quality, while the scarce research published on pest and disease issues has been mostly limited to identification and pathogenicity studies. The sole published manuscript on integrated pest management for wasabi, to the author's knowledge, was for the control of *Phoma* black rot disease in Taiwan (Lo et al. 2002). The bulk of their study investigated the efficacy of fungicides (difenoconazole, thiabendazole, benomyl, and polyoxin), noting that a soaking treatment of vegetatively propagated plantlets reduced disease incidence later in the season. This underscored the importance of using clean material at the beginning of a crop. The second part of

their study recognised the need to prevent secondary infection associated with splashing from irrigation or precipitation after crop establishment, in this case through the use of a polyethylene cover. In British Columbia, polyethylene tunnels are already utilized and there are a number of fungicides in the registration pipeline.

The need for more in depth integrated pest management strategies is of great importance. Investigating true hydroponic or other semi-hydroponic systems that avoid unnecessary leaf moisture may help to solve some of the leaf spot and rot problems currently prevalent in the industry. If the same substrate is to be used over multiple cropping seasons, developing a cost-effective strategy for sterilizing it would be integral to any integrated pest management program, as planting disease-free material is of no benefit if the substrate is harbouring inoculum. Better strategies for managing insect pests may help to alleviate disease pressure if links between them are discovered. It was noted during the course of this project that the parasitoid *Diadegma insulare*, which was released as a biological control for larval stage of *P. xylostella* (diamondback moth), appeared to persist for over 6 months in the wasabi growing environment (author, pers. obs.). More research should be done to verify this and demonstrate any efficacy. The foundation to any integrated pest management program of wasabi, however, will be to establish methods that better control the environmental temperatures and humidity levels to those within the growing parameters of wasabi, and not conducive to those for pathogen development.

1.9 Research Objectives

Commercially grown wasabi is increasing in acreage in the lower mainland and Vancouver Island regions of British Columbia. Because of its relatively new commercial introduction to North America, there is little knowledge of the diseases that can affect wasabi.

Although wasabi has a long history of cultivation in Japan, much of the Japanese knowledge is inaccessible to both industry and academics in Canada. To manage the common and/or destructive pathogens and pests that are present in this region, the first step is to identify those that are affecting wasabi crops in British Columbia. Once identified, an increased understanding of pathogen lifecycles can contribute to an integrated pest management plan and pest control products, thereby increasing marketable yield for growers.

The objectives of this research were to:

- 1) Determine the causal agent of leaf blight on wasabi in British Columbia
- 2) Determine the causal agent of anthracnose leaf spot on wasabi in British Columbia
- 3) Determine the causal agent of white blister rust on wasabi in British Columbia
- 4) Note possible cultural management solutions for these diseases.
- 5) Objectives 1-3 were accomplished through isolation of the possible microbes associated with the symptoms observed, and fulfillment of Koch's postulates where possible through reinoculation of susceptible host plants.

CHAPTER 2: OCCURRENCE OF BOTRYTIS LEAF BLIGHT, ANTHRACNOSE LEAF SPOT, AND WHITE BLISTER RUST ON *WASABIA JAPONICA* IN BRITISH COLUMBIA¹

2.1 Introduction

Wasabia japonica (Miq.) Matsumura (syn. *Eutrema japonicum* Matsum.) (wasabi) is a perennial plant belonging to the Brassicaceae family that is native to Japan, where it grows on the shaded banks of cool streams and springs (Adachi 1987). It is cultivated primarily for its valuable rhizome which is used as a freshly-ground condiment eaten with fish or noodle dishes (Hodge 1974; Chadwick et al. 1993). Wasabi is cultivated in Japan (Follet 1986; Chadwick et al. 1993), Taiwan (Lo & Wang 2000), China, Korea (Choi et al. 2014), New Zealand (Palmer 1990), the USA (Chadwick et al. 1993), and Canada (Rodríguez & Punja 2007; 2009). While wasabi can be cultivated in soil, the rhizomes are generally considered to be of inferior quality compared to those grown in soilless media (Chadwick et al. 1993; Sultana et al. 2003). In British Columbia, most commercial wasabi growers use hydroponic or semi-hydroponic systems, with river rock as the planting substrate (Fig. 2-1a) and overhead misting systems to provide a cool, moist environment (authors, personal observations). All of the production takes place inside greenhouses comprised of double polyethylene plastic (polyhouses) or glass. The plants are harvested after 12-18 months and new plantings are often initiated from vegetative cuttings taken from the axillary shoots, but tissue culture propagation is becoming increasingly popular (authors, personal observations). The two main cultivars grown are ‘Mazuma’ and ‘Daruma’; however, a third cultivar ‘Greenthumb’, has recently been introduced (authors, personal observation).

¹ This chapter has been published in the Canadian Journal of Plant Pathology, available online: <http://www.tandfonline.com/doi/full/10.1080/07060661.2017.1304021> (MacDonald & Punja 2017, Can. J. Plant Pathol. Vol. 39(1): 60 – 71).

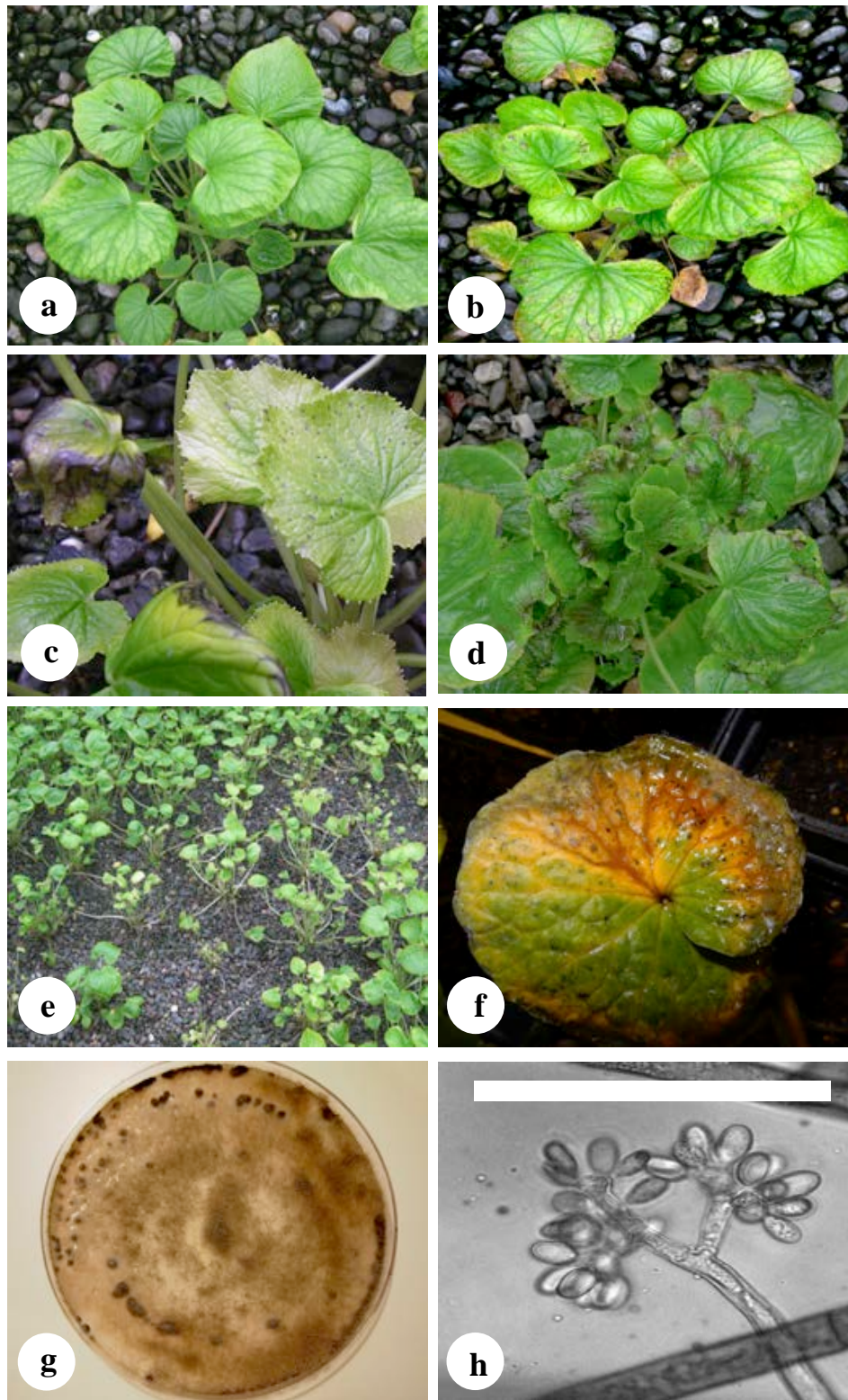
The biggest issues confronting wasabi growers are diseases caused by fungi and bacteria, which are favoured by the cool and humid growing environment inside wasabi greenhouses. The first diseases to be reported on wasabi grown in British Columbia were root rot, caused by *Pythium dissotocum* Drechsler and *P. intermedium* de Bary, and internal vascular blackening of the rhizomes caused by *Pectobacterium carotovorum* (Jones) Waldee subsp. *carotovorum* (Jones) Bengey et al. (Rodríguez & Punja 2007; 2009). Subsequently, phoma leaf spot caused by *Phoma wasabiae* Yokogi (*Leptosphaeria biglobosa* Shoemaker & H. Brun subsp. *occiaustralensis* Vincenot et al.) was identified on wasabi (Punja et al. 2017). With the expanding wasabi production industry in BC, the incidence and severity of other pathogens are also beginning to increase (authors, personal observation). In this study, the occurrence of three previously unreported diseases affecting wasabi in Canada is summarized. The diseases were observed on wasabi plants over a period of two years and the symptoms and results from pathogenicity studies using isolated pathogens are described.

2.2 Materials and methods

2.2.1 Botrytis leaf blight

Wasabia ‘Mazuma’ leaves showing symptoms of yellowing and marginal necrosis were collected from polyethylene greenhouses located at Maple Ridge and Agassiz, British Columbia in June 2013 and July 2015 (Fig. 2-1*b-c*). Small pieces of diseased leaf tissue (5 – 10 mm²) were surface-sterilized in 70% ethanol for 30 s, followed by a dip in sterile distilled water for 1 min before being blotted dry and incubated on potato dextrose agar containing 5 drops of lactic acid per L (APDA) at 25°C for 7 days under ambient light. Ten Petri dishes, each containing three leaf pieces, were used for isolation. The resulting colonies were identified as a *Botrytis* sp. based

Figure 2-1. Symptoms of botrytis leaf blight on wasabi plants in a commercial greenhouse. (a) Healthy plant. (b) Initial yellowing and necrosis on leaf margins. (c) Small flecking on upper leaves of plant. (d) Severe marginal necrosis under conditions of high moisture. (e) Blighting, stunted plants and plant death resulting in gaps in the plant stand. (f) Symptoms on wasabi leaf resulting from inoculation with *Botrytis* isolate. (g) Isolate growing on PDA, with sclerotial formation at the colony margins. (h) Branched conidiophore with conidia from PDA culture. Scale bar = 100 μ m.



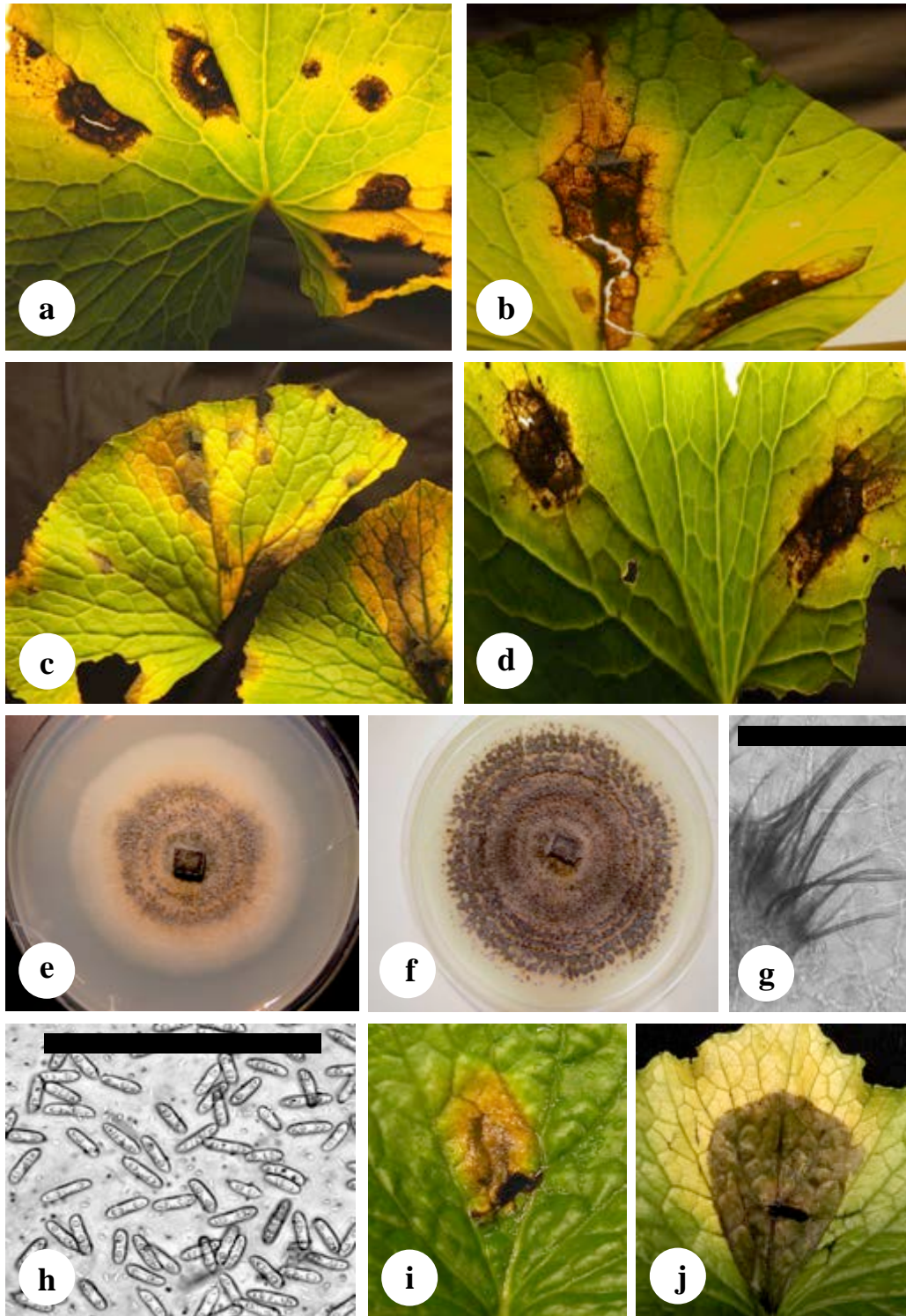
on colony morphology and characteristics of conidia and conidiophores when examined using a Nikon Eclipse Ci microscope at 400 X magnification (Fig. 2-1g). To determine the pathogenicity of two selected isolates, separate conidial suspensions containing $2.0 - 4.0 \times 10^5$ conida/mL prepared from 7-day old cultures grown on PDA were sprayed on to six potted 'Mazuma' plants each until runoff. Control plants were misted with sterile distilled water. The plants were kept in a humidity chamber at room temperature under natural daylight conditions, and assessed regularly for symptom development. After one week, symptomatic leaves were collected and pathogen isolation was conducted as previously described. The two representative isolates were deposited in the Canadian Collection of Fungal Cultures (CCFC), Ottawa (DAOMC 250508 and DAOMC 250509).

2.2.2 Anthracnose leaf spot

Leaves from a commercial wasabi crop grown in a greenhouse in Maple Ridge, BC showing symptoms of leaf spot and blight were collected in June 2014 (Fig. 2-2). Small tissue pieces were excised, surface-sterilized, and placed on APDA as described previously. Twelve Petri dishes, each containing three leaf pieces, were used for isolation. Resulting colonies were identified as a *Colletotrichum* sp. based on colony morphology and characteristics of the conidia, acervuli, and setae in 10-day old cultures.

Five wasabi leaves were excised from healthy plants, and the center of the leaf above the petiole was inoculated with a conidial suspension containing $8.4 - 8.6 \times 10^4$ conidia/mL mixture of two selected isolates to determine pathogenicity. The experiment was repeated on abaxial and adaxial leaf surfaces, and on wounded and non-wounded leaves.

Figure 2-2. Symptoms of anthracnose leaf spot on wasabi and pathogen characteristics. (a–d) Range of symptom development showing dark brown irregular-shaped lesions with visible yellowing. Some lesioned tissues have dropped out, leaving a shot-hole appearance. (e) 10-day-old colony on PDA, showing salmon color and black centre. (f) One-month-old colony on PDA, showing black concentric rings of setae. (g) Close-up of setae. Scale bar = 100 μ m. (h) Conidia of *Colletotrichum* from PDA culture. Scale bar = 100 μ m. (i, j) Development of anthracnose lesions following inoculation of young wasabi leaves and incubation under high humidity conditions for 7 days. Chlorotic areas can also be seen developing around the lesions. Scale bar = 100 μ m



Wounding was performed by piercing a sterile dissecting needle through the center of the leaf at the inoculation site. An equal number of control leaves were treated the same, but with sterile distilled water instead of a conidial suspension. Each leaf was placed in a Petri dish lined with moistened filter paper and incubated at 25 ± 2 °C and observed for symptom development. Pathogen reisolation was completed as described previously and the resulting colonies were compared to the original isolate. A representative isolate was deposited in the CCFC (DAOMC 250510).

Five-mm-diameter mycelial plugs taken from PDA cultures were transferred from the colony margins to fresh PDA dishes, which were then kept on a thermal gradient plate (TGP) in darkness to assess the effect of temperature on colony growth of *Colletotrichum* sp.. The TGP (built by Agriculture and Agri-Food Canada, Saskatoon) was comprised of 176 individually controlled cells with potential ranges of 0 to 40 °C, and controlled by individual thermoelectric pumps. A range of 0 to 35 °C was used for this experiment. After 7 days, maximum colony diameters were measured from 5 replicate dishes. This was repeated three times. To determine the effect of light on spore production, PDA plates were incubated in each of three Conviron units set at 0, 12, or 24 hr photoperiod (8000 ± 250 lux; 25 °C). After 7 and 14 days, colony diameters were measured and spore numbers assessed from five replicate dishes using a hemocytometer, and the experiment was repeated three times. Spore number experiments were analyzed independently and completed in SAS University Edition (v3.4), using log-transformed mean values in a one-way ANOVA (Tukey's HSD, $P = 0.05$).

A host differentiation study was conducted to differentiate between the closely related species *C. destructivum* O'Gara and *C. higginsianum* Sacc.. Mustard (*Brassica juncea* (L.) Czern) 'Southern Giant Curled' and alfalfa (*Medicago sativa* L.) were Czern) 'Southern

Giant Curled' and alfalfa (*Medicago sativa* L.) were selected for inoculation studies. *Colletotrichum destructivum* is reported to be pathogenic to members of Fabaceae (alfalfa) but not Brassicaceae (mustard), and vice versa for *C. higginsianum* (Damm et al. 2014). Ten mustard and ten alfalfa plants were seeded in pots and grown in the greenhouse for 5 weeks then inoculated with a conidial suspension containing 1.8×10^5 conidia/mL. An equal number of control plants received sterile distilled water. Each inoculated plant was misted with water, and covered with a humidity dome for three days. The plants were kept in a greenhouse with a targeted temperature setting of 25°C (set points: heating at 23°C and passive venting at 27°C). Pathogen isolation from developing lesions was conducted as previously described after 3 weeks and the resulting cultures examined after 7 days.

2.2.3 White blister rust

Wasabi plants 'Daruma' showing blister formation and white sori on the abaxial side of leaves were collected from a research polyhouse located in Agassiz, BC in October 2015 (Fig. 2-3). A severely diseased leaf was used to prepare slides for photomicrography and obtain measurements of sporangia at 400 X magnification (Nikon Eclipse Ci microscope, Nikon DS-Fi2 camera). Naturally-infected plants of *Capsella bursa-pastoris* (L.) Medic (shepherd's purse) (Fig. 2-4a) with typical symptoms of white rust (Alexander & Burdon 1984) were collected from two field sites, one adjacent to the polyhouse in Agassiz and one in a field in Abbotsford, BC. A sporangia solution (4.0×10^5 spores/mL) from each host was prepared by suspending sori in sterile distilled water and each was then misted onto four separate healthy, 9-month old 'Mazuma' plants in 10 cm diameter pots until runoff, and then covered with a transparent humidity dome. An equal number of control plants were misted with sterile

Figure 2-3. Albugo white rust symptom development on naturally infected leaves of wasabi. (a) Initial puckering of the upper leaf surface with yellowing. (b) Swellings on the underside of leaves with white mycelium (c) Large swellings that produce gall-like symptoms. (d) Severe infection at the leaf margin, showing leaf curling and development of galls. (e) Close-up of swollen tissues with white sporulation on surface. (f) White spore masses with black sori. (g) Close-up of leaf swelling with spore masses. (h) Sporangiospores as viewed under the microscope. Scale bar = 100 μ m.

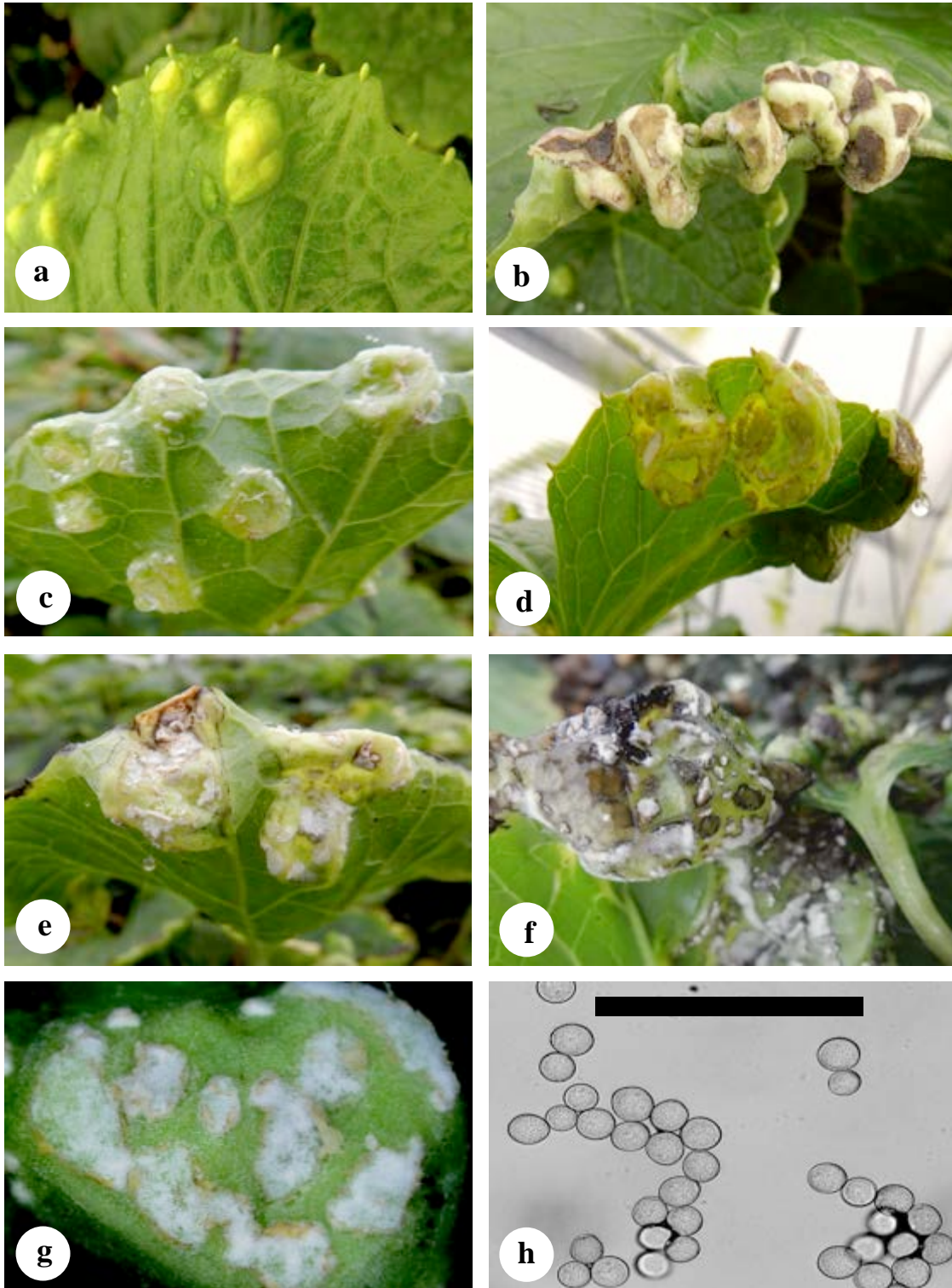
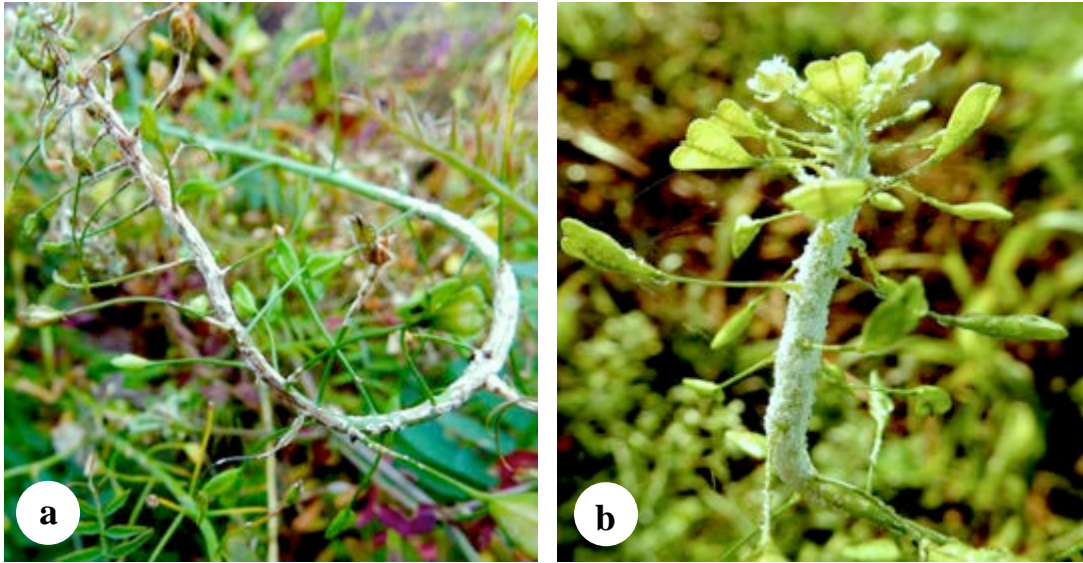


Figure 2-4. *Capsella bursa-pastoris* exhibiting white rust and white-rust like symptoms. (a) White rust on shepherd's purse showing epinasty and white sporulation on stem. (b) Downy mildew (*Hyaloperonospora parasitica*) on shepherd's purse showing masses of sporangia production.



distilled water. After three days, the humidity domes were removed and disease incidence and symptoms were assessed after 7 days. Each inoculation experiment was also repeated on four field-collected shepherd's purse plants that did not have symptoms of white blister rust.

2.2.4 Molecular identification of pathogen isolates

Two cultures each of *Botrytis* and *Colletotrichum* isolates, as well as *Albugo*-infected wasabi and shepherd's purse leaf and stem samples, were sent to the University of Guelph Laboratory Services, Agriculture and Food Laboratory, Guelph, ON where they were subjected to PCR using the primers ITS1F-ITS4 (ITS1-F CTTGGTCATTTAGAGGAAGTAA and ITS4 TCCTCCGCTTATTGATATGC). The corresponding sequences were compared to ITS1-5.8S-ITS2 sequences of *Botrytis cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) (11 isolates), *Colletotrichum destructivum* O'Gara (8 isolates), *Colletotrichum higginsianum* Sacc. (5 isolates), *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore (5 isolates), and *Albugo candida* (Pers. ex Lev) Kuntze (13 isolates) from the National Center for Biotechnology Information (NCBI) GenBank database. Multiple sequence alignment of the respective isolates of each species were made using CLUSTAL W program (<http://www.genome.jp/tools/clustalw>). The sequences were subsequently subjected to the neighbor joining (NJ) method analysis (Saitou & Nei 1987; Tamura et al. 2004) using the software MEGA v. 5 (Tamura et al. 2011). A bootstrap consensus tree was inferred from 1000 replicates to represent the distance. The outgroup used was *Sclerotinia sclerotiorum* (Lib.) de Bary (M96382) and *Hyaloperonospora parasitica* (Pers.) Constant (AY531452). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches as described by Felsenstein (1985).

2.3 Results

2.3.1 Botrytis leaf blight

Initial symptoms on naturally-infected plants were a general yellowing of leaves, small necrotic flecking on the upper leaves, and marginal leaf necrosis (Fig. 2-1*b-e*). With progressive development of the disease, larger necrotic areas developed on the leaves and young plants showed symptoms of blighting and were stunted. Light grey to white mycelial growth was observed under high humidity, together with masses of grey conidia. Over time, infected plants died, leaving gaps in the stand (Fig. 2-1*e*). Two isolates (DAOMC 250508, DAOMC 250509) developed grey- to brown-coloured, fast-growing colonies on PDA, with masses of conidia borne on branched conidiophores (Fig. 2-1*g-h*). The length and width of the conidia of the two isolates measured 14.0 μm long x 8.2 μm wide (8.1 – 18.7 x 6.2 – 11.1) and 12.9 μm x 7.6 μm (9.2 – 17.5 x 5.3 – 9.9), respectively, and were single-celled, colourless, and typically oval in shape. Black sclerotia developed on the colonies at the edges of the Petri dish (Fig. 2-1*g*). These features were used to identify the cultures as *Botrytis* sp. Following inoculation of wasabi leaves, a general yellowing was observed with subsequent flecking and marginal necrosis on wounded or senescing leaves, and abundant grey sporulation characteristic of *Botrytis* was observed (Fig. 2-1*f*). Reisolation from diseased tissues yielded morphologically identical colonies to those used for inoculation.

2.3.2 Colletotrichum leaf spot

Symptoms observed on naturally-infected plants included dark brown lesions that varied in size and shape, and were frequently surrounded by a chlorotic margin (Fig. 2-2*a-d*). Concentric rings of varying shades of brown were present in some lesions. Colonies growing on PDA were

initially light salmon in color with black masses of concentric rings, presumably of conidia production (Fig. 2-2e-f). Acervuli were produced that were cream to apricot coloured, measuring 170 – 790 μm in diameter, and with abundant setae, which were dark brown to black, 65 – 236 μm in length (Fig. 2-2g). Masses of cylindrical-shaped conidia were produced, which were obtuse, hyaline, and granulose and measured 14.2 μm long x 4.3 μm wide (8.1 – 22.3 x 2.8 – 5.6) (Fig. 2-2h). These observations were consistent with the description of O’Gara (1915), Higgins (1917), and Damm et al. (2014) of *Colletotrichum* species. Leaf inoculations with a conidial suspension of a mixture of two isolates resulted in symptoms that were similar to those on naturally infected plants (Fig. 2-2i-j). There were no differences in inoculations regardless of whether they were done on the adaxial or abaxial surface, or whether leaves were wounded or not. After 3 days, small brownish-black lesions formed at the site of inoculation and lesions enlarged and coalesced by 8 days. A yellow chlorotic halo developed in the leaf tissues surrounding the lesions. Cultures obtained from inoculated leaves were morphologically identical to the original culture.

Colonies grew best at 25 and 30°C, and growth was reduced at higher and lower temperatures (Fig. 2-5). At 5°C, colonies showed very little growth. Photoperiod had no effect on colony diameter; however, morphology of colonies was different under varying light regimes. Colonies were white under darkness, a deep salmon color under 12 h light per day, and a faded salmon colour under 24 h light per day. Conidia production was affected by different photoperiods after 7 days. In the presence of light (12 and 24 h photoperiods), fewer conidia were produced, while colonies grown under darkness produced the most conidia (Fig. 2-7). However, after 14 days, there was no difference in conidial production under different light regimes (data not shown).

Figure 2-5. Mean diameter of colonies of *C. higginsianum* after 7 days when grown on PDA at 5, 10, 15, 20, 25, 30, and 35°C. Data are from three different experiments (n=35) with standard deviation.

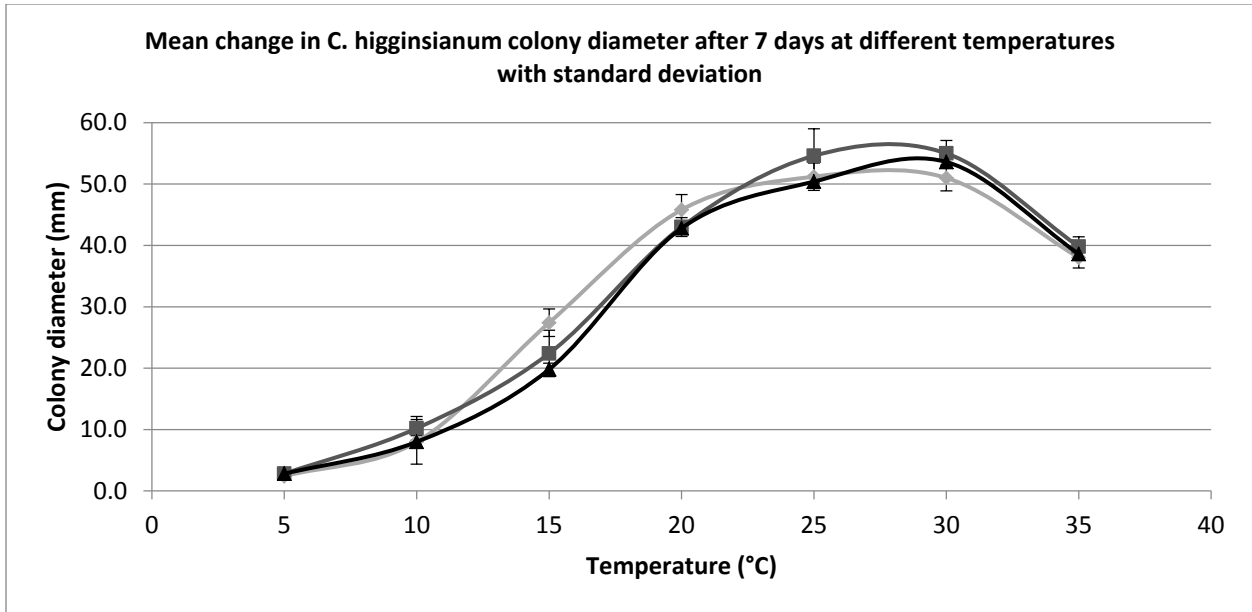


Figure 2-6. Cultures of *C. higginsianum* grown on PDA under different daily photoperiods for 7 days. (a) 0-hrs of light/24-hrs of darkness. (b) 12-hrs of light/12-hrs of darkness. (c) 24-hrs of light/0-hrs of darkness.

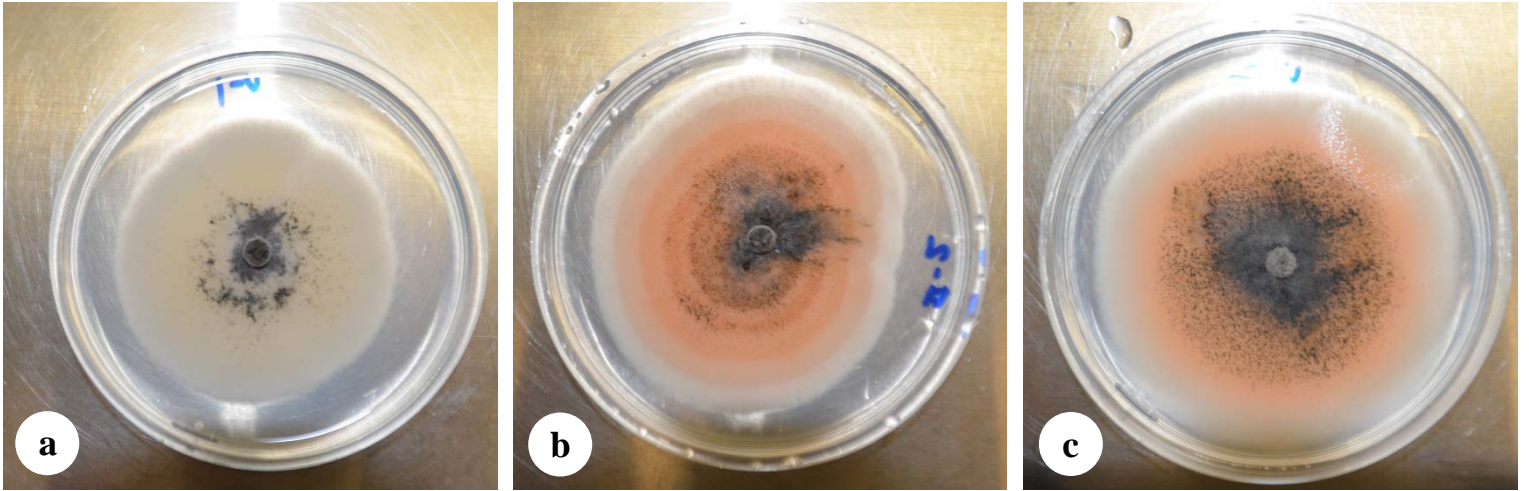
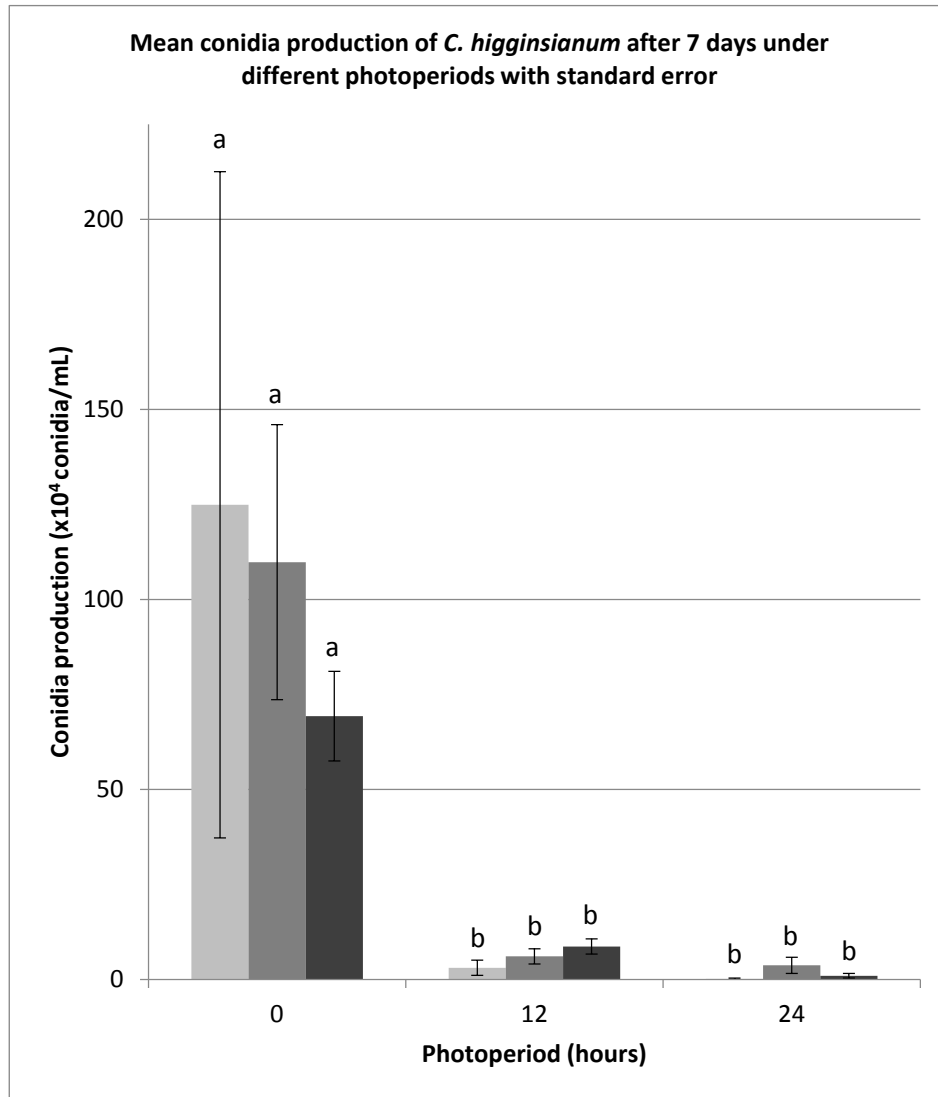


Figure 2-7. Mean conidia production in *C. higginsianum* cultures after 7 days when grown on PDA under 0, 12, and 24-h photoperiods. Data are from three different experiments (n=15) except experiment 2 (dark grey shade) where one significant outlier (value = 643) was excluded from the 24-h photoperiod. Each letter represents a significance difference in the Log-transformed values in a one-way ANOVA (Tukey's, $P=0.05$).



Lesions developed on three of the ten mustard plants, but not on any alfalfa plants. Following isolations from diseased tissues, a *Colletotrichum* sp. was identified on three of the six dishes.

2.3.3 Albugo white blister rust

About 50% of wasabi plants naturally developed symptoms of white blister rust, which ranged from small (<1 cm) swellings with no chlorosis, with sori developing on the underside, to large swellings and distortions enveloping the circumference of a leaf (Fig. 2-3a-e). Frequently, a black rot, with cream-coloured sori and blistering on the abaxial surface, developed with chlorotic symptoms on the adaxial leaf surface (Fig. 2-3f-g). Sporangia appeared white to the naked eye. Sporangiospores were globose or sub-globose and hyaline with a cell wall of uniform thickness, and measured 15.8 μm x 17.1 μm (12.4 – 18.9 x 14.3 – 19.9) (Fig. 2-3h). The symptoms and microscopic observations matched those of wasabi white blister rust caused by *Albugo* sp. in Korea (Choi et al. 2014). After 7 – 10 days, the abaxial side of a single wasabi leaf inoculated with sporangiospores collected from shepherd's purse produced blisters. These were similar to those observed on naturally infected leaves, although they were much smaller in size, possibly due to the environmental conditions during infection. Inoculations using sporangiospores collected from wasabi did not confirm pathogenicity on shepherd's purse or wasabi plants.

2.3.4 Molecular identification of pathogen isolates

The two isolates of *Botrytis* were identified as *B. cinerea* (Fig. 2-8). They grouped together with isolates from a range of substrates and hosts and sequences of the ITS1-5.8S-ITS2 rDNA region were 99-100% identical. The isolates of the *Colletotrichum* sp. from wasabi were

grouped within a large cluster of isolates identified as *C. destructivum* (Fig. 2-9). These isolates were also grouped together with *C. higginsianum* and could not be separated from them in the phylogenetic analysis. However, isolates of *C. truncatum* formed a separate group (Fig. 2-9). The isolates of the *Albugo* sp. from wasabi and shepherd's purse collected from two field sites, one near the wasabi polyhouse, were identified as *A. candida* and grouped together with isolates from a range of hosts and geographic regions (Fig. 2-10). One isolate collected from shepherd's purse 60 km from the polyhouse was identified as *Hyaloperonospora parasitica* (Fig. 2-4b), the causal agent of downy mildew.

2.4 Discussion

The high humidity environment required for commercial production of wasabi provides ideal conditions for the development and spread of plant pathogens. Grey mold caused by *B. cinerea* was observed as a weak pathogen on wasabi plants, causing yellowing, necrotic lesions and stunted growth under high humidity conditions. *Botrytis cinerea* is known to be an opportunistic necrotrophic pathogen that infects over 200 host plant species world-wide (Williamson et al. 2007). The pathogen can persist in plant debris as mycelia, conidia, or sclerotia (Williamson et al. 2007). With the extended growing period required for wasabi (18 months), and the wide host range and abundance of *Botrytis* sp. in the environment, reducing disease incidence would require growers to clean and/or sterilize wasabi growing materials between crops in order to avoid losses to leaf blight. Interestingly, wasabi defensin genes have been the focus of a number of studies in transgenic crops which have been used to successfully introduce resistance or partial resistance to *B. cinerea* in potato and tobacco plants (Khan et al. 2006; Kiba et al. 2007; Hoshikawa et al. 2012). The fact that leaf blight has been observed at low levels consistently throughout the growing period in wasabi plantings reinforces the need to

Figure 2-8. A phylogenetic tree constructed with ITS1-5.8S-ITS2 rDNA sequence of the two *Botrytis* isolates from this study (“wasabi-1” and “wasabi-2”), and other isolates of *Botrytis* retrieved from GenBank. *Sclerotinia sclerotiorum* was used as the out-group taxon. Number of bootstrap support values $\geq 50\%$ based on 1000 replicates.

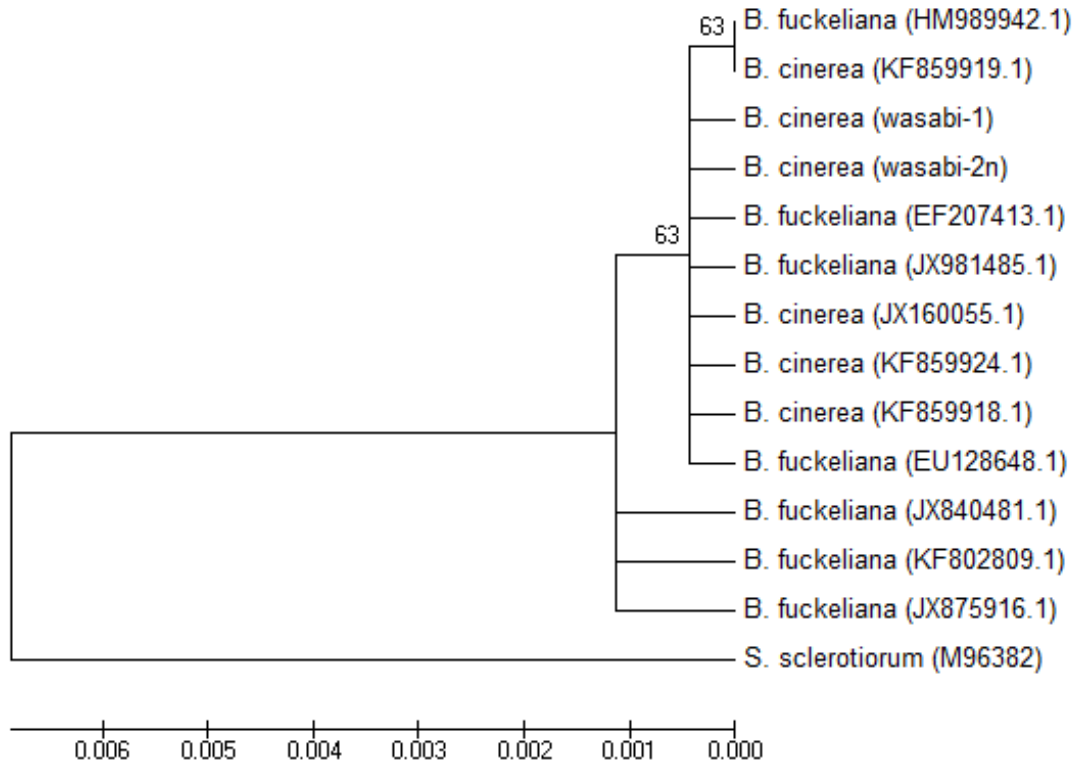


Figure 2-9. A phylogenetic tree constructed with ITS1-5.8S-ITS2 rDNA sequence of the two *Colletotrichum* isolates from this study (“wasabi-3” and “wasabi-4”), and other isolates of *Colletotrichum* retrieved from GenBank. *Sclerotinia sclerotiorum* was used as the out-group taxon. Number of bootstrap support values $\geq 50\%$ based on 1000 replicates.

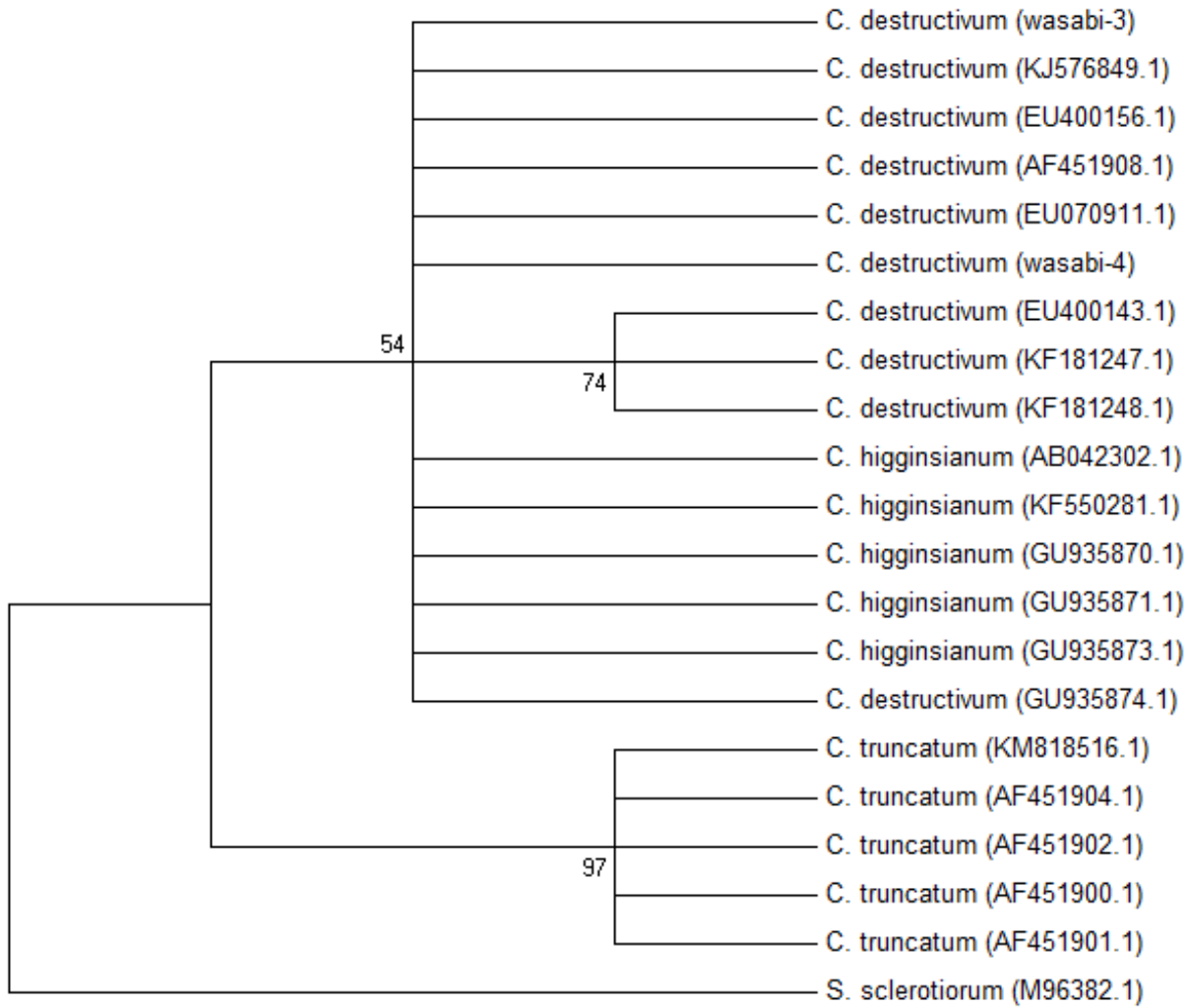
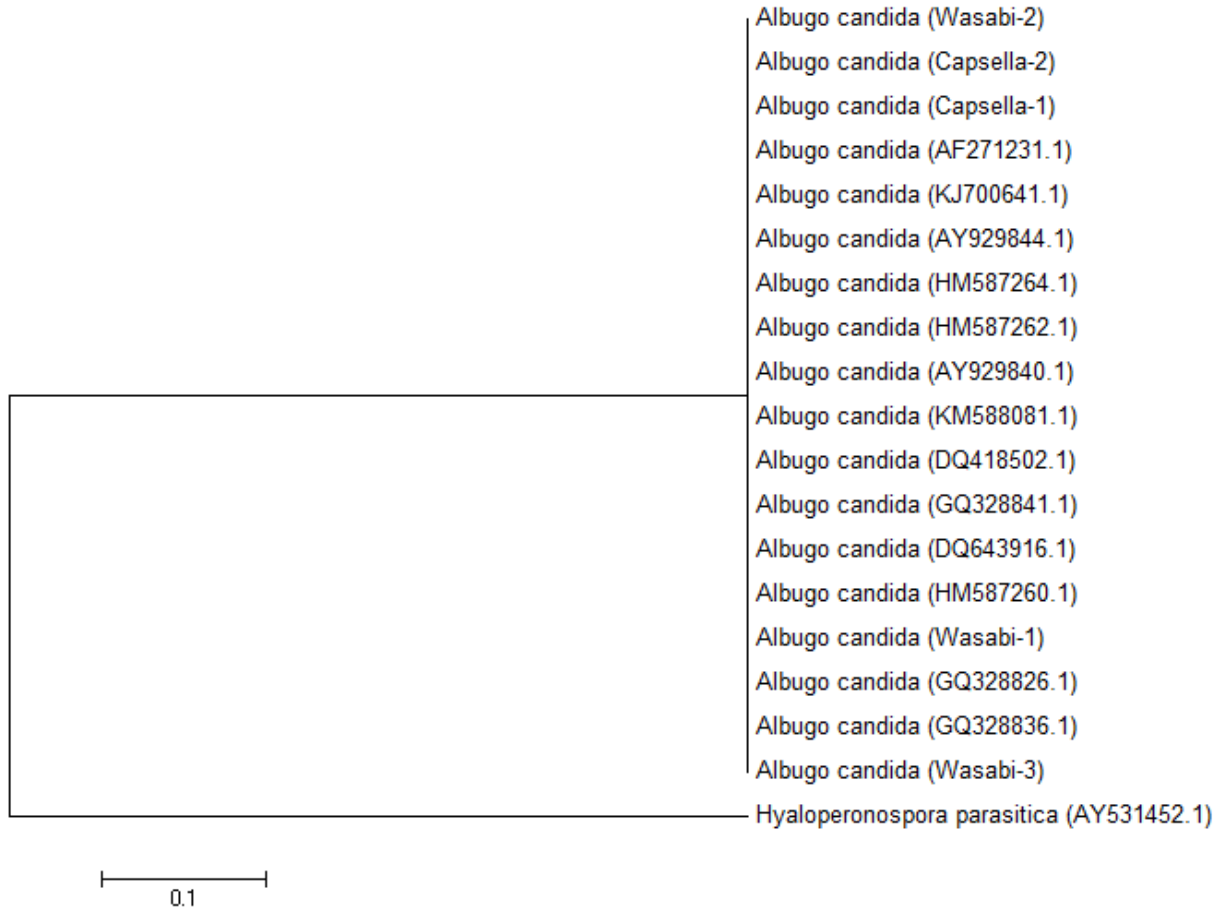


Figure 2-10. A phylogenetic tree constructed with ITS1-5.8S-ITS2 5 rDNA sequence of the five *Albugo* isolates (“Wasabi-1,” “Wasabi-2,” “Wasabi-3,” “Capsella-1,” and “Capsella-2”) from this study, and other isolates of *Albugo* retrieved from GenBank. *Hyaloperonospora parasitica* was used as the out-group taxon. Number of bootstrap support values $\geq 50\%$ based on 1000 replicates.



prevent leaf damage and avoid conditions which would allow *Botrytis* to establish in stressed plants (authors, personal observation).

The *Colletotrichum* sp. causing anthracnose symptoms on wasabi plants was grouped with previously identified isolates of *C. destructivum* or *C. higginsianum* in the phylogenetic analysis. *Colletotrichum destructivum* infects host plants from at least 11 different families (Damm et al. 2014), including alfalfa in Canada (Boland & Brochu 1989), while *C. higginsianum* has been implicated as the most common causal agent of Brassica anthracnose, and was tentatively named *C. brassicae* (Higgins 1917). Our sequence analysis using the ITS1-5.8S-ITS2 rDNA region placed the wasabi isolates within the same clade that contained *C. destructivum* as well as *C. higginsianum*, making it difficult to confirm the identity of the species causing anthracnose. If the host of origin has precedence on the species identification, the causal organism on wasabi would be *C. higginsianum*, which is a part of the *C. destructivum* species complex (Damm et al. 2014). The preliminary results of the mustard and alfalfa inoculations support the identification of the wasabi anthracnose as *C. higginsianum*, since it is known to be pathogenic on mustard but not alfalfa, and vice versa for *C. destructivum*. This agrees with a previous note of anthracnose on wasabi reported to be caused by *C. higginsianum* in New Zealand by Martin & Deo (2000); however, their method for species identification was not provided .

White blister rust was observed on wasabi in this study and has been previously reported to occur on wasabi. The causal pathogen was reported as either *Albugo wasabiae* Hara (Lo & Wang 2000; Joshi & Jeffries 2010) or *A. candida* (Choi et al. 2014). The sequence analysis of the isolates collected from wasabi and from shepherd's purse in this study showed that *A. candida* was the causal agent. The species designation as *A. wasabiae* based on host of origin (wasabi) is

not supported. *Albugo candida* is a commonly-occurring pathogen of shepherd's purse world-wide (Choi et al. 2007) and infects many other members of the Brassicaceae (Saharan & Verma 1992; Choi et al. 2009). However, most of the reports from Canada were restricted to crop species of economic importance in the genus *Brassica* (Pidskalny & Rimmer 1985; Rimmer et al. 2000). Spores collected from shepherd's purse in this study, when inoculated onto wasabi leaves, caused small galls to form, although the susceptibility of shepherd's purse plants to the wasabi isolate could not be confirmed. The wasabi production guide (Miles & Chadwick 2008) states that white rust infection by *A. wasabiae* occurs at 45 – 68°F (7 – 20°C) (Adachi 1987); therefore, our inoculation experiments may have been carried out under temperature conditions unsuitable for infection, and resources limited our ability to repeat the experiment under different conditions. It is conceivable that inoculum for initiating white blister rust on the wasabi plants originated from naturally-infected shepherd's purse plants that were growing in the vicinity of the greenhouse. Pathogen spread was rapid and wasabi plants developed severe disease symptoms, showing they were highly susceptible at all stages of growth. Recent outbreaks of white blister rust have been reported in several greenhouses during fall 2016 (authors, unpublished observations), indicating this is a disease that has the potential to limit wasabi production.

An increased occurrence of diseases of wasabi is becoming apparent with increased intensity of wasabi production and use of vegetatively-propagated plants that could harbour inoculum. The diseases that affect wasabi in British Columbia include some of the most common and destructive pathogens described in other parts of the world, including *A. candida* (Choi et al. 2014) and *L. biglobosa* (Punja et al. 2017), as well as this first report of *Colletotrichum* causing anthracnose of wasabi in Canada. The use of overhead misting, vegetative propagation, continual

and long-term planting of a few cultivars, and use of recycled planting medium is likely contributing to the increased occurrence of these diseases. While ‘Daruma’ may be less susceptible to anthracnose than ‘Mazuma’, it is highly susceptible to white blister rust infection in addition to ‘Greenthumb’ (authors, personal observation).

Disease management practices for wasabi pathogens include fumigating of the growing medium between crops (Chadwick et al. 1993), restricting the use of offshoots to 2 – 3 generations (Adachi 1987), and using clean tissue cultured plants to repropagate a planting. In Canada, there currently are no fungicides registered on wasabi. Monitoring of Brassica plant species, both weedy species and cultivated crops, will become increasingly important, as observations from this study suggest that infected weedy species may produce inoculum that can spread to nearby wasabi plantings. While the range of pathogens capable of infecting wasabi therefore remains unknown, widespread diseases that affect Brassicaceae crops in BC, such as clubroot and alternaria blight, are likely to emerge on wasabi plants in the future.

CHAPTER 3: GENERAL DISCUSSION AND CONCLUSIONS

3.1 Identification of pathogens

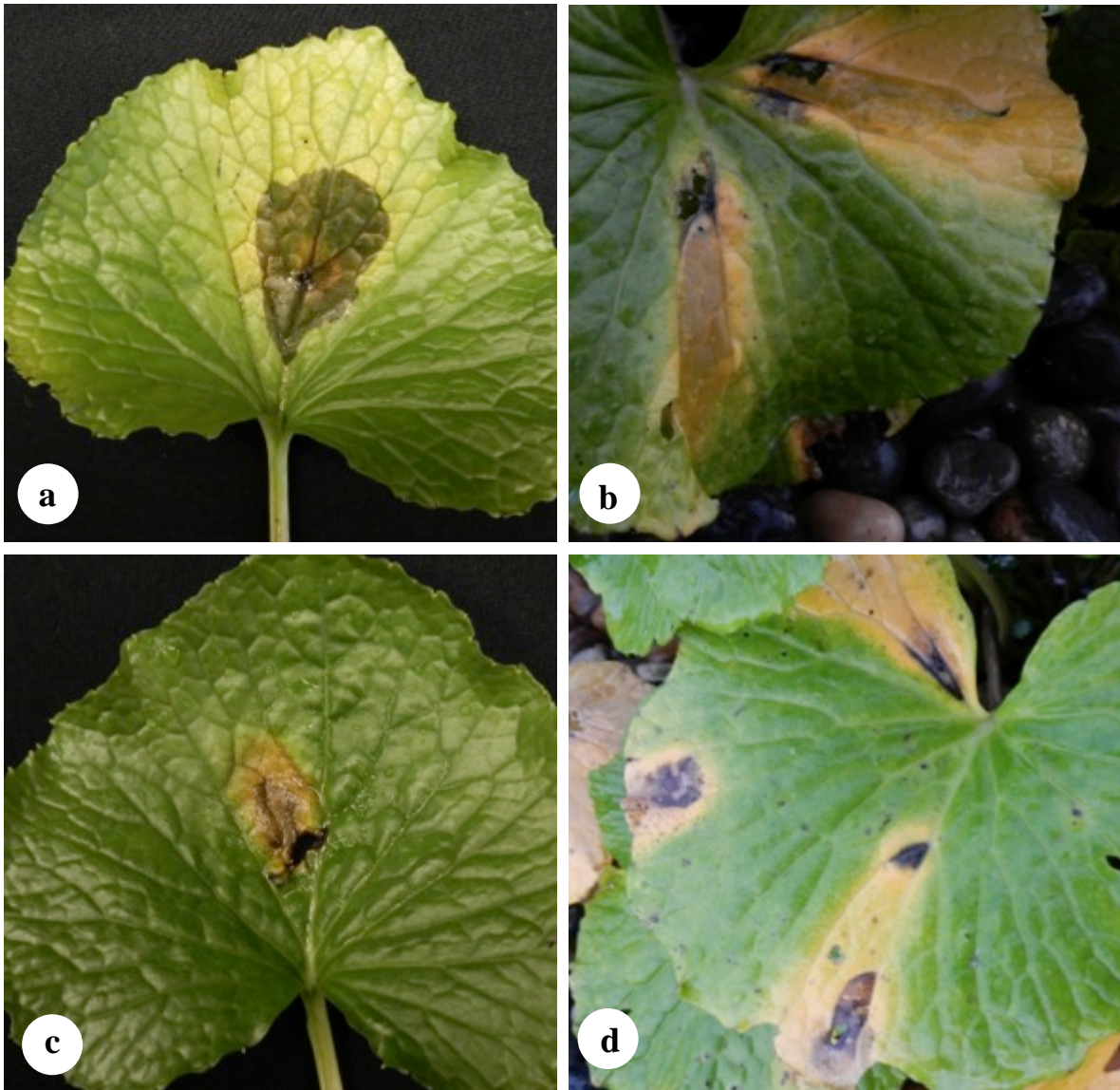
3.1.1 General remarks

The identification and characterization of the disease symptoms of three new pathogens to wasabi in British Columbia nearly doubles the known pathogens of the region. Prior to 2017, *Pythium dissotocum*, *P. intermedium* (Rodríguez & Punja 2007), and *Pectobacterium carotovorum* subsp. *carotovorum* (Rodríguez & Punja 2009) were the only pathogens identified in the literature of wasabi in North America. In addition to these aforementioned pathogens, Punja et al. (2017) have also described *Leptosphaeria biglobosa* subsp. *occiaustralensis* (*Phoma wasabiae*), which coincidentally was also isolated from our research crops during the course of this research. These identifications bring the total number of known pathogens of wasabi in North America from four to seven.

3.1.2 Identification of *Botrytis cinerea*

B. cinerea causing leaf blight has not been listed to be of concern to wasabi, even anecdotally, that the author is aware of. Regardless, it is not entirely unexpected to be pathogenic to wasabi, as it is known to be a global, multi-host species (Williamson et al. 2007). Its role as an opportunistic pathogen of stressed or otherwise weak or senescing plant material is well documented (Williamson et al. 2007). Regardless, understanding the pressure this pathogen can place on a crop may lead to healthier plants that are better suited to tolerate other disease pressures.

Figure 3-1. Anthracnose of *W. japonica* caused by *C. higginsianum* and leaf spot caused by *L. biglobosa*. (a,c) Symptoms of anthracnose on wasabi leaves after inoculation with *C. higginsianum*. (b,d) Leaf spot and blight on wasabi leaves naturally infected with *L. biglobosa*.



3.1.3 Identification of *Colletotrichum higginsianum*

Although anecdotally known in the literature (Martin & Deo 2000), this is the first confirmed report of *C. higginsianum* causing anthracnose of wasabi. This identification and description of disease symptoms is important, as leaf spot of wasabi caused by *Leptosphaeria biglobosa* subsp. *occiaustralensis*, as described by Punja et al. (2017) and observed within our own research crops, presents itself in a noticeably similar fashion (Fig. 3-1). The only known method to differentiate between the two causal agents is to isolate the pathogen from infected plant material and identify it either morphologically or molecularly. This insight leads to the questions: how often has the causal agent of anthracnose been misdiagnosed and how has this affected the efficacy of current management efforts? Understanding their differences in biology could lead to better production and management practices.

3.1.4 Identification of *Albugo candida*

Growers are aware of white blister rust on wasabi, and generally it is not considered a damaging pathogen here (D. Groenendale, Western Wasabi, Washington, USA, pers. comm.). A number of researchers have also noted white blister rust on wasabi in British Columbia, stating that the symptoms they observed did not appear to be as severe as those presented here, but that the disease is not uncommon and still causes damage to leaves (Georgina Rodríguez and Emily Betz, Simon Fraser University, Burnaby, BC, pers. comm.). However, in parts of South East Asia, the disease has been known to be of particular concern to growers (Choi et al. 2014). The white blister rust that was observed in our research plot caused significant and extensive damage, providing evidence that when conditions are right, it can be very economically damaging to wasabi crops here as well, and warrants attention in determining good management practices.

3.2 Areas for future research

While there is potential for insect pests to cause economic damage directly or indirectly by vectoring pathogens (Appendix B), the primary problem wasabi growers continue to face is diseases. The wasabi pathogens *B. cinerea*, *C. higginsianum*, and *A. candida* are all of economic concern to growers, and each on their own can cause crop loss or reduced marketability. More work should be conducted to understand the prevalence of these and other emerging diseases in British Columbia. The recent occurrences of newly identified causal agents of disease on wasabi in British Columbia (Betz et al. 2016) suggests that other pathogens already known to other wasabi-growing regions of the globe may also be present, only remaining unidentified due to lack of time and/or resources. It would not be surprising for pathogens such as *Erysiphe* sp. or *Alternaria* sp. to become of concern to the industry in the near future.

Continuing to promote clean growing conditions should be of particular importance, especially until efficacious pest control products can be identified and registered. This can be achieved by buying plant stock from tissue culture suppliers, and by doing thorough cleaning of polyhouses and planting substrate after harvest but between crops, as well as having staff follow standard greenhouse biosecurity practices. Encouraging growers to alter production strategies for their wasabi crops, supported by education on relevant fungal pathogens, could be a very useful tool to decrease the disease pressure on commercial operations. Identifying alternative temperature management methods for polyhouse and river rock substrate operations to avoid overhead misting could drastically reduce foliar pathogens. Site selection that includes naturally shaded or otherwise cool environmental conditions, such as from stands of mature trees, may be an option at some locations. Besides maintaining clean plant material, reducing plant surface moisture throughout the entire crop life, while retaining adequate root irrigation, and maintaining

temperatures within the optimal range for wasabi growth (6 – 20 °C) could have a significant role in reducing disease incidence. Additional areas for research include selecting biological control agents for disease management and identifying potentially resistant germplasm through screening of different sources of wasabi.

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APPENDIX A: PRELIMINARY LEAF DISC BIOASSAY COMPARISON OF TOLERANCE OF *WASABIA JAPONICA* CV. DARUMA AND CV. MAZUMA TO *COLLETOTRICHUM HIGGINSIANUM* INOCULATION

A.1 Introduction

Colletotrichum higginsianum Sacc. is the causal agent of destructive anthracnose of wasabi leaves, with symptoms very similar to those caused by *Leptosphaeria biglobosa* Shoemaker & H. Brun (Punja et al. 2017). Currently, there are no chemical control options available to growers for managing anthracnose, and as the first in-depth investigation into the infection of wasabi by *C. higginsianum* was only recently published (MacDonald & Punja 2017), cultural control will be the first line of defense. It is accepted among growers that Daruma is generally more tolerant to most diseases than Mazuma; however, there have been no investigations into the dynamics between *C. higginsianum* and these cultivars. This experiment provides preliminary evidence of differences in cultivar response to *C. higginsianum* infection to assist in the disease management problems growers face.

A.2 Materials and methods

A.2.1 Plant material and inoculation

Forty, 2.5 cm diameter, undamaged, and otherwise healthy looking leaf discs were excised from both Daruma and Mazuma leaves from a 9-month old research planting at the Agassiz Research and Development Centre (AAFC), using a Precision Leaf Punch (Rabbit Tool, Rock Island, IL). The discs were surface sterilized with 70% EtOH for 30 s, then rinsed three

times in sterilized distilled water for 1 min, then blotted dry. Two leaf discs of each cultivar were placed in each of 20 90x15 mm Petri dishes lined with damp, sterilized paper towel.

A 10.7×10^5 conidia/mL spore solution was prepared from a 10-day old culture of *C. higginsianum* originally isolated from wasabi and grown on PDA (DAOMC 250510). One disc of each cultivar received a 1 mL droplet of spore solution, and the other received 1 mL of sterile distilled water, before the Petri dish was covered with its lid. A marking above each leaf disc denoted its treatment (M = Mazuma, MI = Mazuma inoculated, D = Daruma, DI = Daruma inoculated). All Petri dishes were placed in a dark box for three days, then removed and kept in ambient light conditions, and were maintained at room temperature for fourteen days.

A.2.2 Disease severity index and percent leaf area showing disease

After removal from the dark-box, each leaf disc was assessed daily. A disease severity index (DSI), adapted from work by Boland & Brochu (1989) on infection of alfalfa by *C. destructivum*, was used to assess severity (Table 2), and a simple scale of the percentage of leaf surface showing symptoms was also created (percent surface area (PSA)(Table 3)). Statistical analysis of the results was conducted with SAS University Edition (v3.6, SAS Institute Inc., Cary, NC, USA) software, using mean values in a one-way ANOVA (Tukey's HSD, $P = 0.05$). Each assessment date was analyzed independent of the others. This experiment was not repeated.

A.3 Results

One day after the leaf discs were removed from the dark box, the first symptoms began to show on both inoculated treatments. On average, the DSI and PSA values of the inoculated

Mazuma leaf discs immediately began to increase, continuing to do so until the last assessment on day 14.

The inoculated Mazuma maintained significantly higher means of both DSI (Fig. A-1) and PSA (Fig. A-2) than the non-inoculated Daruma throughout the experiment. A number of leaf discs progressed from a DSI of 2 to a DSI of 5 without producing acervuli or any visible spores. Inoculated Daruma DSI and PSA means also increased, although less so than the inoculated Mazuma, as did the non-inoculated Mazuma.

A.4 Discussion

Mazuma appears to be more susceptible to *C. higginsianum* infection than Daruma. The similar results between the non-inoculated Mazuma (M) and the inoculated Daruma (DI) could be the consequence of a number of factors, including: 1) a pre-existing infection from the research plot (the author did not confirm cleanliness of plant material), 2) secondary infection from being in proximity to inoculated leaf discs, 3) a natural degradation of leaf material, of which perhaps Mazuma expresses faster than Daruma and in a similar fashion to anthracnose, 4) or another, unconsidered factor. The PSA is likely more relevant in this case; however, in full-plant, simulated field trials, where environmental factors may be more conducive to disease development, the DSI would be an important assessment consideration.

For growers with less environmental control of their polyhouses, especially their ability to cool, and with increased periods of leaf surface wetness, such as from overhead misters, cultivar selection is important. The susceptibility of Mazuma, coupled with the long growing period and lack of effective fungicides, suggests that cultural control will continue to be a driving force for successful wasabi production. This experiment needs to be repeated and the

Table 2. Disease severity index (DSI) of *C. higginsianum* on *W. japonica* leaf discs.

Disease severity index	
1	No or very few pin-prick sized leaf lesions
2	Leaf disc with elongated black or water-soaked lesions but without acervuli
3	Leaf disc with long, wide lesions and with acervuli present
4	Large, coalescing and sporulating lesions
5	Leaf disc wholly rotted or otherwise symptomatic

Table 3. Scale of the percent of leaf disc area (PSA) showing symptoms of *C. higginsianum* infection.

% of leaf surface with symptoms			
0	0-5%	6	56-65%
1	6-15%	7	66-75%
2	16-25%	8	76-85%
3	26-35%	9	86-95%
4	36-45%	10	96-100%
5	46-55%		

assessments refined before any definite conclusions can be reached. It should also incorporate the newest cultivar to local commercial growers, cv. Greenthumb, and be repeated on intact plants with other common leaf pathogens of wasabi such as *L. biglobosa* (Punja et al. 2017), *B. cinerea*, and *A. candida* (MacDonald & Punja 2017) if it is to be meaningful to the current industry.

Figure A-1. Daily means of disease severity index (1 – 5) assessments on non-inoculated Mazuma, Mazuma inoculated with *C. higginsianum*, non-inoculated Daruma, and Daruma inoculated with *C. higginsianum* from 4 days after inoculation to 14 days after inoculation. Results are the means of 20 replicates, $n=80$ with standard error. Letters denote significance differences in a one-way ANOVA (Tukey’s HSD, $P = 0.05$).

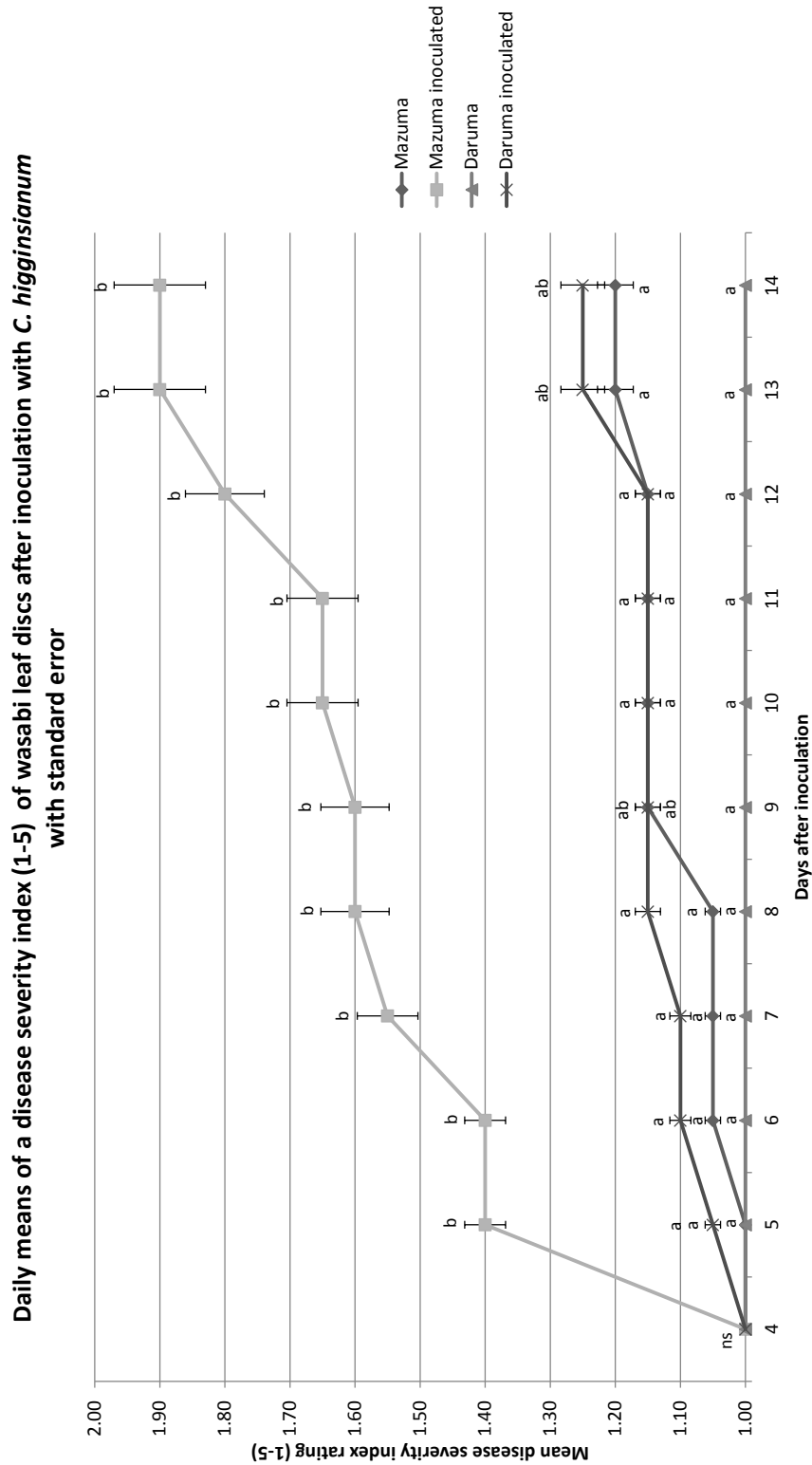
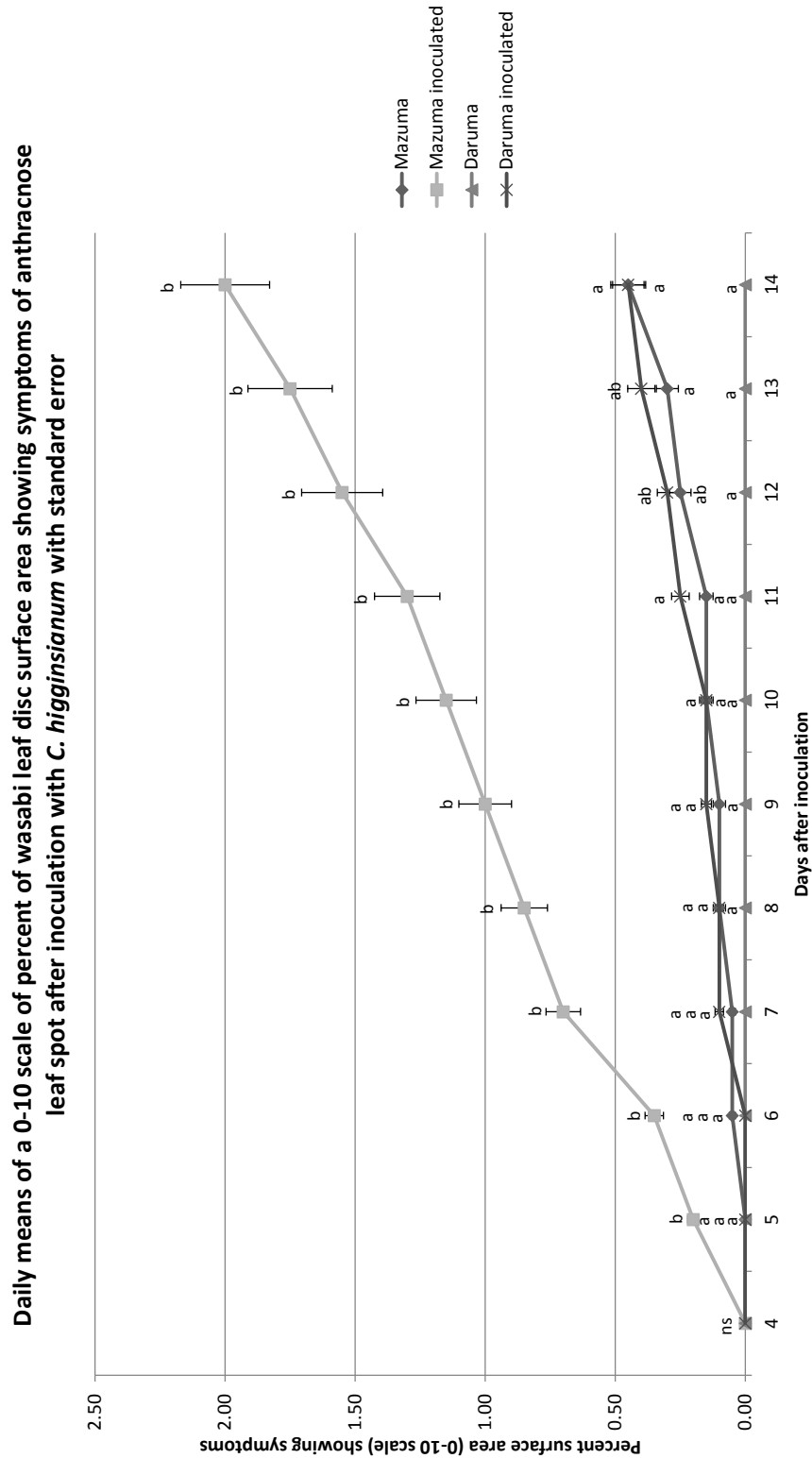


Figure A-2. Daily means of assessments of the percent of leaf disc area displaying symptoms of anthracnose (0 – 10 scale) on non-inoculated Mazuma, Mazuma inoculated with *C. higginsianum*, non-inoculated Daruma, and Daruma inoculated with *C. higginsianum* from 4 days after inoculation to 14 days after inoculation. Results are the means of 20 replicates, $n=80$ with standard error. Letters denote significance differences in a one-way ANOVA (Tukey's HSD, $P = 0.05$).



APPENDIX B: FIRST REPORT OF APHID AND DIAMONDBACKMOTH POPULATIONS ON WASABI IN BRITISH COLUMBIA²

B.1 Introduction

Wasabi (*Wasabia japonica* (Miq.) Matsumura) (Brassicaceae) is native to Japan, where it grows in shaded stream environments (Adachi 1987). It is currently cultivated in Asia, Australasia, and North America for its valuable rhizome, which is used as a freshly-ground condiment eaten with traditional Japanese meals (Hodge 1974; Chadwick et al. 1993). It can fetch US\$150-300/kg on the international market. Although the rhizome is the primary plant part for culinary use, the leaves can also be used to flavour soups or salads (Chadwick et al. 1993). In B.C., there is an estimated 5-10 acres of commercial wasabi in production using hydroponic or similar systems in polyethylene tunnels (polyhouses) or traditional glass greenhouses. Plants are typically grown in river rock substrate, as plants grown in soil are thought to produce an inferior quality rhizome (Chadwick 1993; Sultana et al. 2003). It takes 12 – 18 months before plants are of marketable quality, and due to the humid growing environment and propagation from axillary shoots, disease issues are the most common reason for crop loss in British Columbia (Rodríguez & Punja 2009; Punja et al. 2017; MacDonald & Punja 2017). To date, little research has been directed toward arthropod pests, and all reports are anecdotal. We report the first occurrence of insect pests on wasabi in North America at a research planting in Agassiz, British Columbia.

B.2 Materials and methods

Two polyhouses, each planted with 500 tissue cultured (cv. Daruma) and 500 auxiliary-shoot propagated (cv. Mazuma) wasabi plantlets, were established in January 2015 at the Agriculture and Agri-Food Canada (AAFC) Agassiz Research and Development Centre in

² Appendix B has been accepted to the Journal of the Entomological Society of British Columbia (MacDonald, Maw & Clarke 2017).

Agassiz, BC. Prior to transplant into the polyhouses, the plants were maintained in a production greenhouse for one month. A commercial nutrient growing system was used, with overhead misters fertigating at regular intervals or when triggered by a photosensor. Plants were grown in ~20 cm of 2-3 cm diameter river-rock. From April to October, 70% shade cloth covered the polyhouses to reduce direct exposure to UV radiation. Weekly or bi-weekly inspections were conducted by trained staff to identify pests and for treatment recommendations. Aphid populations were treated with imidacloprid. Two releases of *Diadegma insulare* Cresson parasitoids were conducted weekly to manage feeding diamondback moth larvae, followed by treatment with flubendiamide.

Leaves, petioles, and roots were visually inspected at random throughout each planting. Approximately 5 infested plants with representative pest populations from either leaves and petioles or roots were selected for each sample. A soft paintbrush was used to gently brush specimens off of plant material into vials of 95% ethanol (EtOH) for identification. Aphids were identified by morphological determination or by sequencing mitochondrial cytochrome C oxidase, subunit 1 (“DNA barcoding”), and adult diamondback moths by morphology under a binocular microscope. Assessments were carried out until harvest, after 15 months.

B.3 Results and discussion

B.3.1 Poplar petiole gall aphids

In January 2015, *W. japonica* ‘Daruma’ plantlets grown in soil-less plugs were uprooted for transplant into polyhouses and a heavy root aphid infestation was noted. Aphids were present throughout the crop and each root system, and a characteristic white waxy secretion was visible (Fig. B1a). No alates were present. The following January to March (2016) on the same crop of

Daruma and a neighbouring crop of Mazuma, identical root aphid populations were again found. In both cases treatment with imidacloprid appeared to provide control. All populations were identified as *Pemphigus populitransversus* Riley by sequence matching to specimens collected from galls on *Populus deltoides* (Fig. B1b).

Root aphids have been implicated as pests of wasabi historically (Miles & Chadwick 2008; Chadwick et al. 1993) but identified only once, in New Zealand, as *P. bursarius* Linnaeus (Douglas & Follett 1992); that population was difficult to control. *Pemphigus populitransversus* is known to alternate hosts between roots of various Brassicaceae, sometimes as a significant pest (for example Chen et al. 2009), persisting by parthenogenesis, and a sexual stage on *Populus* spp. trees, where they overwinter as eggs and form galls on the petioles of the leaves the following spring (Jones & Gillette 1918). Aphid damage to roots and rhizomes may be an important pathway for pathogens such as *Pectobacterium carotovorum* subsp. *carotovorum*, which has been found to cause vascular blackening of the rhizome after entry through small wounds (Rodríguez & Punja 2009). This is the first published report of *P. populitransversus* in B.C. that the authors are aware of, although there are specimens of unidentified *Pemphigus* species from wasabi collected in Aldergrove and Langley in 1997 and 1998 in the Canadian National Collection of Insects, Arachnids and Nematodes.

B.3.2 Turnip aphids

In spring of 2016, aphids were found predominantly on leaves of one-quarter of the affected polyhouse and identified as *Lipaphis pseudobrassicae* Davis (n = 62 specimens). *Macrosiphum euphorbiae* Thomas (n = 2) and *Myzus persicae* Sulzer (n = 1) were also present in the sample (Fig. B1c-d).

The most serious issue associated with aphids on wasabi is their ability to transmit viruses (Douglas & Follett 1992). Wasabi is susceptible to *tobacco mosaic virus* (TMV), *turnip mosaic virus* (TuMV), and *cucumber mosaic virus* (CMV) (Chadwick et al. 1993; Wilson 1998), and although problematic elsewhere, no viruses have been identified on wasabi in B.C. Should these diseases be reported locally, *L. pseudobrassicae* should be assessed as a potential vector of TuMV and CMV (Chan et al. 1991).

B.3.3 Diamondback moth

In June 2015, a heavy infestation of diamondback moth, *Plutella xylostella* Linnaeus, and associated ‘shothole’ damage on leaves was found. Adults were prevalent and flew as plants were disturbed.

Diamondback moth is the most destructive pest of Brassica crops worldwide. It has been reported on wasabi crops in Japan (Hodge 1974; Adachi 1987; Chadwick *et al.* 1993; Miles & Chadwick 2008). Due to successful management of the infestation with flubendiamide, it is unclear whether *D. insulare* releases were effective. Interestingly, a single mobile parasitoid adult was photographed almost 10 months later in a neighbouring polyhouse which had no previous biocontrol releases, suggesting the population persisted.

B.4 Conclusion

This first survey of insect pests of commercial wasabi production suggests that there is considerable potential for economic damage. Currently, no insecticides are registered in the United States for use on wasabi and only one synthetic insecticide is registered in Canada (permethrin). Although there are a number of biopesticides available in Canada, these may not be sufficient if the aphids are vectors for viruses. Investigations into the relationship between

aphids and pathogens (such as *P. carotovorum*), or as vectors of viruses, may generate interest in an integrated management approach, as well as the registration of additional control products for resistance management for use in commercial wasabi crops.

Figure B-1. Aphids identified feeding on *W. japonica*. (a) Parthenogenic *P. populitransversus* population with waxy exudate in the root mass of a *W. japonica* plant grown in a plug-tray. (b) Apterous *P. populitransversus* with proboscis in *W. japonica* root. (c) *L. pseudobrassicae* colony consisting of different instars on a *W. japonica* leaf. (d) *M. euphorbiae* aptera with proboscis in a *W. japonica* leaf.

